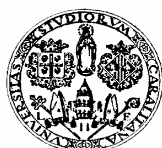

Università degli Studi di Cagliari

DOTTORATO DI RICERCA

In TOSSICOLOGIA

Ciclo XXVI

TITOLO TESI

T3-induced hepatocyte proliferation requires β-catenin and is protein kinase A dependent

Settore scientifico disciplinare di afferenza

MED/04

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Esame finale anno accademico 2012 – 2013

Acnowledgments

I would like to express my sincere gratitude to Prof. Amedeo Columbano and Prof. Giovanna Maria Ledda who placed their trust in me by giving me the opportunity to work on the project of this thesis in collaboration with Dr. Satdarshan Pal Singh Monga and the Department of Pathology of the University of Pittsburgh (USA). Thanks to the joint work of the two groups, this study has been published in the journal Hepatology (Fanti et al., Hepatology, Oct. 2013).

I am deeply grateful to Dr. Monga, a mentor and a friend, for his guidance in science, motivation and enthusiasm. He has made me a more confident person in my work and in my life.

The experience at Pittsburgh was made special and unforgettable due to the presence of very nice friends like Enza and Rocco Di Domenico. They supported me as my family and always made me feel at home. I will never forget it.

I would also like to thank my colleagues and in particular Dr. Monica Pibiri. I really appreciate her help, advice and all her contribution of time in the writing of this thesis.

Lastly, I would like to thank my family and my friends for all their love, patience and encouragement during all these years.

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ABSTRACT

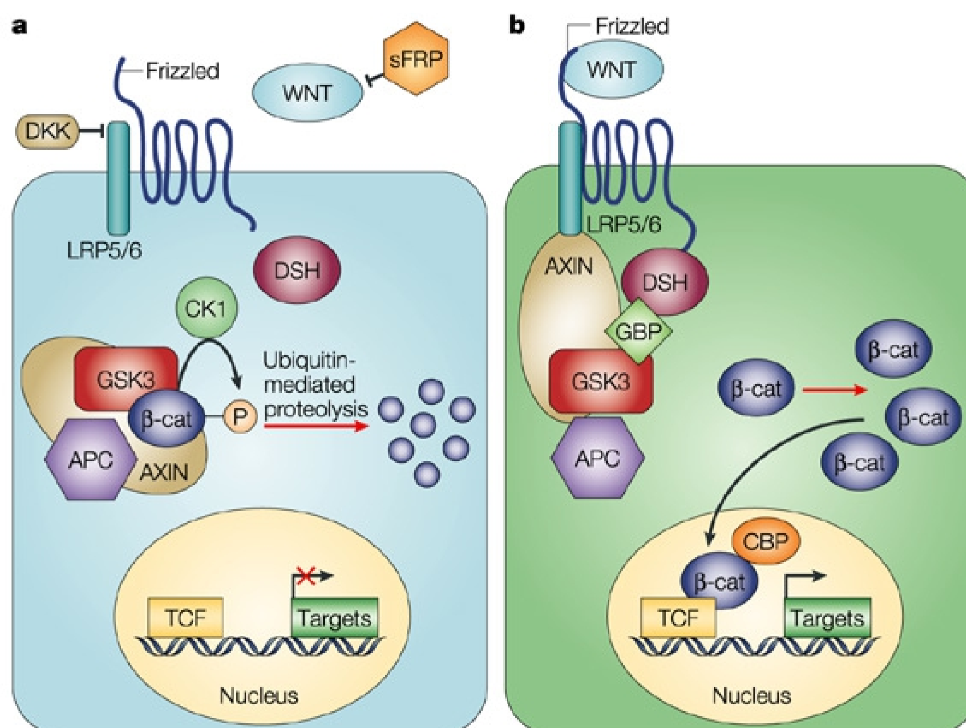
Thyroid hormone (T3), like many other ligands of the steroid/thyroid hormone nuclear receptor superfamily is a strong inducer of liver cell proliferation in rats and mice. However, the molecular basis of its mitogenic activity, which is currently unknown, must be elucidated if its use in hepatic regenerative medicine is to be considered. F-344 rats or C57BL/6 mice were fed a diet containing T3 for 2-7 days. In rats, administration of T3 led to an increased cytoplasmic stabilization and nuclear translocation of β-catenin in pericentral hepatocytes with concomitant increase in cyclin-D1 expression. T3 administration to wild-type (WT) mice resulted in increased hepatocyte proliferation, however no mitogenic response in hepatocytes to T3 was evident in the hepatocyte-specific β-catenin knockout mice (KO). In fact, T3 induced β-catenin-TCF4 reporter activity both *in vitro* and *in vivo*. Livers from T3-treated mice demonstrated no changes in *Ctnnb1* expression, activity of Glycogen synthase kinase-3β known to phosphorylate and eventually promote β-catenin degradation, or E-cadherin-β-catenin association. However, T3 treatment increased β-catenin phosphorylation at Ser675, an event downstream of protein kinase A (PKA). Administration of PKA inhibitor during T3 treatment of mice and rats as well as in cell culture abrogated Ser675-β-catenin and simultaneously decreased cyclin-D1 expression to block hepatocyte proliferation. Conclusion: We have identified T3-mediated hepatocyte mitogenic response to be mediated by PKA-dependent β-catenin activation. Thus, T3 may be of therapeutic relevance to stimulate β-catenin signaling to in turn induce regeneration in selected cases of hepatic

insufficiency.

INTRODUCTION

β-catenin is an important nuclear effector of the Wnt signaling pathway that is involved in the establishment of the dorsoventral axis or the segmentation pattern in embryos [1]. Wnt proteins are a large family of secreted glycoproteins which, upon receptor binding, mediate extracellular signaling initiating a signaling cascade, which results in activation of β-catenin, the central player in the canonical Wnt pathway. In absence of Wnt ligands, intracellular levels of β-catenin are kept low by ubiquitin-dependent proteasomal degradation, set in motion by a multicomponent degradation complex, consisting of Axin, caseine kinase 1α (CK1α), Adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). Binding of Wnt proteins to the seven transmembrane (7-Tm) Frizzled (Fz) receptor and low density lipoprotein receptor-related protein co-receptor 5/6 (LRP5/6), triggers activation of the canonical Wnt pathway. Dishevelled (Dvl) is recruited to the Frizzled receptor [2], and the Fz/Dvl complex in turn relocates Axin to LRP5/6 [3]. Axin-bound GSK-3β and CK1 then phosphorylate LRP5/6 [4,5], which leads to inactivation of GSK-3β [6]. The absence of phosphorylation, releases β-catenin from the Axin/APC/GSK3 complex, resulting in accumulation of cytoplasmic and active β-catenin. Although β-catenin lacks a nuclear localization sequence, it then translocates to the nucleus, through an unknown mechanism, where it acts as a transcriptional co-activator for transcription factors of the Tcell factor/lymphoid-enhancing factor (TCF/LEF) family, among others [7,8]. Additional co-activators, such as B-cell lymphoma 9 (BCL9), cAMP response element-binding protein (CREB)-

Binding Protein (CBP)/p300 and brahmarelated gene 1 (BRG1) bind the β-catenin-TCF complex and ensure cell- and tissue-specific activation or suppression of transcription of numerous Wnt responsive genes [2,3, 9–12].



Nature Reviews Genetics ***5****, 691-701 (September 2004)*

There is a growing evidence of the involvement of the Wnt/β-catenin pathway in liver biology [13]. In adult liver, β-catenin is observed localizing mostly at the hepatocyte membrane [14], while it is present in the cytoplasm in the biliary epithelial and endothelial cells where its role remains unknown. During development β-catenin has been implicated in biliary

differentiation [15–17] and transgenic mice overexpressing β-catenin resolve intrahepatic cholestasis more efficiently than wild type controls [18]. Similarly, recently has been reported that Wnt2/Fz4/β-catenin axis seems to be important in sinusoidal endothelial cell proliferation [19].

Wnt/β-catenin signaling has also been identified in hepatic stellate cells, where has been implicated either in the maintenance of stellate cell quiescence [20], as in stellate cell activation [21,22]. It will be highly relevant to conclusively address the role of Wnt in stellate cell biology in order to modulate its expression as a means to inhibit hepatic fibrosis.

A key role of Wnt/β-catenin pathway was identified in adult liver zonation. Interacting with HNF4α and Ras/MAPK/ERK signaling, β-catenin signaling appear to dictate pericentral versus periportal gene expression in hepatocytes, thus determining their molecular heterogeneity based on their location within the hepatic lobule [20,23–25]. In pericentral hepatocytes, in addition to membranous localization, β-catenin is expressed both in cytoplasmiand nucleus, and it regulates the expression of genes that encode for enzymes critical in ammonia and xenobiotic metabolism such as those encoding for glutamine synthetase, (GS), glutamate transporter (Glt1) and cytochrome P450 family members CYP2E1 and CYP1A2. On the opposite, the hepatocytes in the periportal zone show lower unbound, dephosphorylated β-catenin, due to the higher APC protein content, and express genes encoding for ammonia metabolism (such as arginase 1 and carbamoyl-phosphate synthase).

β-catenin also plays a pivotal role in the maintenance of cell-cell adhesion. In fact, it is an essential part of the adherens junctions in epithelial cells, forming a bridge between the actin cytoskeleton and E-cadherin [26–28]. Phosphorylation of β-catenin at a specific tyrosine residue (Y654) [29] causes dissociation of the complex and subsequent degradation of E-cadherin [30]. This results in a disruption of adherens junctions and, in the case of hepatocytes, impairment in the apical trafficking of specific proteins [31]. Loss of cadherin function is involved in processes such as development and turnover of adult tissues, and may also contribute to motility and metastasis in the presence of aberrant growth [32].

An important association between β-catenin and several receptor tyrosine kinases has also been observed in hepatocytes. The effect of hepatocyte growth factor (HGF), a known liver mitogen, motogen, and morphogen [33], is mediated through the binding to its receptor, Met, a tyrosine kinase ubiquitously expressed in hepatocytes, that results in phosphorylation of Met which, in turn, phosphorylates β-catenin at tyrosine residues 654 and 670, promoting its nuclear translocation and subsequent upregulation of target genes [34-36]. The Met/β-catenin pathways operate synergistically to induce hepatocyte proliferation in normal and dysregulated growth.

Active β-catenin promotes migration and cell dedifferentiation in nonconfluent cells cultured in the presence of HGF [37]. Opposite, in matrigel-induced hepatocyte re-differentiation, there is an inverse correlation

between Met and β-catenin association and the amount of nuclear and tyrosine-phosphorylated β-catenin [38]. In human tumor cell lines, β-catenin has also been shown to be tyrosine phosphorylated upon stimulation with HGF [39]. β-Catenin contributes to HGF-induced hepatomegaly in mice through loss of Met/β-catenin association and subsequent activation of β-catenin and increased cyclin-D1 expression [40], shown to be an important mediator of β-catenin and Met induced HCC in genetic models [41]. Further, simultaneous activation of Met and a mutated active form of β-catenin have been found in a subset of human hepatocellular carcinomas [42].

β-catenin also appears to be phosphorylated by ErbB2, a member of the epidermal growth factor receptor (EGFR) family, whose ligand, EGF, is another known hepatocyte mitogen. Phosphorylation of β-catenin at tyrosine-654 [43] and constitutive tyrosine phosphorylation of β-catenin by ErbB2 has been implicated in cancer metastasis [44,45]. EGFR has also been identified as a transcriptional target of Wnt/β-catenin signaling [46], able to enhance and prolong β-catenin signaling and potentiate its mitogenic effect.

