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β-Catenin Regulates Vitamin C Biosynthesis and Cell Survival in Murine Liver*

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Because the Wnt/β-catenin pathway plays multiple roles in liver pathobiology, it is critical to identify gene targets that mediate such diverse effects. Here we report a novel role of β-catenin in controlling ascorbic acid biosynthesis in murine liver through regulation of expression of regucalcin or senescence marker protein 30 and L-gulonolactone oxidase. Reverse transcription-PCR, Western blotting, and immunohistochemistry demonstrate decreased regucalcin expression in β-catenin-null livers and greater expression in β-catenin overexpressing transgenic livers, HepG2 hepatoma cells (contain constitutively active β-catenin), regenerating livers, and in hepatocellular cancer tissues that exhibit β-catenin activation. Interestingly, coprecipitation and immunofluorescence studies also demonstrate an association of β-catenin and regucalcin. Luciferase reporter and chromatin immunoprecipitation assays verified a functional TCF-4-binding site located between −163 and −157 (CTTTGCA) on the regucalcin promoter to be critical for regulation by β-catenin. Significantly lower serum ascorbate levels were observed in β-catenin knockout mice secondary to decreased expression of regucalcin and also of L-gulonolactone oxidase, the penultimate and last (also rate-limiting) steps in the synthesis of ascorbic acid, respectively. These mice also show enhanced basal hepatocyte apoptosis. To test if ascorbate deficiency secondary to β-catenin loss and regucalcin decrease was contributing to apoptosis, β-catenin-null hepatocytes or regucalcin small interfering RNA-transfected HepG2 cells were cultured, which exhibited significant apoptosis that was alleviated by the addition of ascorbic acid. Thus, through regucalcin and L-gulonolactone oxidase expression, β-catenin regulates vitamin C biosynthesis in murine liver, which in turn may be one of the mechanisms contributing to the role of β-catenin in cell survival.

The Wnt/β-catenin signal transduction pathway, which is essential for normal development and tissue regeneration, also has a well characterized role in tumorigenesis in many tissues (1). Mutations in β-catenin lead to aberrant signaling, which causes stabilization and nuclear translocation of β-catenin, along with subsequent activation of target genes such as c-*myc* and cyclin D1 (2–4). In the liver, mutations in the β-catenin gene are primarily involved in the pathogenesis of HCC[2] (5, 6). To fully comprehend the role and extent of Wnt/β-catenin signaling in the liver, it will be helpful to identify novel targets of this pathway. Regucalcin, also known as SMP30 (senescence marker protein-30) (7), is a Ca^{2+}-binding protein that has been implicated in cell homeostasis and function (8). Regucalcin activates plasma membrane Ca^{2+}-pumping ATPases to regulate the cytosolic calcium levels (9, 10). Regucalcin may also regulate the transcriptional process by binding protein and DNA in the nucleus (11). Much of the characterization of regucalcin has occurred in the context of the liver. Regucalcin was shown to play an important role in the regulation of Ca^{2+} signaling in the proliferating cells of regenerating rat liver (8, 12). During regeneration, regucalcin has been suggested to suppress protein synthesis (13) and DNA synthesis activity (14, 15). The human hepatoma cell line HepG2 expresses regucalcin, and its expression could be further stimulated by insulin (16). Regucalcin overexpression also had a suppressive effect on apoptosis induced by tumor necrosis factor-α or thapsigargin (17). More recently, the role of regucalcin or SMP30 was reported as a gluconolactonase (18). Conversion of L-gulonate to L-gulonolactone is the penultimate step in the biosynthesis, the final step being oxidation to L-ascorbic acid by L-gulonolactone oxidase (19). It should be noted that it is the inactivity of the latter enzyme due to mutations that has led to the inability of humans, guinea pigs, and other species to synthesize vitamin C. In summary, regucalcin is thought to have a diverse role in proliferation, survival, and differentiation of cells (12).