The genes activated by Wnt/β-catenin signaling are diverse and include target genes, such as Met, Jagged, gastrin, MMP7, survivin, and various FGFs [47–54], cell-cycle regulators important in proliferation, such as cyclin-D1 [55,56], as well as oncogenes such as c-myc [57]. The majority of Wnt target genes, however, appear to be cell-type specific and are thus regulated both temporally and contextually. In the liver, these genes include glutamine synthetase, cyclin-D1, several cytochrome P450s (2e1, 1a2),

EGFR and leukocyte cell-derived chemotaxin 2 (LECT2), as well as regucalcin or senescent marker protein-30 [46,58–64]. Interestingly, some of the target genes of the pathway are components of the Wnt signaling pathway itself. For example, β-catenin can activate expression of repressors, such as Axin2, Tcf1, and Dkk1 [53,65,66], or suppress pathway activators, such as Frizzled and LPR6 [67,68], indicating the presence of a negative feedback loop to trigger or suppress Wnt signaling. It is still unknown whether β-catenin activation in the absence of mutations in the β-catenin gene, *CTNNB1,* or components of degradation complex such as *AXIN1/2* will determine any significant target gene expression, given the presence of several negative feedback opportunities in this signaling cascade. In the same way, heterogeneity is evident in β-catenin activation due to mutation sites within the exon-3 of *CTNNB1*, *AXIN1/2* mutations as well as additional modes in HCC such as by TGFβ or receptor tyrosine kinases, determining variations in target gene expression, which eventually result in a distinct tumor phenotype [69–73]. Interestingly, Wnt/β-catenin signaling also can induce the expression of genes which enhances and prolongs the signal, such as LEF1, indicating the presence of a feed-forward mechanism which can be exploited by carcinoma cells [74,75].

Despite its aberrant accumulation due to mutations in hepatocellular cancer (HCC), β-catenin is crucial for cell cycle regulation during embryonic liver development and liver regeneration after partial hepatectomy (PH) [76,77]. In liver development across various species,

through regulation of cell proliferation, differentiation, and maturation, β-catenin directs foregut endoderm specification, hepatic specification of the foregut, and hepatic morphogenesis [77].

The Wnt/β- catenin pathway also plays a key role in postnatal liver growth, its nuclear translocation correlating with an increase in cell proliferation evident in livers between 5–20 postnatal days [78]. Further, conditional hepatocyte β-catenin knockout (KO) mice older than two months [63,64], showed a significant decrease in the liver weight/body weight ratio (15–25%), due to a basal decrease in hepatocyte proliferation as a result of lower expression of cell cycle regulators, such as cyclin-D1, in the KO livers.

Direct overexpression of β-catenin has also been shown to enhance liver growth and regeneration. Transgenic mice overexpressing liver-specific wild-type β-catenin showed a 15% increase in liver size compared to normal wild-type aged-matched controls, due to increased proliferation [46]. Liver hyperplasia and hepatomegaly was also described in a transgenic mouse expressing an oncogenic form of β-catenin lacking the N-terminus, involved in protein stabilization [79]. Another report described enlarged livers shortly after adenoviral infection in a mouse strain carrying a mutant inducible form of β-catenin [80]. It's noteworthy that none of the mouse models described above developed spontaneous hepatic tumors, demonstrating that mutation of β- catenin alone is insufficient to cause tumorigenesis and suggesting that mutant-β-catenin promote tumorigenesis only after a "first hit", such as chemical induction or mutation of another

oncogene.

During liver regeneration the steady-state kinetics of β-catenin changes dramatically. Adult liver represents a useful model to study organ regeneration and controlled growth having the unique capacity to regenerate after insult and loss of liver mass. A phenomenon unique to liver regeneration is that repopulation of the organ occurs almost exclusively through proliferation of mature cell populations, including hepatocytes, biliary epithelial cells, endothelial cells, and stellate cells [81]. Remarkably, throughout the regenerative process, the liver still performs all the essential functions needed for organism homeostasis, without loss of function and in absence of inflammation or damage to surrounding tissues. The most common method of inducing regeneration experimentally is surgical removal of three of five lobes from the rodent liver, namely 2/3rd or partial hepatectomy (PHx) [82]. In these conditions, the remaining two lobes grow until complete restoration of the liver mass, approximately 7 days in the rat [83].

Partial hepatectomy triggers a series of cell signaling pathways and cascades that are very tightly regulated. One of the earliest events, within 30 minutes after PHx, is the induction of “immediate early genes”, including members of the jun, c-fos, and myc families. Transcription of these genes are the result of rapidly activated transcription factors, preexisting in a latent form, such as Stat3 and NF-κB, which are, in turn, activated by cytokines, such as TNF-α and IL-6 [84]. Concomitantly with cytokine

stimulation, or immediately after this period, in response to growth factors, such as HGF and EGF [85,86] genes important for regulation of cell cycle entry are also transcribed. Interestingly, fetal markers such as alpha-fetoprotein are also upregulated during this time [87], suggesting that regeneration may recapitulate development to some extent.

The hallmark of liver regeneration is proliferation of adult hepatic cell types. The first peak of DNA synthesis occurs in hepatocytes at 24 hours in the rat and at approximately 36 hours in the mouse [83]. Hepatocyte DNA synthesis and proliferation proceed in a zonal manner through the hepatic lobules, from periportal to pericentral areas [88]. Rat liver is able to continue to regenerate even after 12 sequential hepatectomies [89], which suggests that unlike other mature cells in the body, hepatocytes are not terminally differentiated and can divide continuously when challenged with appropriate stimuli [81]. Proliferating hepatocytes also produce growth factors for other cell types, including stellate cells and endothelial cells, in a paracrine fashion. These cells, in the rat, undergo DNA synthesis 24 hours after hepatocytes, peaking at 48 hours after PHx [90]. Given that cyclin-D1 is a critical gene for initiation of cell proliferation and as β-catenin is a key driver of cyclin-D1 it is logical to assume that β-catenin plays a role in liver regeneration after PHx.

In the rat, a 2.5-fold increase in β-catenin protein, due to decreased Ser phosphorylation and subsequent decreased protein degradation, was observed during the early minutes of liver regeneration induced by PHx, concomitant with translocation to the nucleus [91]. Further,

this initial increase in β-catenin protein was immediately countered by a downregulation of this pathway and activation of the destruction complex, so that β-catenin activation after partial hepatectomy was transient, and protein levels returned to normal 48 hours post-surgery. The early increased nuclear localization of β-catenin during liver regeneration contributes to cyclin-D1 and c-myc expression increase observed previously after PHx; thus, β-catenin is a positive modulator of cellular proliferation [91]. As mentioned above, β-catenin was also identified to be a downstream effectors of HGF, a significant player in liver regeneration [34,36,92].

Since conventional β-catenin knockout shows early embryonic lethality [93], the importance of β-catenin to liver regeneration has been elucidated by several studies in which β-catenin is removed or absent from the liver. Ablation of β-catenin transcription by administration of an antisense oligonucleotide simultaneous with PHx resulted in a significant decrease in liver weight/body weight ratio as late as 7 days after PHx, which was a result of decreased hepatocyte proliferation [94]. Accordingly, conditional knockout (KO) mice containing a hepatocyte-specific disruption of the β-catenin gene, showed a significant and sustained decrease in liver weight/body weight ratio (15–25%) [63,64]. Additionally, when subjected to partial hepatectomy, these mice displayed a 2-fold decrease in the number of cells in S-phase at the time of hepatocyte proliferation peak in wild-type (40 hours) associated to deficient expression of cyclins involved in G1 to S transition, including cyclin-A, D, and E [64]. Interestingly, an increase in hepatocyte proliferation was

noted in the β-catenin-null mice 3 days after PHx, indicative of a delayed regenerative onset, and a second, smaller proliferation peak at day 14, perhaps as a compensatory event to ongoing apoptosis. This biphasic trend in proliferation allows for delayed but sufficient regeneration of β-catenin knockout livers after PHx [64]. Another laboratory utilized liver-specific β-catenin knockout mice to confirm a delayed onset of DNA synthesis and hepatocyte proliferation after partial hepatectomy, which is likely due to a lack of cyclin-D1 induction [95].

The KO mice were also investigated for addressing the role of β-catenin in toxicant-induced liver regeneration. Sublethal doses of acetaminophen in mice leads to hepatic injury immediately followed by enhanced regeneration. An early β-catenin stabilization and not injury following acetaminophen exposure was identified as a mechanism of hepatocyte proliferation [96].

While loss of β-catenin leads to defects in liver regeneration, activation of this pathway through gain-of-function mutants, has demonstrated the positive effect of Wnt signaling on liver regeneration. Two studies have demonstrated the effect of β-catenin stabilization indirectly through ablation of the pathway negative regulator APC gene. In the first study, Apc-inactivated mice display both a clear increase in liver size and a high incidence of spontaneous hepatocellular cancer [97]. In the second study is reported that regeneration was accelerated in $Apc^{+/-}$ zebrafish after 1/3 PHx [98], while inhibition of β-catenin transcription resulted in impaired

liver regeneration as expected, confirming the requirement for Wnt/β-catenin signaling after liver resection. These authors further supported this finding by demonstrating enhanced kinetics of β-catenin expression and proliferation in APC-mutant mice livers after PHx [98]. Finally, it has also been demonstrated that transgenic (TG) mice expressing Ser45 mutated β-catenin in hepatocytes show a growth advantage both *in vitro* and during liver regeneration through cyclin-D1 regulation. The growth advantage of S45D TG hepatocytes after PHx may be attributed to acceleration of Met/β-catenin dissociation, phosphorylation, and nuclear translocation 40 hours after partial hepatectomy.

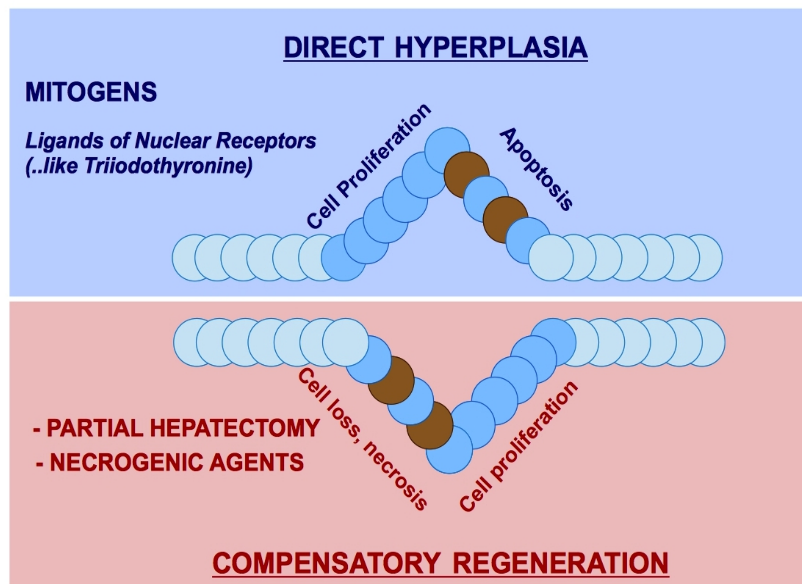
Liver regeneration is a compensatory response to injury, in which proliferation is essential to restore hepatic mass and function. In contrast, numerous primary mitogens induce hepatocyte proliferation (direct hyperplasia) without causing liver injury. Unlike liver regeneration, *direct hyperplasia*, results in an excess of hepatic DNA and liver mass [99].

Such mitogens include the peroxisome proliferators (PPs), retinoic acids (RA), thyroid hormone triiodothyronine (T3), and the halogenated hydrocarbon 1,4-bis[2-(3,5 dichloropyridyloxy)]benzene (TCPOBOP), which are all ligands of nuclear receptors of the steroid/thyroid superfamily [100].

As heterodimers with RXRα, these receptors function as ligand-activated transcription factors and regulate genes involved in lipid metabolism, adipogenesis, xenobiotic detoxification, and differentiation. Among these nuclear receptor ligands, T3 is particularly interesting since its mitogenic

activity is associated with regression of preneoplastic lesions induced by chemical carcinogens [101,102].

Hepatocyte proliferation



T3 is known to influence a variety of physiological processes, including cell growth and metabolism in mammals, metamorphosis in amphibians, and development of the vertebrate nervous system. Most of these effects are mediated by thyroid hormone nuclear receptors, (TRs), which act as transcription factors [103, 104]. Due to the ubiquitous nature of TRs, T3 has been shown to induce proliferation in several organs including liver, kidney, pancreas, heart and intestine, although its effect on liver and pancreas is predominantly via TRβ [105].

In the liver, T3-induced proliferation lacks many early events thought to be

critical in liver regeneration, such as activation of latent transcription factors (AP1, NFκB) or increased expression of immediate-early transcription factors (c-fos, c-myc) and c-jun [106, 107]. These differences suggest that the signaling pathways activated by T3 via TRs may be different from those activated in liver regeneration. Interestingly, T3 has been shown to induce cyclin-D1 expression [108]. Since cyclin-D1 is regulated by the Wnt/β-catenin signaling in the liver during proliferative states, we asked if the effect of T3 on cyclin-D1 expression and eventually on hepatocyte proliferation was indeed β-catenin-dependent [108-110].

AIM OF THE STUDY

Based on the notion that cyclin-D1 is regulated by the Wnt/β-catenin signaling, we asked if the effect of T3 on cyclin-D1 expression and eventually hepatocyte proliferation could be β-catenin-dependent [108-111]. To this aim, we analyzed the role of β-catenin in T3-induced hepatocyte proliferation *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Animals

In order to evaluate the proliferative response of hepatocytes to T3 treatment, 8 week old male F-344 rats (Charles River, Milan, Italy) were maintained on a standard laboratory diet (Ditta Mucedola, Milan, Italy) or fed a diet supplemented with *3,3′,5-Triiodo-L-thyronine* (T3, 4 mg/kg of diet, Sigma Chemical Co., St Louis, MO) for 2 or 4 days.

8-10 week old male β-catenin KO mice or sex-matched littermate controls obtained from Jackson Laboratories (Bar Harbor, ME) were fed a basal or a T3-supplemented diet (4 mg/kg of diet) for 1 week. To obtain this transgenic strain, homozygous floxed β-catenin mice (C57BL/6 strain) were bred to Albumin-Cre mice (C57BL/6 strain) and the offspring carrying a floxed β-catenin allele and albumin-Cre were again bred to the homozygous floxed β-catenin mice. This led to a floxed allele and a floxdel-deleted (floxdel) allele of Ctnnb1 and are referred to as Ctnnb1$^{loxp/loxp}$; Alb-Cre$^{+/-}$ or knockout (Ko) mice throughout the manuscript. C57BL/6 WT mice were also fed T3 or basal diet for 1 week to harvest livers for addressing molecular changes. C57BL/6 WT mice were also used in the experiments with PKA inhibitor (see below).

To label the cells, *5-bromodeoxyuridine* (BrdU, 1mg/ml, Sigma Chemical Co., St Louis, MO) dissolved in drinking water was given to all animals throughout the experimental period.

In each experiment, the animals were given food and water *ad libitum* with a 12h light/dark daily cycle. After sacrifice, liver sections were paraffin embedded for immunoistochemistry or frozen at −80°C for total RNA

isolation, and analysis.

All studies on mice and rats were performed in strict accordance with the Institutional Animal Use and Care Committee at the University of Pittsburgh, the University of Cagliari and the National Institutes of Health guidelines.

Administration of H89, a PKA inhibitor

In order to evaluate the involvement of PKA pathway in hepatocyte proliferation T3-dependent, H89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl), a potent selective inhibitor of cAMP dependent protein kinase A (PKA), was tested in three different protocols:

Experimental protocol 1

3-5 month old male mice (C57BL/6 strain) (n≥3) were fed a T3 diet (4mg/kg/diet) or a basal diet for 3 days. H89 dissolved in saline (200μg/100g/bw, Merck, Billerica, MA) was injected intraperitoneally (IP) 1 hour prior to T3-feeding and 2 hours before sacrifice.

Experimental protocol 2

This protocol was essentially similar to Experimental Protocol 1 except that H89 was injected IP every 24 hours for 5 days. The animals received BrdU dissolved in drinking water (1mg/ml) during the entire experimental period.

Experiment protocol 3

7 week old male F-344 rats were given a single IP dose of T3 dissolved in saline (20μg/100g/bw) 30 minutes after H89 (200μg/100g/bw, IP). Rats were killed 24 hours after treatment. The animals received BrdU in drinking water (1mg/ml) during the entire experimental period.

Isolation and culture of primary hepatocytes

Rat or mouse hepatocytes were isolated by adaptation of the calcium 2-step collagenase perfusion technique as previously described [112]. Hepatocytes were plated on a single layer of collagen gel and left to attach for 2 h. Six-well cluster plates (9.8 square centimeters per plate) from Coming (Ithaca, NY) were used. Unless otherwise specified, 10,000 hepatocytes per square centimeter surface were inoculated for all the experiments described. The medium was changed at 2h after cells were plated.

Immunohistochemistry

Liver sections were analyzed by immunohistochemistry for β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin-D1 (Thermo Scientific, Freemont, CA), glutamine synthetase (GS, Santa Cruz Biotechnology), β-galactosidase (Rockland Immunochemicals, Gilbertsville, PA), BrdU (Becton Dickinson, San Jose, CA).

Briefly, formalin-fixed sections were deparaffinized. Endogenous peroxide was inactivated using 3% hydrogen peroxide (Sigma, St. Louis, MO).

For β-catenin, cyclin-D1, β-galactosidase and GS staining, slides were

microwaved in citrate buffer for 20 minutes followed by blocking in the blue blocker (Shandon Lipshaw, Pittsburgh, PA). Sections were then incubated with secondary anti-mouse horseradish-peroxidase–conjugated antibody (Chemicon, Temecula, CA) for 30 minutes and the signal was detected using the ABC Elite kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions.

BrdU incorporation into nuclei was determined immunohistochemically by the avidin-biotin-peroxidase complex (ABC) method using a mouse anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems, San JoseÁ, CA) and Vectastain ABC kit (PK-4002, Vector Laboratories Inc., Burlingame, CA). In details, formalin-fixed sections were deparaffinized, exposed to 0.3% hydrogen peroxide in ethanol for 10 min to block endogenous peroxidase, treated with 2N HCl for 1 hour, incubated with trypsin 0.1% for 20 min and then with normal horse serum for 20 min at room temperature. Sections were then incubated for 2h with an anti-BrdU monoclonal antibody, followed by biotinylated horse anti-mouse IgG and avidin-biotin peroxidase complex. Sites of peroxidase binding were detected with diaminobenzidine (DAB), and the sections were counterstained with hematoxylin. A segment of duodenum, an organ with a high rate of cell proliferation, was included from each animal to confirm delivery of the DNA precursor. At least 5000 hepatocyte nuclei per animal were scored.

The labeling index (LI) was calculated as BrdU or cyclin-D1-positive hepatocyte nuclei/100 nuclei. At least 5000 hepatocyte nuclei per liver were

scored.

Protein Extraction and Western Blot Analysis

Whole-cell lysate preparation and protein extraction from primary hepatocyte cultures or frozen liver tissue were performed using RIPA buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L mono- basic sodium phosphate, 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO).

The concentration of the protein in the lysates was determined by the bicinconinic acid protein assay with bovine serum albumin as a standard. Concentration of the samples ranged from 25 to 40 μg/μL. Aliquots of the samples were stored at −80°C until use.

To perform the Western Blot, proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis using the mini-PROTEIN 3-electrophoresis module assembly (BioRad, Hercules, CA) and transferred to immobilonpolyvinylidene difluoride membranes; proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography. These were scanned and densitometric analysis was performed by NIH Image software (Bethesda, MD) for quantitative assessment.

Antibodies:

The primary antibodies used were against β-catenin, GSK3β, pSer9GSK3β, Gapdh (Santa Cruz Biotechnology, Santa Cruz, CA), pSer675β-catenin (Cell Signaling Technology, Danvers, MA), cyclin-D1 (Thermo Scientific), p133CREB (Cell Signaling Technology, Danvers, MA), and β-actin (Sigma). Horseradish peroxidase–conjugated secondary antibodies were purchased from Chemicon. The proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography.

Immunoprecipitation

In order to assess the association between E-cadherin and β-catenin, immunoprecipitation was performed using 500μg of protein extract and β-catenin-conjugated A/G agarose beads (Santa Cruz). Blots were probed for E-cadherin (BD Transduction Labs) and β-catenin (Santa Cruz).

Equal pull down was verified by probing the immunoblot for β-catenin.

β-catenin/Tcf Transcription Reporter Assay

Hepatocytes were plated on collagen coated six-well plates (Becton Dickinson) at 300,000 cells/well and transfected with 2.5μg of the plasmids TOPFlash (Upstate Biotechnology, Lake Placid, NY) at 80% of confluency. TOPFlash has three copies of the Tcf/Lef sites upstream of a thymidine kinase (TK) promoter and the firefly luciferase gene. All transfections were

performed with Lipofectamine2000 as previously described [113]. To normalize transfection efficiency, cells were co-transfected with 0.5μg of the internal control reporter Renilla reniformis luciferase driven under the TK promoter (pRL-TK; Promega, Madison, WI). Cells have been treated with or without 100nM of T3 in medium (Sigma). T3 was administered in two doses, once every 24h. Forty-eight hours after transfection the cells were harvested and lysed with Passive lysis buffer (Promega). Luciferase assay was performed using the Dual Luciferase Assay System kit according to the manufacturer's protocols (Promega). Relative luciferase activity was reported as fold induction after normalization for transfection efficiency. Experiments were repeated at least three times with 6 samples per experiment.

Real-time PCR

Total RNA was extracted by homogenizing frozen liver tissue in Trizol® reagent (Invitrogen, Carlsbad, CA). Two microgram of total RNA from each sample was reverse-transcribed after DNase treatment using Super Script III first strand kit (Invitrogen). Real-time PCR was performed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using specific Taqman Gene Expression assays (Applied Biosystems) for TRα and TRβ, and the relative expression levels were calculated after normalization to 18S. For β-catenin gene expression, Sybr green assay was used (Applied Biosystems) and the values were normalized to Cyclophilin expression.

Statistics

Data are presented as mean ± standard error (S.E.). Comparison between treated and control groups were performed by the ANOVA and Student's *t* test. $p<0.05$ (*) was considered significant throughout the study.

RESULTS

T3-fed rats show stabilization of β-catenin and increased expression of its targets in the liver.

Previous studies have shown T3 to be a powerful liver mitogen in rats [101]. To investigate if the Wnt/β-catenin pathway is altered after T3 treatment and contributes towards hepatocyte proliferation, we performed immunohistochemical studies on liver sections from rats sacrificed 2 and 4 days after T3-feeding. Normal untreated livers, referred henceforth as control livers, show β-catenin staining only at the hepatocyte membrane (Fig. 1). T3 treatment led to increased cytoplasmic accumulation of β-catenin at day 2 followed at 4 days by enhanced cytoplasmic and nuclear localization. GS-positivity was limited to hepatocytes in the pericentral area in controls and T3-treated rats after 2 days; however, a modest increase in the number of GS-positive hepatocytes was observed in the perivenular area at 4 days after T3 treatment (Fig. 1). Control livers were negative for cyclin-D1, another target of β-catenin and a cell cycle regulator, but increased specifically after 2 and 4 days of T3 treatment (Fig. 1). The enhanced cyclin-D1 expression in response to T3 was evident in pericentral hepatocytes at 2 days and it expanded to additional zones at 4 days. Thus, T3 induces β-catenin activation in the rat liver.

β-catenin is an absolute requirement for T3 mitogenic action in mouse liver.

Based on the above findings, we next asked whether β-catenin plays an essential role in T3-induced mitogenesis. To this end we administered T3 to Ctnnb1$^{lox/lox}$; Alb-Cre$^{+/-}$ mice (KO) and wild-type littermate control mice (WT). While WT mice fed T3 diet for 1 week exhibited several BrdU-positive hepatocytes and hence a high LI (19.8%), almost no hepatocyte proliferation was evident in KO mice (1.2%) (Fig. 2A, 2B). No significant difference in the LI was observed between untreated WT or KO mice. Non-parenchymal cells however did show BrdU incorporation in the hepatocyte-specific β-catenin KO mice in response to T3. T3 is also mitogenic for the pancreatic acinar cells [113]. To eliminate the possibility that the discordant proliferative effects elicited by T3 in WT versus KO mice could be due to differences in T3 metabolism or systemic effects, we next examined the LI of BrdU in the pancreatic acinar cells, which in the same animals do express normal β-catenin. The analysis clearly indicates that the pancreatic acinar cells in the KO and WT mice respond robustly and comparably to T3 (Fig. 2C, 2D).