Our laboratory reported the characterization of the β-catenin conditional knock-out animals, which is an excellent resource for identification of novel targets of β-catenin in the liver (20). Regucalcin was identified as one of the genes most affected by the loss of β-catenin. Here we report that regucalcin or SMP30 is indeed a downstream target of β-catenin signaling in the liver. The expression of SMP30 mirrored the activation of β-catenin during liver growth, regeneration, and in hepatocellular cancer. The promoter region of regucalcin was also characterized for β-catenin/TCF4 binding. Finally, we addressed the role of regucalcin as a target of Wnt/β-catenin signaling in the hepatocyte, which along with L-gulonolactone oxidase plays an important role in vitamin C biosynthesis and also regulates

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[2] The abbreviations used are: HCC, human hepatocellular carcinoma; RT, reverse transcription; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; WT, wild type; KO, knock-out; FBS, fetal bovine serum; IHC, immunohistochemistry; TUNEL, deoxynucleotidyltransferase-mediated dUTP nick-end labeling; NAC, *N*-acetylcysteine; PHx, partial hepatectomy; GS, glutamine synthetase; EMEM, Eagle's minimum essential medium; TCF, T cell factor.

cell survival. Thus, regulation of SMP30 and L-gulonolactone oxidase expression by β-catenin contributes to a broader role of Wnt/β-catenin in liver homeostasis.

EXPERIMENTAL PROCEDURES

Tissues, Animals, and Cell Lines—Nineteen HCC from two different groups were used for this study. The first group (group 1, $n = 9$) had known activating β-catenin mutations (21), and for the second group (group 2, $n = 10$), the β-catenin mutational status was unknown. Normal livers (from donor liver tissue) were available from four cases. Tissues from first group were processed for mRNA isolation and RT-PCR, whereas samples from the second group were processed for histology.

Mouse models used in this study include a conditional β-catenin knock-out described previously (20) and a stable β-catenin overexpressing transgenic mouse similar to one described previously (22). For the conditional knock-out, homozygous floxed β-catenin mice were bred to albumin-cre mice (both on a C57BL/6 background); the resulting mice, after another backcrossing to homozygous floxed β-catenin mice, resulted in conditional null mice with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{+/-}$ (referred to in this paper as knock-out or KO mice) and controls with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{-/-}$ or $Ctnnb1^{loxp/Wt}$; and $Alb\text{-}Cre^{-/-}$ (referred to in this paper as wild-type or WT mice). The mice were sacrificed at 1-, 3-, and 6-month time points, and the livers were utilized for subsequent analysis. The β-catenin overexpressing transgenic mice (FVB strain) were generated similar to the albumin/β-catenin transgenic mice characterized previously (22), except in this strain, β-catenin carries a point mutation at serine 45 thus rendering it more stable overall.

Rat liver tissue was obtained from partial hepatectomy studies, as described previously (20). Frozen livers from partial hepatectomy studies were harvested for protein.

For hydrodynamic delivery of naked DNA, 1 μg/g of body weight of Wnt-1 plasmid (Millipore, Billerica, MA) or pcDNA3 control plasmid (Invitrogen) was administered via hydrostatic tail vein injection to CD-1 mice weighing around 18 g, as described previously (23, 24). Eighteen hours after the injection, the mice were subjected to PHx. Mice were sacrificed at 30 h after PHx, and livers were processed for total and nuclear protein. Cytoplasmic and nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagent kit as per the instructions (Thermo Fisher Scientific, Rockford, IL).