As expected, a strong nuclear staining for cyclin-D1 was observed as early as 4 days after T3 treatment in the hepatocytes in the WT mice; *au contraire*, almost no cyclin-D1-positive hepatocytes were seen in the KO mice (Fig. 2E). These results suggest that lack of β-catenin impairs cyclin-D1 expression and hence affects T3-induced hepatocyte proliferation.

Finally, to establish whether the lack of response of KO mouse hepatocytes to T3 could be due to down-regulation of liver thyroid hormone receptors, we performed qRT-PCR. The results show no significant difference in the mRNA levels of either TRα or TRβ between the WT and KO mice (Fig. 2F). This was further validated by examining the previously published microarray analysis of untreated WT and KO livers for thyroid receptor targets [64]. No notable differences in the expression of several relevant target genes of thyroid receptor signalling [114] were obvious representing three pooled livers for each group. Thus it is unlikely that the impaired response of KO to T3 is due to decreased transcriptional activation of thyroid hormone receptor genes in the absence of β-catenin in the hepatocytes.

T3 treatment induces β-catenin activity both *in vitro* and *in vivo* in the liver cells*.*

To establish whether T3 could directly induce β-catenin activation, we measured luciferase activity in TOPFlash plasmid-transfected mouse and rat primary hepatocytes as described in the methods. T3 treatment promoted binding of β-catenin to the TCF-binding elements leading to an increase in the luciferase activity in primary hepatocytes in both species suggesting that T3 can indeed activate β-catenin signaling (Fig. 3A, 3B). A control FOPFlash reporter that contains mutated TCF-binding elements showed no luciferase activity in absence or presence of T3 (data not shown).

Next, TOPGAL reporter mice that harbor a transgene comprising β-galactosidase gene (LacZ) downstream of TCF binding elements were fed T3-diet for 4 or 7 days. Livers sections from these mice were stained for β-galactosidase by indirect immunostaining. While TOPGAL mice on basal diet showed β-galactosidase expression only in pericentral hepatocyte rim as reported elsewhere [23], T3-feeding led to expansion of β-galactosidase-positive hepatocytes to additional few layers around the central vein, which is suggestive of enhanced *in vivo* β-catenin activation in response to T3 (Fig. 3C).

Mechanism of β-catenin activation brought about by T3-treatment *in vivo*.

To address the mechanism by which T3 induces β-catenin activation, livers from T3-fed mice for 4 days were assessed. qRT-PCR analysis of β-catenin mRNA levels did not show any difference between T3-treated and untreated mice (Fig. 4A). While cyclin-D1 protein was consistently increased after 4 days of T3 treatment, no notable changes total β-catenin or GSK3β-Ser9 (inactive form) were observed suggesting that β-catenin activation downstream of T3 may not be via canonical Wnt pathway (Fig. 4B). Yet another pool of β-catenin is at the membrane where it associates to E-cadherin and is part of the adherens junction [115]. T3-treatment did not alter E-cadherin-β-catenin association as shown in a representative immunoprecipitation analysis (Fig. 4C).

Futher supporting the notion the T3-induced β-catenin activation does not occur via Canonical Wnt pathway, interestingly, a striking increase in the levels of Ser675-β-catenin was found in the T3-treated mouse livers (Fig. 4D). To verify if T3 also induced Ser675-β-catenin phosphorylation *in vitro,* primary cultures of mouse hepatocytes were treated with T3 for 30 minutes. Analysis of whole cell lysates from these cells also showed a clear increase in Ser675-β-catenin levels (Fig. 5A). Phosphorylation at Ser675 has been identified as a mechanism of β-catenin activation downstream of cyclic AMP-dependent protein kinase A (PKA) [116, 117]. Since CREB is a known

substrate of PKA (118), we next asked if T3 also induced CREB-phosphorylation. Indeed increased pSer133-CREB was observed by western blot analysis after 30 minutes of T3 treatment (Fig. 5A). Eventually, to directly address if T3-induced serine phosphorylation of β-catenin and CREB were PKA-dependent, primary hepatocytes were treated with T3 in the presence of H89, a small molecule inhibitor of PKA as indicated in the methods [119]. H89 prevented any increase in Ser675-β-catenin and pSer133-CREB in primary hepatocytes in response to T3 treatment (Fig. 5A).

H89 blocks T3-induced β-catenin activation, cyclin-D1 expression and hepatocyte proliferation in mice and rats.

To determine relevance of PKA in T3-mediated β-catenin activation and hepatocyte proliferation *in vivo,* mice were fed T3 diet along with H89 administration as discussed in methods (see Experimental protocol 1). Total liver lysates from such treatment show that H89 successfully abrogated T3-mediated increase in Ser675-β-catenin in mice, which was also accompanied by decreased levels of cyclin-D1 (Fig. 5B). Immunohistochemistry confirmed a significant decrease in the number of cyclin-D1-positive hepatocytes in mice that were administered H89 in addition to T3 (Fig. 5C). Intriguingly, BrdU incorporation in hepatocytes in mice undergoing H89 and T3 treatment showed a decrease as compared to T3 only fed mice, although it missed statistical significance due to variation in the T3-fed animals (Fig. 5D). Since an appreciable mitogenic effect of T3 in mouse liver is achieved between 4-5 days [116], we modified the protocol by administering both T3 and H89 for 5 days (see Experimental protocol 2). This modification led to a more uniform BrdU incorporation in control mice and an almost complete abrogation of T3-induced hepatocyte proliferation in T3+H89 group of mice (LI was 4% in animals receiving H89+T3 *vs* 16% in mice treated only with T3) (Fig. 5E).

Since T3 also induced β-catenin activation, cyclin-D1 expression and hepatocyte proliferation in rat livers as shown in Fig. 1, we investigated if this effect was via PKA. It is known that, in rats, a single dose of T3 induces a

peak of hepatocyte proliferation 24 hours after treatment [107]. Therefore, in this study we administered the PKA inhibitor 30 minutes prior to T3 treatment and sacrificed the animals 24 hours thereafter (Experimental protocol 3). PKA inhibition almost completely prevented BrdU incorporation (LI of H89+T3 was 1.8% *vs* 14.0% of the T3 group) (Fig. 6A, 6B). In concordance, cyclin-D1 nuclear expression was also significantly reduced in this group of animals (Fig. 6C, 6D). To address the efficacy of PKA inhibition in decreasing β-catenin activity brought about by T3, Ser675-β-catenin levels were assessed. Indeed, PKA blockade before T3 administration led to a significant decrease in Ser675-β-catenin levels as assessed at 90 minutes after H89 or 60 minutes after T3 treatment (Fig. 6E, 6F).

DISCUSSION

The unique capability of the liver to regenerate is known since 1930s [82]. Vast body of literature has led to an improved understanding of the cellular and molecular basis of liver regeneration and it has become clear now that tremendous redundancy exists in these processes that enable successful initiation and execution of the liver regeneration process [83]. This is highly relevant since liver is located strategically to perform key functions indispensable to survival but at the same time is the portal of entry to toxins and other harmful molecules through the portal circulation. Due to its critical function in synthesis, metabolism and detoxification, and its rather vulnerable location, liver is bestowed with a capacity to regenerate to maintain hepatic health and homeostasis on a day-to-day basis. An overwhelming insult or any mechanism that may impair the liver regeneration process due to acute or chronic injury, may lead to the end stage liver disease (ESLD). The majority of these patients with ESLD may require a liver transplant, which despite being a major advance still faces issues of donor organ scarcity and associated morbidity. Indeed major efforts are underway to discover not only improved transplantation technologies, but also research is underway to assess alternate strategies such as cell therapy, stem cell differentiation and transplantation, tissue engineering and regenerative therapies. Thus it is of significance to discern the signaling pathways naturally activated to enhance liver regeneration, which may be further stimulated through use of naturally occurring or synthetic agents as basis for regenerative therapies [120].

The role of the Wnt/β-catenin signaling in liver regeneration has now

been identified in multiple species ranging from zebrafish to patients and in various models ranging from PH to toxicant-induced liver injury [91, 94-96, 98, 109]. β-catenin's stabilization and nuclear translocation is an early event in rat and mouse liver regeneration [110, 91, 94]. Acetaminophen-induced liver regeneration also leads to β-catenin stabilization and absence of β-catenin impaired regenerative response to an equitoxic injury in the KO mice [96]. The major basis of β-catenin's role in liver regeneration is its ability to induce expression of cyclin-D1, a major G1 to S phase regulator [64, 110]. Indeed loss of β-catenin in hepatocytes led to delay in liver regeneration due to decreased cyclin-D1 levels [64, 95]. Conversely, transgenic expression of N-truncated β-catenin results in increased proliferation and hepatomegaly, while mice expressing point mutant of β-catenin display accelerated regeneration after PH [58, 108]. Also Wnt-1 gene therapy promoted liver regeneration after PH due to enhanced cyclin-D1 expression [108]. Thus it appears highly relevant to find a suitable modality to stimulate the Wnt signaling pathway as a means of regenerative therapy for the liver.

In the current study, we followed up on the observation where T3-induced hepatocyte proliferation also depended solely on the induction of cyclin-D1 expression [107]. T3's ability to stimulate hepatocyte proliferation was in fact reported in two models of impaired liver regeneration [101]. T3 stimulated cyclin-D1 expression and liver regeneration in old rats subjected to PH. It also improved BrdU LI via increased cyclin-D1 expression in rats subjected to 90% hepatectomy. However, how T3 stimulated cyclin-D1 expression has

remained an enigma and the current study demonstrates an imperative role of β-catenin in this process.

In the current study, we report that the absence of β-catenin in hepatocytes impairs the ability of T3 to induce cyclin-D1 expression and hence T3 was unable to stimulate hepatocyte proliferation in the KO mice. Further studies demonstrate that T3 was able to cause direct activation of the β-catenin signaling. However, this effect was not through the canonical Wnt signaling pathway or through disruption of adherens junctions, which is yet another independent pool of β-catenin in a cell [121]. Since T3 is known to have both genomic and non-genomic effects [122], we interrogated if T3 could impact gene expression of *CTNNB1*. The genomic effects of T3 are mediated by recruitment of thyroid hormone receptor-associated protein (TRAP)/Mediator (Med) complex and histone acetyl transferases such as p300 and p160/steroid receptor coactivator to the promoters of various genes [123]. Notably, recent studies have shown that intestinal epithelial cell proliferation is controlled by thyroid hormones, and that TRα directly controls transcription of the β-catenin gene in these cells, suggesting a direct correlation between T3, β-catenin and a positive regulation of proliferation-controlling genes, such as type D cyclins [124]. However, no difference in the mRNA expression of β-catenin in the liver was observed secondary to T3-treatment, *in vivo* or *in vitro*. It should be mentioned that hepatocytes are known to express higher levels of TRβ and selective agonists for this receptor subtype are in clinical trials for their ability to decrease blood lipids through reverse cholesterol

transport in hepatocytes [105, 125]. Intriguingly, β-catenin phosphorylation at Ser675 site was greatly induced by T3. This site has been shown to cause β-catenin activation through second messenger cAMP-mediated PKA activation [116, 117]. Indeed one of the major non-genomic effects of thyroid hormone is via cAMP-dependent protein kinase activation [126]. Thus it appears that T3 activates PKA to in turn induce β-catenin activation. These findings were strengthened by inhibition of PKA through application of a small molecule H89, which abrogated T3-induced PKA activity leading to an impairment of CREB (c-AMP response element-binding protein) and β-catenin's phosphorylation both *in vitro* and *in vivo* [118, 127]. Simultaneously, H89 administration to T3 fed mice or rats blocked cyclin-D1 expression and resulted in dramatically lowering hepatocyte proliferation. It was interesting to note that T3 treatment led to a discordant β-catenin activation, since its two downstream targets were not comparably induced. While cyclin-D1 was notably enhanced, GS was only modestly increased. The molecular basis of this observation remains obscure and may be due to the co-factor function of β-catenin, which may be binding to a set of specific transcription factors and other regulatory proteins upon specific signaling. Indeed, other studies have demonstrated such partial and discordant activation, for example in the context of Lgr5-positive stem cell activation in the liver [128].

Thus T3 administration is a plausible and practical modality to induce β-catenin signaling as a potential regenerative therapy. Additionally, selective TRβ agonists may possess a similar property without any untoward side ef-

fects associated with TRα activation predominantly in the heart [102, 105, 125]. This is of even greater relevance since TRβ-agonists induced regression of preneoplastic lesions in rodent livers [102]. Regenerative therapies depend on stimulation of surviving hepatocytes in a liver after acute or chronic insult and can be envisioned to be of benefit in live donor setting, small for size syndrome, and even toxicant induced liver injury such as acetaminophen overdose [120]. Indeed, in another study from our lab, β-catenin-positive hepatocytes that existed in KO mice due to leaky albumin-cre, showed growth and survival advantage in the face of chronic liver injury brought about by administration of 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidin diet [129]. These β-catenin-positive hepatocytes eventually repopulated the adult KO liver to maintain hepatic function and survival. Thus, stimulation of β-catenin signaling through T3 or other analogues may be of translational relevance in the setting of liver transplantation, cell therapy and in artificial liver devices and hepatic tissue engineering [13].

FIGURES AND LEGENDS

Figure1.

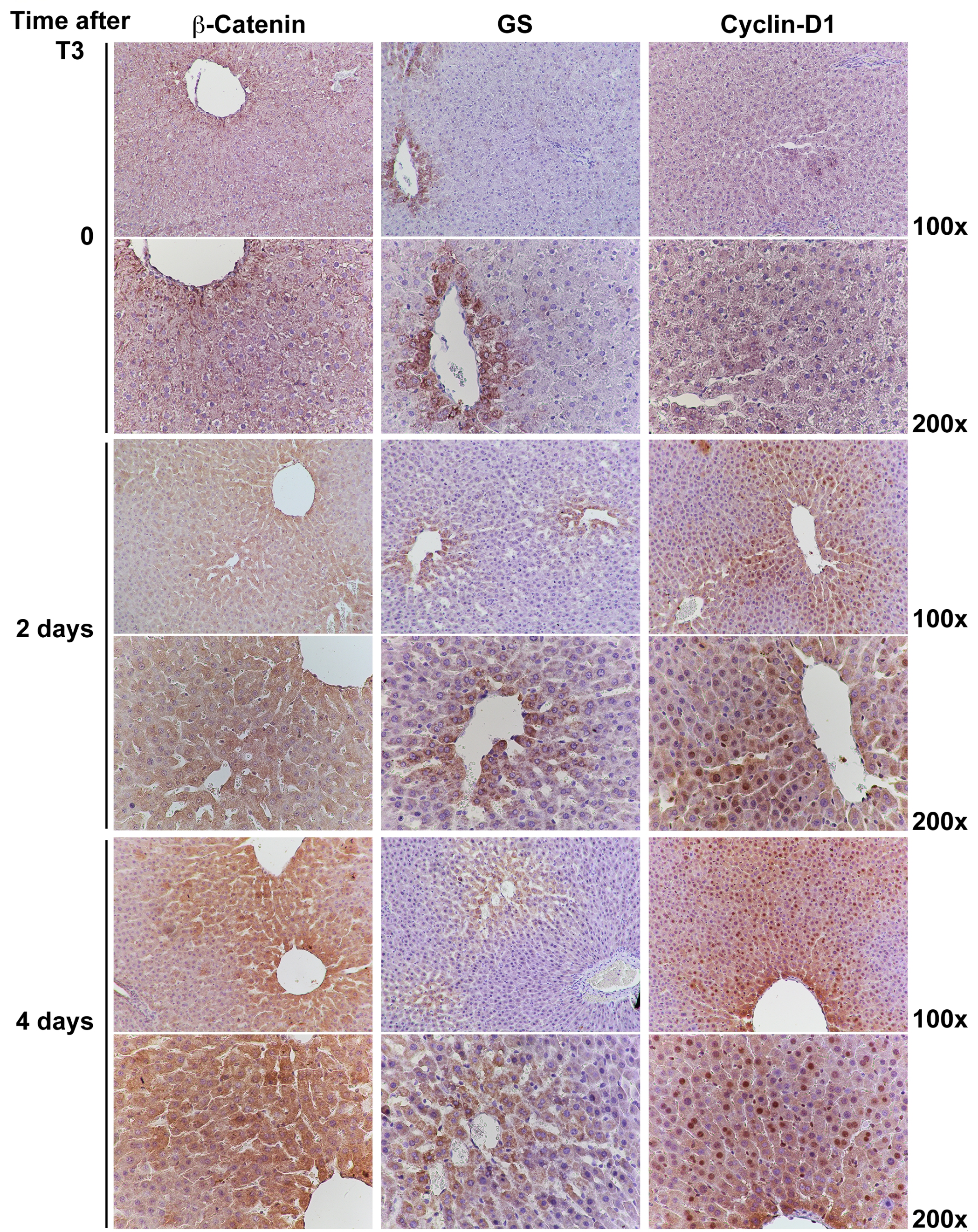


Figure1. Activation of β-catenin signaling in rat livers after T3-feeding.

Immunostaining shows β-catenin localizing to the hepatocyte membrane in the control livers, while it accumulates in the hepatocyte cytoplasm in T3-fed rats at 2 days. A progressive shift of stabilization from zone I towards zone II along with nuclear translocation of β-catenin-positive is observed at day 4 of T3 feeding. A concomitant increase in the number of GS-positive cells around central vein is observed at 4 days after T3. Progressive cyclin-D1 nuclear staining is evident in zone I at 2 days, and in zone I and II at 4 days of T3 feeding as compared to the control livers. (4 rats/group were used)

Figura 2.

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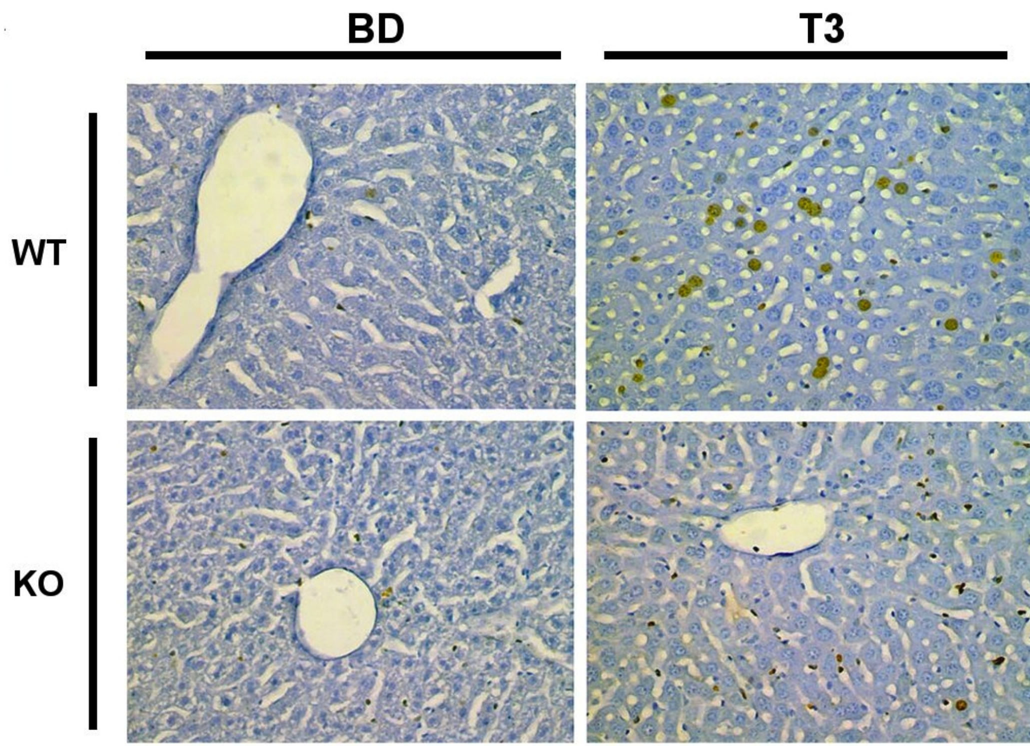

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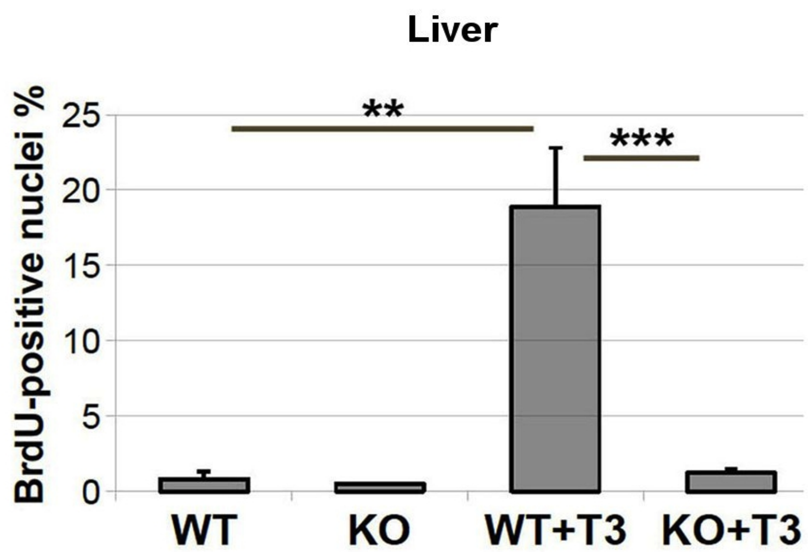

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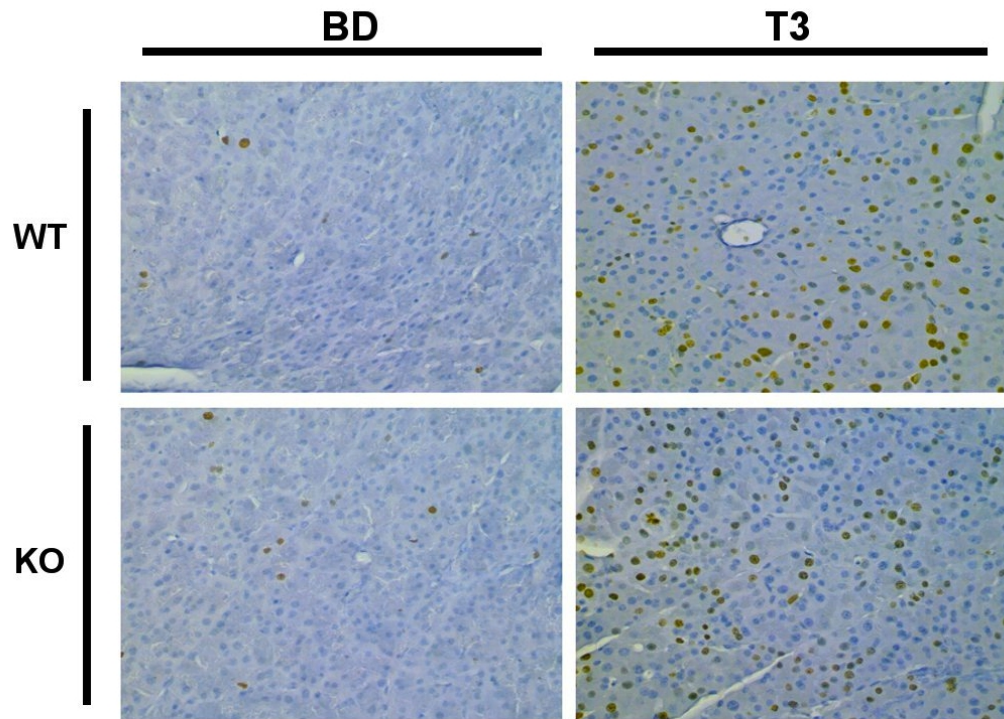