Hep3B and HepG2 human hepatoma cell lines described previously (25) were obtained from American Type Culture Collection (Manassas, VA) and cultured in EMEM (Cambrex, East Rutherford, NJ) with 10% FBS (Mediatech, Herndon, VA) in a humidity-saturated incubator with 5% CO_2 maintained at 37 °C.

mRNA Isolation and Real Time PCR—mRNA was isolated and purified from 4- to 7-month-old WT and AFP/Alb/Ctnnb1 KO mice frozen livers using TRIzol (Invitrogen). RT-PCR was performed as described elsewhere (26). The forward (F) and reverse (R) primers for mouse regucalcin and L-gulonolactone oxidase were selected using Primer Express software (PE Applied Biosystems) and produce a 413-bp product. Regucalcin F was 5′-GAT GCC CCA GTC AGT TCA GT-3′ and R was 5′-ATT CTG CGG TTG GAA ATC TG-3′; L-gulonolactone oxidase F was 5′-AAA ACT GGG CGA AGA CCT ATG-3′ and R was 5′-CCT TGT TGG TGT GAT CTT GGT-3′; β-actin primers were used as an internal control as F 5′-CAG CTG AGA GGG AAA TCG TG-3′ and R 5′-CGT TGC CAA TAG TGA TGA CC-3′ and produced a 150-bp product. PCR amplification was carried out as follows: initial denaturation at 94 °C for 2 min, followed by 35 or 28 cycles (for semi-quantitative PCR) of denaturation at 94 °C for 1 min, annealing 55 °C for 1 min, 72 °C for 1 min, and 10 min of final extension at 72 °C.

For real time PCR, following DNase treatment, mRNA was converted to cDNA (23). Real time PCR was performed as described previously (27). TaqMan Gene Expression Assays for human regucalcin and β-actin were obtained from Applied Biosystems (Foster City, CA). The standard conditions used for real time PCR were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 15 s of denaturation at 95 °C and 1 min of annealing/elongation at 60 °C. TaqMan signal (Applied Biosystems, Foster City, CA) was measured in each step. Changes in regucalcin mRNA were normalized to actin mRNA for each sample and presented as fold-change over the average from four normal livers. In hepatoma cell lines, the expression of regucalcin gene was expressed as the $\Delta\Delta Ct$ method (28). Each sample was run in triplicate.

Protein Extraction, Western Blots, and Immunoprecipitation—Whole-cell lysates from mouse livers, cell cultures, and human tumor tissues were prepared and assayed in RIPA buffer as described elsewhere (29). Immunoprecipitation studies with regucalcin/SMP30 antibody and β-catenin antibody were done using standard protocol discussed elsewhere (29). Proteins were electrophoresed and blotted as described previously (29). Membranes were stained with Ponceau-S solution to confirm transfer and equal loading. Primary antibodies used in this study were against regucalcin/SMP30 (K-18 used at 1:500; C-16 used at 1:150), β-catenin (used at 1:200), GS (used at 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), and actin (used at 1:5000) (Chemicon, Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies used in the study included goat anti-mouse (1:10,000), donkey anti-rabbit (1:10,000), and donkey anti-goat (1:2500) (Chemicon, Temecula, CA). Proteins were detected by application of the Super-Signal West Pico chemiluminescent substrate to the membranes (Pierce). Blots were stripped with IgG elution buffer (Pierce) for re-probing. Densitometry analysis was performed using Image J version 1.38. All experiments were performed in triplicate, and representative results are shown.

Immunohistochemistry (IHC) and Immunofluorescence—IHC on paraffin-embedded sections was performed on mouse livers and human tumors as described elsewhere (30). Primary antibodies used were against β-catenin (1:200), SMP30/regucalcin (1:100), GS (1:200) (Santa Cruz Biotechnology), and β-catenin (1:50) (BD Biosciences). Secondary antibodies were horse anti-mouse (Vector Laboratories, Inc., Burlingame, CA), goat anti-rabbit, and donkey anti-goat (Chemicon, Temecula, CA), all used at a 1:500 dilution. IHC was performed using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burl-