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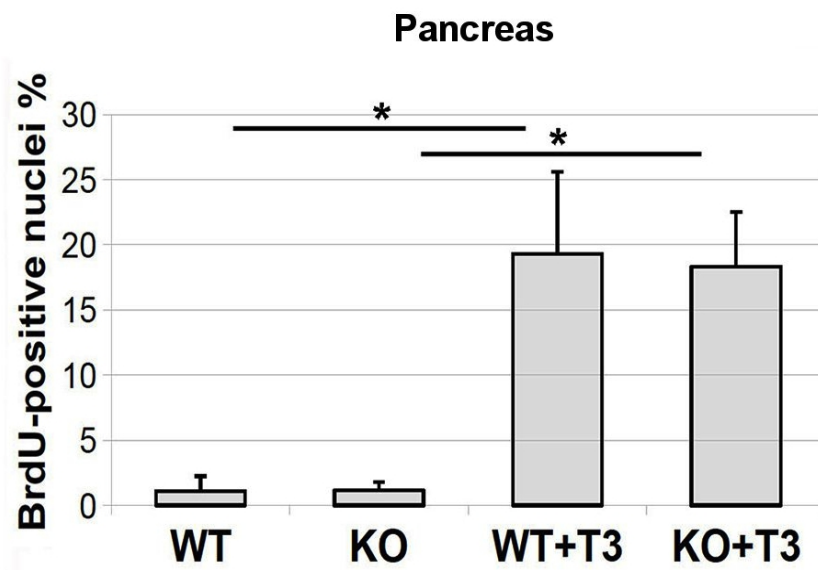

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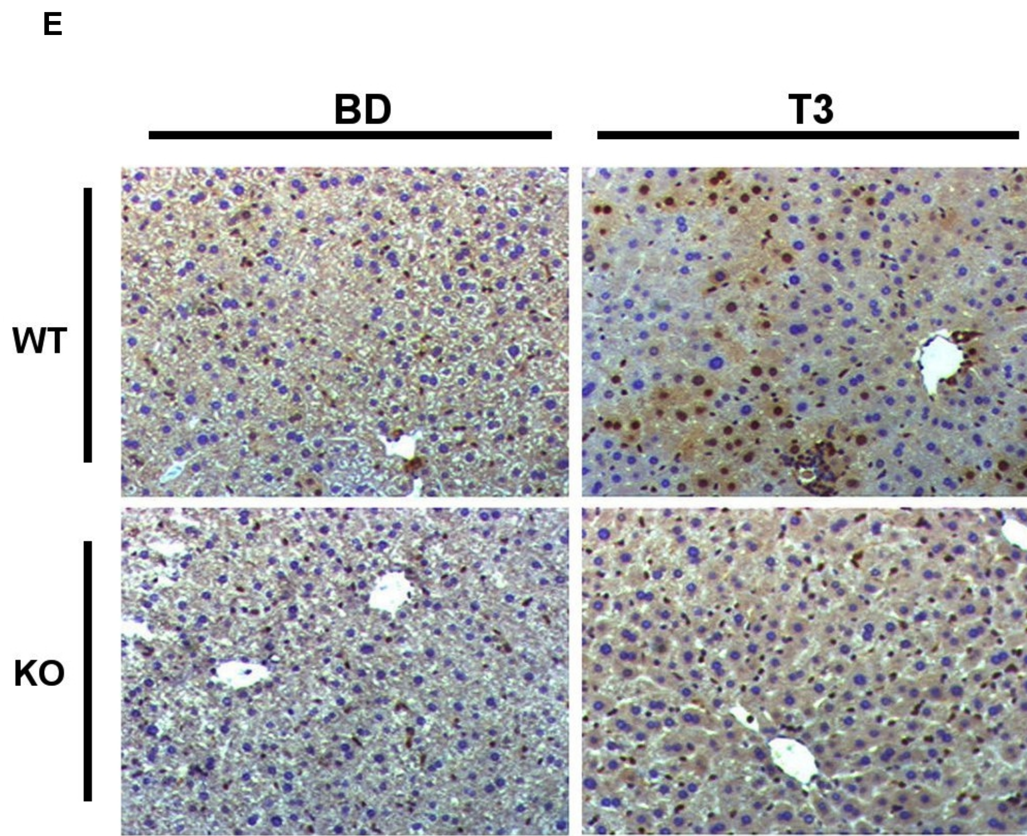

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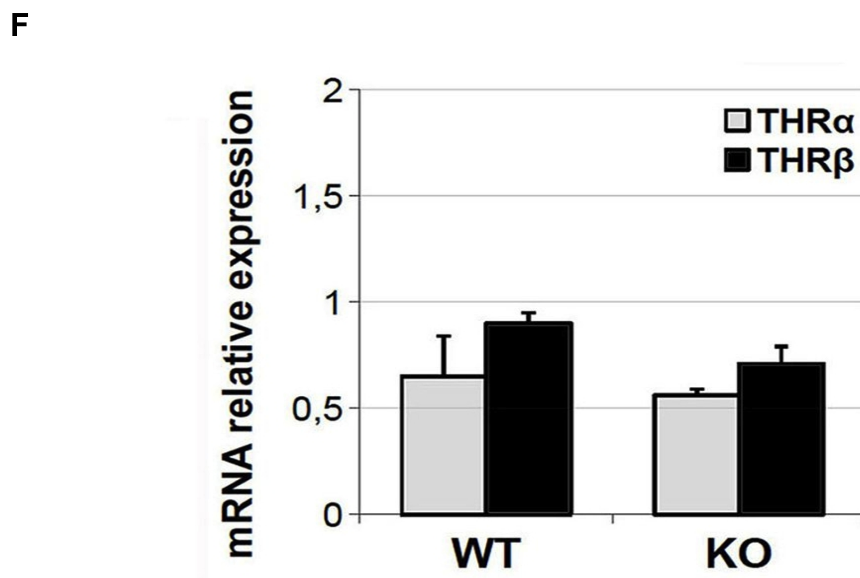

Figure 2. Lack of β-catenin in hepatocytes impairs hepatocyte proliferation and cyclin-D1 expression in response to T3 diet.

A) Representative microphotographs showing immunohistochemical staining for BrdU in the WT and KO livers after T3-treatment for 4 days (200X). Several BrdU-positive hepatocyte nuclei are observed in livers of WT mice fed T3 for 4 days. KO after T3 treatment lack BrdU staining in the hepatocytes although BrdU-positive non-parenchymal cells are evident, similar to WT livers.
B) A significant (*$p<0.05$) decrease in BrdU LI of hepatocytes in KO versus WT mice after T3 treatment. While T3 stimulated BrdU incorporation in the WT hepatocytes, no significant increase was evident in KO hepatocytes. At least 5000 hepatocyte nuclei per liver were scored. The LI is expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Results are expressed as mean ± standard error (S.E.) of 3 or more mice per group.
C) LI of pancreatic acinar cells in WT and KO mice shows no significant difference between the two groups following treatment with T3-supplemented diet (4 mg/kg) for 7 days. T3 stimulated BrdU incorporation in pancreatic acinar cells comparably and significantly (*$p<0.05$) in WT and KO. At least 2000 acinar cell nuclei per pancreas were scored. LI was expressed as number of BrdU-positive acinar cell nuclei/100 nuclei and results expressed as mean ± standard error (S.E.) of 3 or more

mice per group.

D) Representative microphotographs showing labeling of BrdU in the pancreas of WT and KO mice treated with T3 for 4 days (200X).

E) Representative microphotographs of cyclin-D1 staining in the WT and KO livers after 4 days of T3-feeding (200x). Absence of cyclin-D1 immunoreactivity in KO hepatocytes nuclei is clearly evident despite T3 feeding as compared to the WT.

F) qRT-PCR analysis of TRα and TR β expression in livers of untreated WT and KO mice. 18S was used as endogenous control. Error bars represent the standard error (S.E) of TaqMan RT-PCR performed in triplicates.

Figure 3.

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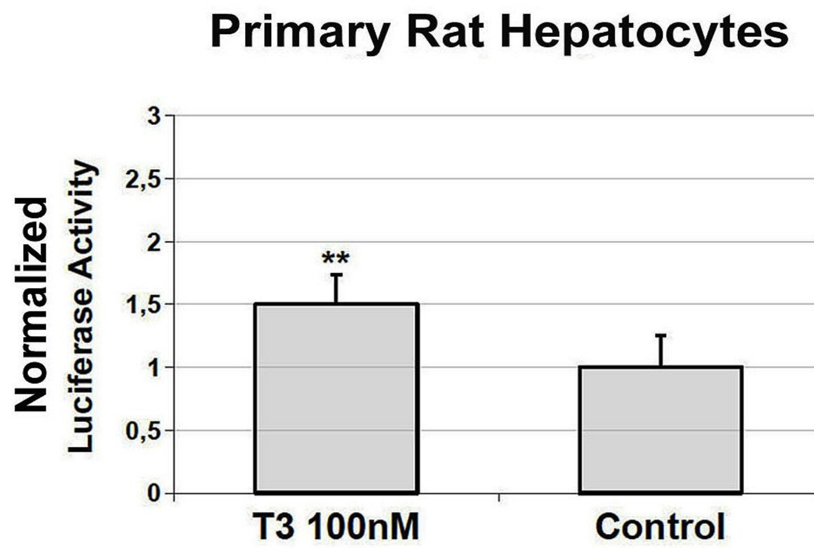

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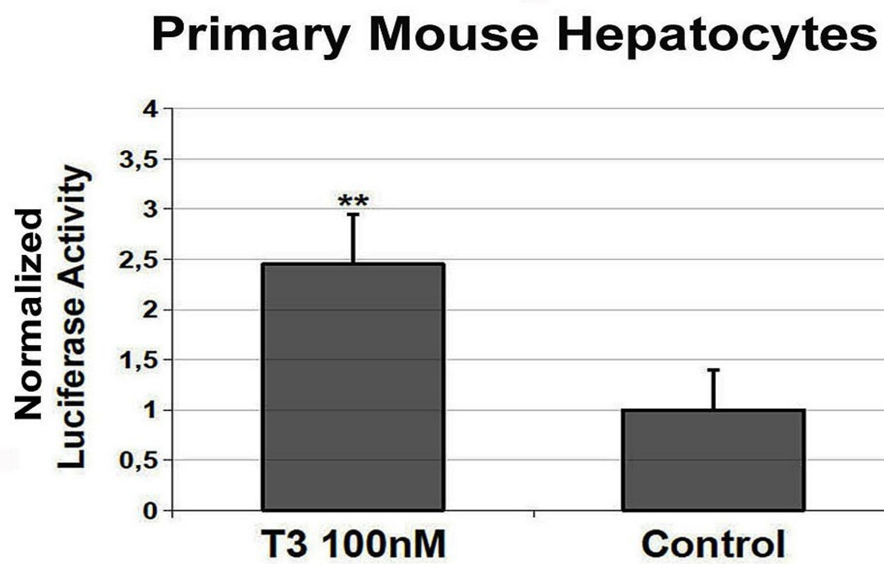

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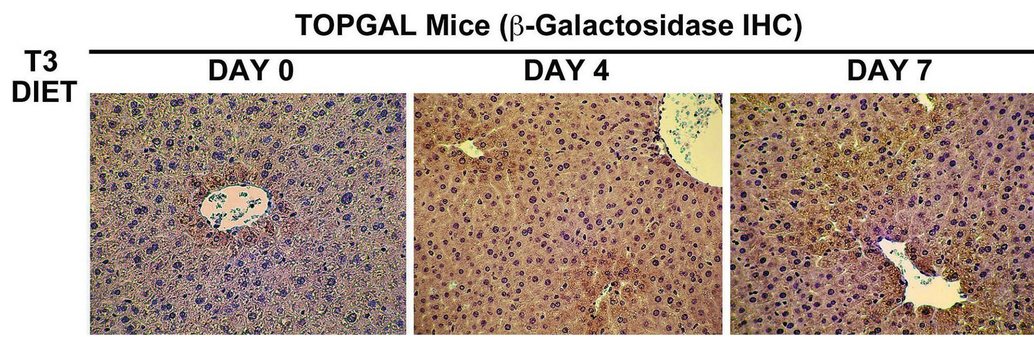

Figure 3. T3 induces β-catenin activation in vitro and in vivo.