TABLE 1
Primer pairs used in the construction of regucalcin promoter fragments

	Forward primer	Reverse primer
A fragment, −864/+102	5′-GATCATGAGCTCGTAGAGACGGGGTTTCACCA-3′	5′-TCTAGACTCGAGCAGTCGCCTTCTGGGAGAT-3′
B fragment, −864/−307	5′-GATCATGAGCTCGTAGAGACGGGGTTTCACCA-3′	5′-TCTAGACTCGAGTTAAACCCAAACAGGTTAAAAAG-3′
C fragment, −676/+102	5′-GATCATGAGCTCTCACTTTGTTGCCAGGTTCA-3′	5′-TCTAGACTCGAGCAGTCGCCTTCTGGGAGAT-3′
D fragment, −306/+102	5′-CATAGTGAGCTCCCGTCCCAGTCTCTCTCTGT-3′	5′-TCTAGACTCGAGCAGTCGCCTTCTGGGAGAT-3′
E fragment, −103/+102	5′-CATAGTGAGCTCCATGCCATCCACCTGCCAAGC-3′	5′-TCTAGACTCGAGCAGTCGCCTTCTGGGAGAT-3′

ingame, CA) and developed using either 3,3′-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA) or 3-amino-9-ethylcarbazole substrate (Scytek Laboratories, Logan, UT). The slides were counterstained with aqueous Gill's hematoxylin and mounted with either crystal mount or cytoseal (Biomeda Corp., Foster City, CA). For β-catenin, GS, and regucalcin expression in HCC, each sample was allocated an arbitrary IHC score on the basis of the intensity of the expression of β-catenin, GS, and regucalcin in the nucleus and/or cytosol as compared with the normal liver samples. Each stain was scored as − (absent staining), + (positive, similar to normal), and ++ (strongly positive or greater than normal). For β-catenin IHC, even a subset of HCC cells showing nuclear and/or cytoplasmic localization was assigned + score.

Coverslip-plated HepG2 or Hep3B hepatoma cells were processed for immunofluorescence as described previously (31). Primary antibodies against β-catenin (1:100) and SMP30 (also known as regucalcin) (1:100) (Santa Cruz Biotechnology) were utilized. Secondary antibodies included donkey anti-goat Alexa 488 and donkey anti-mouse Cy3 at either 1:500 or 1:1000 dilutions (Molecular Probes, Eugene, OR). Hoechst stain was used as nuclear counter stain. All fluorescence labeling was imaged under Zeiss Axiovert 40 CFL microscope, and photographs were obtained by Zeiss Axiocam MRC5 digital camera.

Construction of the Reporter Gene Plasmid—The reporter gene plasmids were generated by cloning restriction fragments, which were isolated from the 5′-flanking region of the human regucalcin gene. The DNA fragments have an average size of 900 bp and typically cover the sequence from 864 bp upstream to 102 bp downstream of the transcription start site (*Homo sapiens* regucalcin mRNA, NM_004683) in *H. sapiens* chromosome X (NC_000023). The genomic DNA was extracted for normal human liver tissues using DNAzol (Molecular Research Center Inc., Cincinnati, OH) as described previously (32). Genomic DNA was used as a template for PCR amplification, and primer pairs were used to obtain the following eight fragments: A fragment, −864/+102 (four TCF-4E-binding sites); B fragment, −864/−307 (three TCF-4E-binding sites); C fragment, −676/+102 (one TCF-4E-binding site); C1 fragment, the C fragment cut with SmaI, which removes 109 bp (from −180 to −71) (no TCF-4E-binding sites); D fragment, −306/+102 (one TCF4E-binding site); D1 fragment, the D fragment cut with SmaI, which removes 109 bp (from −180 to −71) (no TCF-4E-binding sites); E fragment, −103/+102 (no TCF-4E-binding sites); control, no DNA fragment (luciferase only). Primers for all the fragments are shown in Table 1. The sense primers were designed to contain a SacI restriction enzyme cutting site (GAGCTC) and the antisense primers to contain an XhoI restriction enzyme cutting site (CTCGAG). After PCR amplification and digestion with the restriction enzymes, the DNA fragments were purified and ligated into the pGL3-Basic vector containing the firefly luciferase gene. Further digestion with SmaI was used to create the C1 and D1 fragments.