A) TOPflash reporter assay shows an increase in luciferase activity 48 hours after T3 treatment of primary rat hepatocytes. A vector containing renilla luciferase was used as an internal control for transfection efficiency, and results are expressed as relative firefly/renilla luciferase activity. The results presented are the mean ± standard error (S.E.) for three experiments; $*p < 0.05$.

B) TOPflash reporter assay shows an increase in luciferase activity 48 hours after T3 treatment of primary murine hepatocytes. A vector containing renilla luciferase was used as an internal control for transfection efficiency, and results are expressed as relative firefly/renilla luciferase activity. The results presented are the mean ± standard error (S.E.) for three experiments; $*p < 0.05$.

C) IHC showing increased β-galactosidase expression in TOPGAL mice fed T3 diet for 4 and 7 days. At baseline β-galactosidase expression was detected in pericentral hepatocytes only, whereas T3 feeding led to widening of the expression to several hepatocytes layers around the central vein demonstrating an increase in the activity of β-catenin-TCF complex.

Figure 4.

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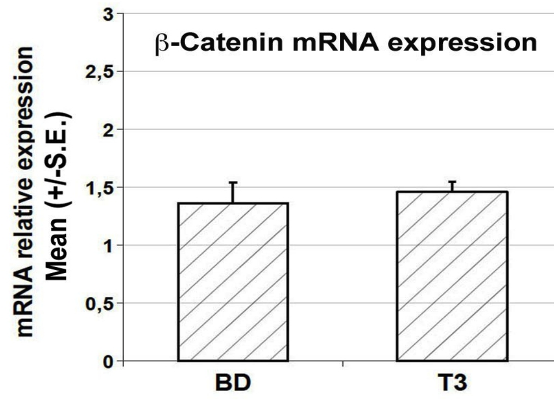

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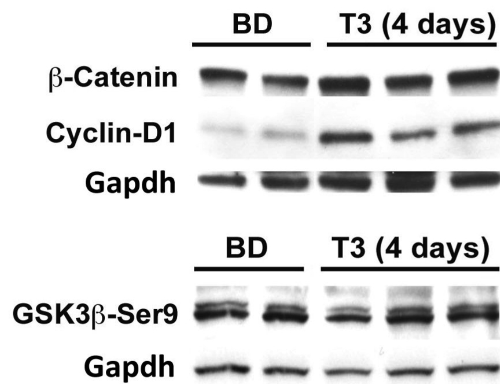

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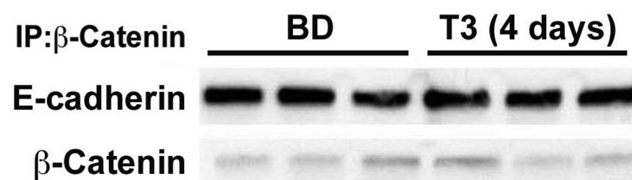

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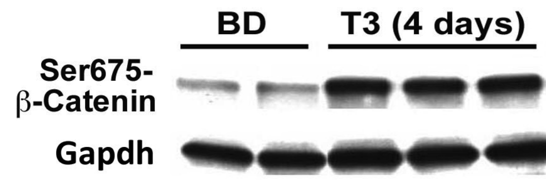

Figure 4. T3 induced β-catenin activation via Ser675-phosphorylation.

A) qRT-PCR analysis of β-catenin expression (normalized to cyclophilin A) in C57BL6 mice treated with T3 for 4 days shows no change. Error bars represent the standard error (S.E.) of TaqMan RT-PCR performed in triplicates.

B) Representative western blots show T3 feeding for 4 days does not lead to a notable increase in total β-catenin when compared to basal diet fed mice. However cyclin-D1 levels are consistently increased. GSK3β-Ser9 levels remain unaffected at 4 days of T3 treatment. Gapdh verifies equal loading. (Each lane represents a single sample).

C) Immunoprecipitation studies from three representative livers show no change in association of β-catenin and E-cadherin in the livers of 4 days T3- versus basal diet-fed C57BL/6 mice.

D) Representative western blot shows a noteworthy increase in pSer675-β-catenin levels in 4 days T3-fed as compared to control diet-fed C57BL/6 mice. Gapdh verified equal loading.

Figura 5.

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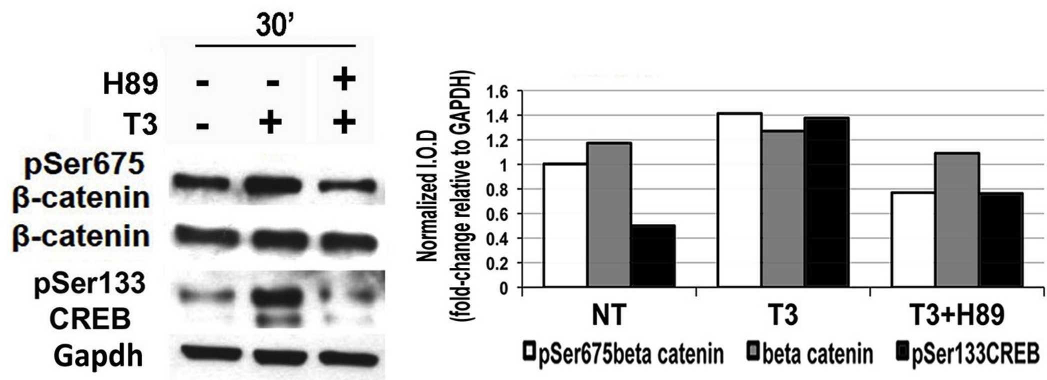

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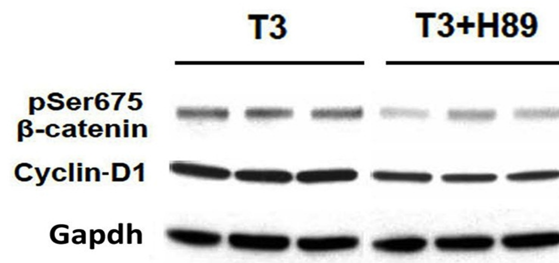

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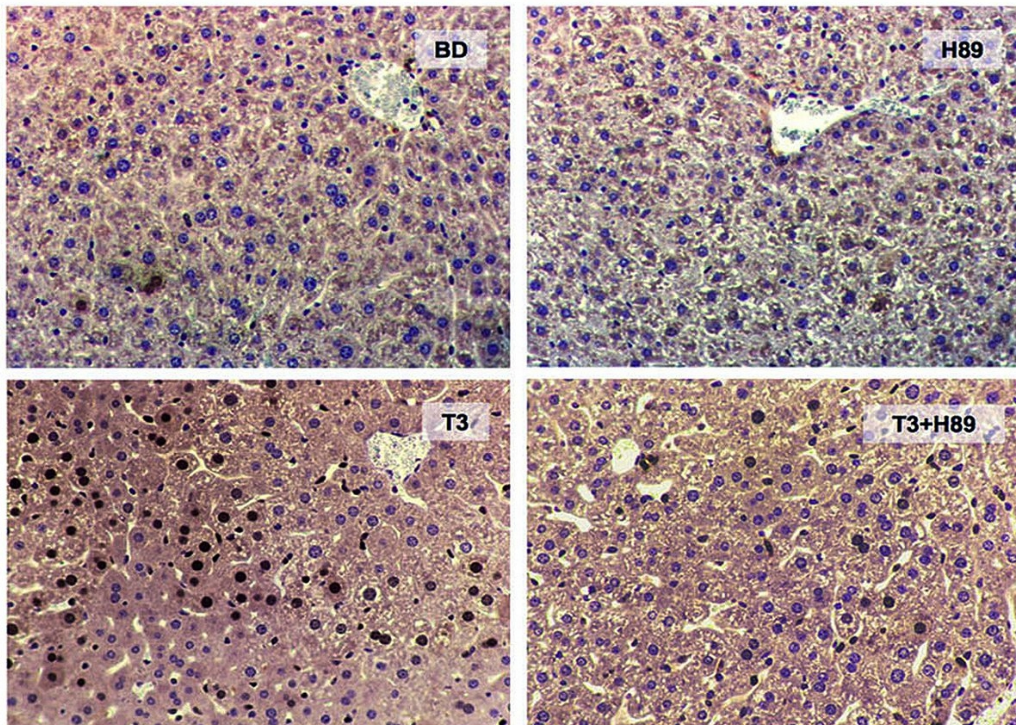

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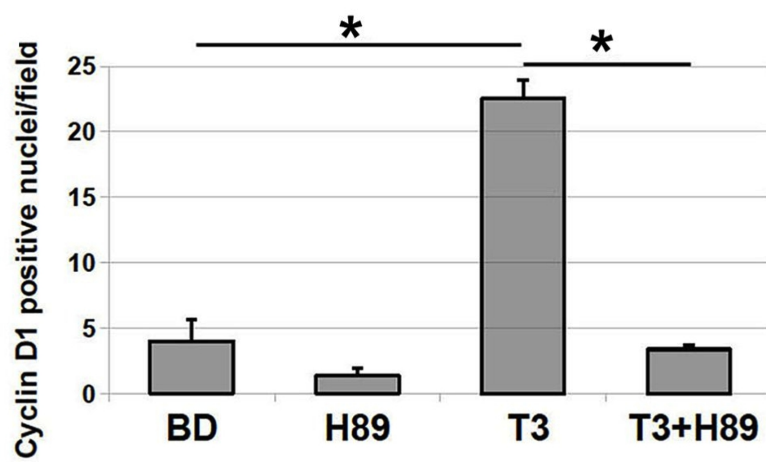

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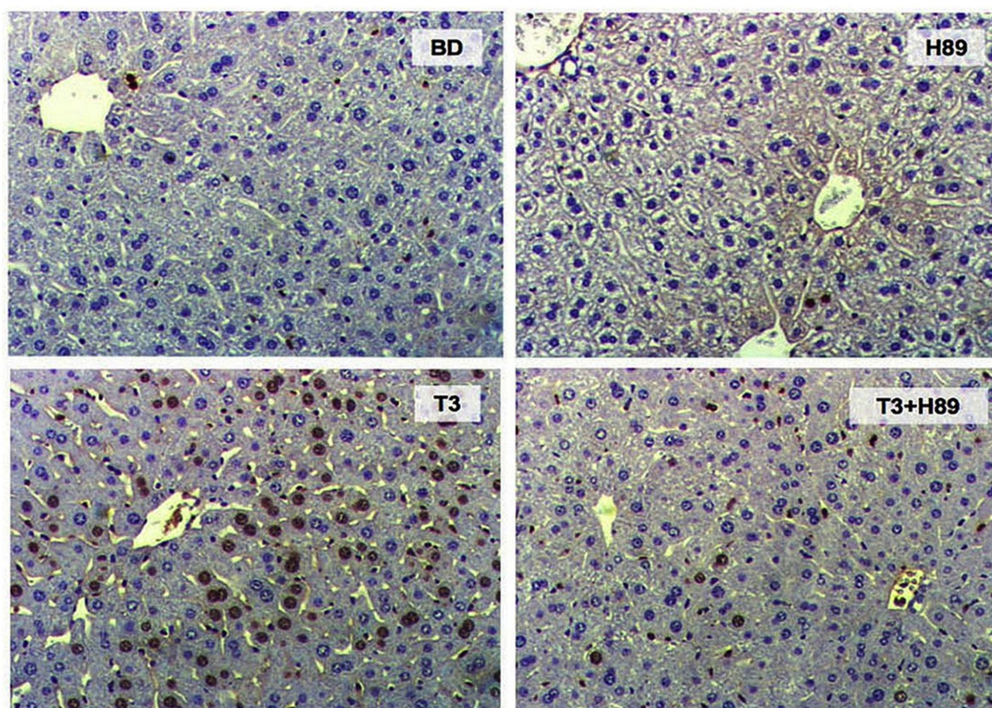

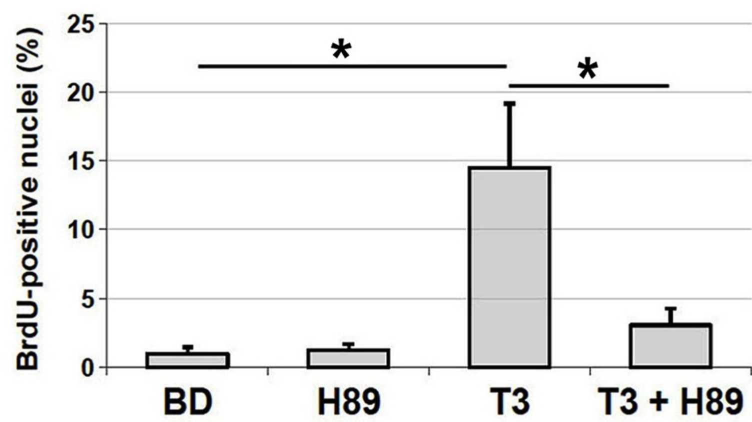

Figure 5. Blockade of Protein Kinase A impairs T3's effect on β-catenin, cyclin-D1 and hepatocyte proliferation in mice.

A) A representative western blot using pooled samples from three wells per condition (upper panel) shows increased levels of pSer675-β-catenin and pSer133-CREB in primary mouse hepatocytes after 30 minutes of T3 treatment. Inclusion of PKA inhibitor H89 (100nM) in the media 30 minutes prior to the addition of T3 (100nM) showed a notable decrease in pSer675-β-catenin and pSer133-CREB levels. Densitometry on the representative WB (lower panel) shows an increase in pSer675-β-catenin and pSer133-CREB after T3 treatment, which was blocked by H89 treatment. (I.O.D. – integrated optical density).

B) A representative western blot shows a decrease in the hepatic levels of pSer675-β-catenin and cyclin-D1 when H89 was injected twice IP in 3-day T3 fed mice as compared to 3 day T3 only group. Gapdh verifies equal loading. Each lane represent a single sample.

C) A representative micrograph (200x) illustrates a decrease in the number of cyclin-D1-positive hepatocytes when H89 was injected twice to the 3-day T3-fed mice as compared to T3 only group (upper panels). Quantification of the cyclin-D1-positive hepatocytes shows a significant decrease in positive cells in H89+T3 as compared to T3 only group (*$p<0.05$) (lower panel). Three or more mice /group were used.

D) BrdU LI was after day of T3-feeding was significantly increased as

compared to basal diet or H89 treatment (*$p<0.05$). BrdU LI was lower in livers when H89 was injected twice to the 3-day T3-fed mice as compared to T3 only group, however it missed statistical significance due to variation in the number of BrdU-positive hepatocytes in the T3 only group. Three or more mice /group were used.

E) A representative microphotographs (200x) illustrates a noteworthy increase in BrdU uptake by the hepatocytes in mice after 5 days of T3 feeding, which was dramatically decreased in animals simultaneously administered H89 IP every 24 hours (left panels). Quantification of BrdU positive hepatocytes shows a significant (*$p<0.05$) decrease in the LI in T3+H89 group as compared to T3 only. Three mice/group were used.

Figure 6.

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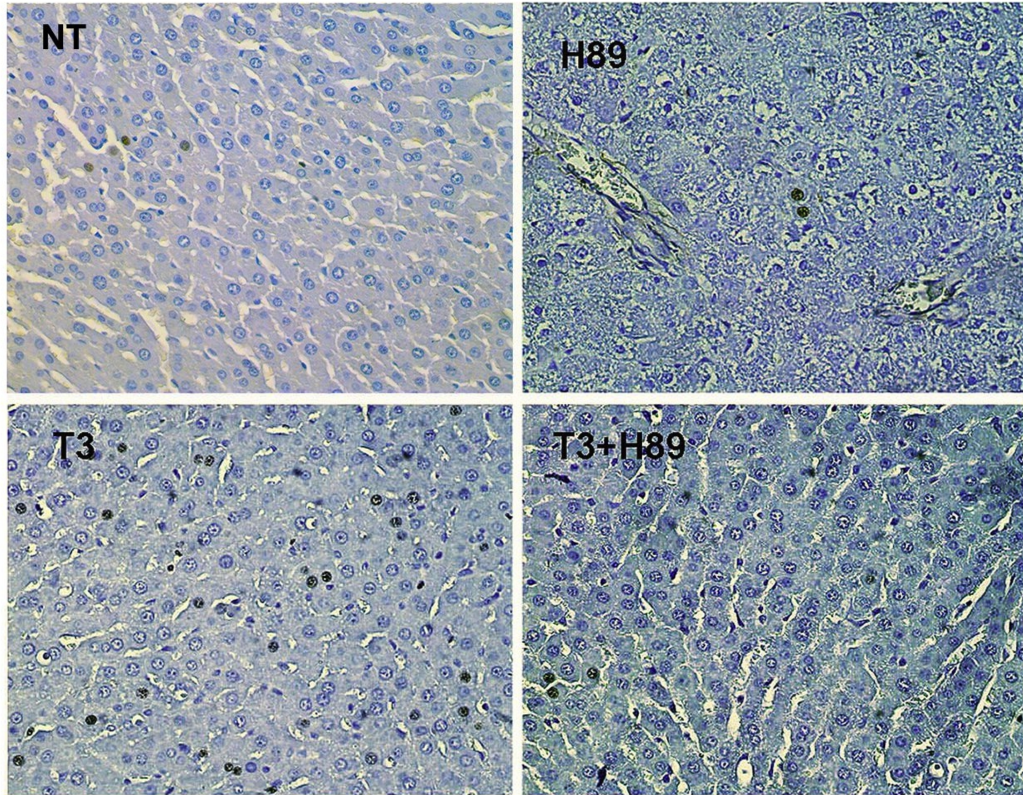

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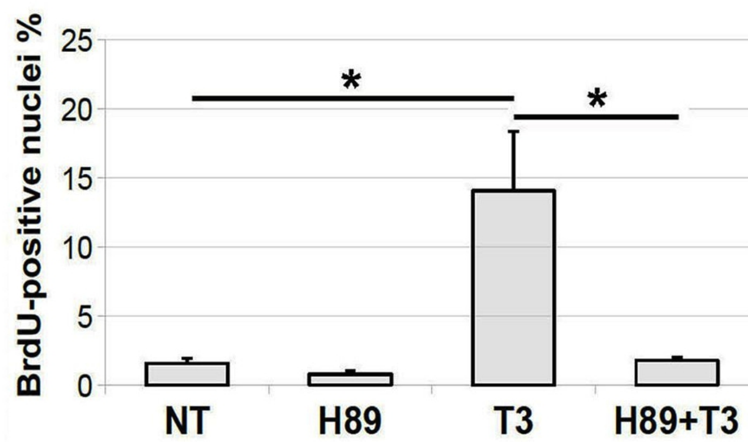

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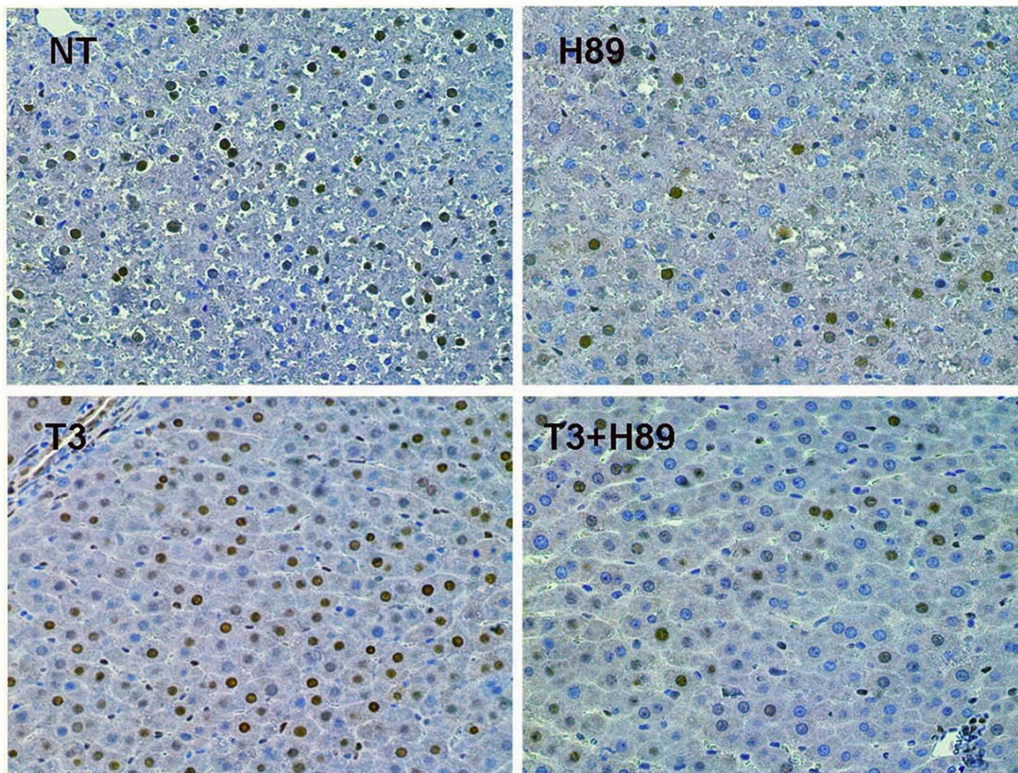

D

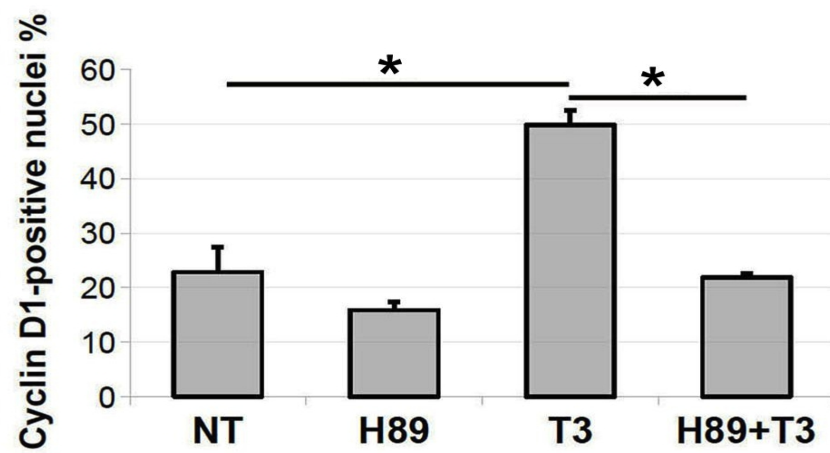

Figure 6. Blockade of Protein Kinase A impairs T3's effect on β-catenin, cyclin-D1 and hepatocyte proliferation in rats

A) Representative microphotographs (200x) illustrate the effect of H89 on T3-induced rat hepatocyte proliferation by BrdU immunohistochemistry. H89 was given 1 hour prior to a single dose of T3 (20 ug/100 g) and the rats were sacrificed 24 hours later.

B) Quantification of BrdU positive hepatocytes in A shows a significant (*$p<0.05$) increase in the LI after T3 treatment, which was significantly abrogated in the presence of H89 (*$p<0.05$). Four to five rats/group were used.

C) Representative microphotographs (200x) show increased nuclear cyclin-D1 expression in hepatocyte following a single injection of T3, which was decreased in the group that simultaneously received H89 as well.

D) Quantification of cyclin-D1-positive hepatocytes to calculate LI shows a significant increase after T3 treatment (*$p<0.05$), which was reduced significantly in the H89 pretreatment group (*$p<0.05$). Four to five rats/group were used.

E) A representative western blot shows the effect of H89 on T3-induced Ser675-β-catenin levels in rat liver. Gapdh depicts protein loading.

F) Densitometric analysis of E (Ser675-β-catenin normalized to Gapdh) using the ImageJ software shows a significant (*$p<0.05$) decrease in

Ser675-β-catenin levels in T3+H89 as compared to T3 only group. (I.O.D. – integrated optical density).

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