For the transfection experiments, human HepG2 cells were grown on 6-well plates to ~70% confluence and transiently transfected. A total of 2 μg of the pGL3-Basic vector containing the DNA fragments was cotransfected with the internal control reporter *Renilla reniformis* luciferase into HepG2 cells using FuGENE HD reagent (Roche Applied Science) (33). 48 h after transfection, luciferase activity was assayed using the Dual Luciferase assay system kit according to the manufacturer's protocol (Promega, Madison, WI). Relative luciferase activity (arbitrary units) was reported as fold induction after normalization for transfection efficiency.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed according to the chromatin immunoprecipitation kit manual (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, HepG2 and Hep3B cells (2×10^7 cells) were fixed with 1% formaldehyde and then neutralized by adding 0.125 M glycine. Cells were collected and lysed in Cell Lysis buffer and then sonicated to obtain soluble chromatin with an average length of 1000 bp. After a 1:10 dilution, chromatin solutions were precleared by protein G-agarose and then incubated with no antibody (control), anti-β-catenin antibody (Santa Cruz Biotechnology), or anti-TCF-4 antibody (Upstate) at 4 °C overnight on a rotating platform. The immune complexes were recovered with protein G-agarose. After extensive washing, the bound DNA fragments were eluted and subjected to PCRs using the following primer pairs located in the regucalcin promoter: 5′-CCG TCC CAG TCT CTC TCT GT-3′ (−306 to −287) and 5′-CCG CGG CTG GAA GAA TCC TG-3′ (−139 to −158). The products were separated by electrophoresis on a 2% agarose gel and visualized.

Primary Hepatocyte Culture—Hepatocytes from β-catenin KO mice were isolated as described previously and seeded on chamber slides wet-coated with collagen at a density of 100,000 cells per ml in minimal essential media supplemented with 10% FBS (34). After 2 h, media were changed to a chemically defined minimal essential media-based growth medium containing insulin/transferrin/selenium (1 g/liter), dexamethasone (10^{-7} M), hepatocyte growth factor (40 ng/ml), and epidermal growth factor (25 ng/ml) as described elsewhere (35). The cells were cultured for 48 h in the presence or absence of ascorbic acid at 0.2 mM final concentration (36). TUNEL analysis is described below.

Rat hepatocytes were isolated and plated as discussed elsewhere (35). After 2 h, media were replaced with basal media and 5 ng/ml recombinant mouse Wnt3a (R & D Systems, Minneapolis, MN) or phosphate-buffered saline. Plates were harvested for nuclear protein 24 h after treatment.

Transfection of siRNA—Hep3B and HepG2 human hepatoma cell lines were seeded onto either 100-mm dishes or 6-well plates and maintained until the cells reached 50–60% confluence in EMEM (ATCC, Manassas, VA) with 10% FBS. Two independent siRNAs were utilized for transfection. Cells were transfected with siRNA purchased from either Dharmacon (Lafayette, CO) or Applied Biosystems (Foster City, CA). 100 nM control siRNA or siRNA against human regucalcin mRNA from Dharmacon was transfected into cells using Oligofectamine transfection reagent (Invitrogen) in the absence of serum for 4 h. After incubation with siRNA, EMEM with 10% FBS was added to the cells without removal of the siRNA mixture. Media were changed 24 h after transfection, and fresh EMEM with 10% FBS was added. Plates were harvested for protein 48 h after addition of siRNA. Cells were transfected with 5 nM of either control siRNA or siRNA against human regucalcin mRNA from Applied Biosystems using Lipofectamine 2000 transfection reagent (Invitrogen) in the presence of serum. Media were changed 24 h after transfection, and fresh EMEM with 10% FBS was added. Plates were harvested for protein and RNA 48 h after addition of siRNA. Experiments were done in triplicate, and representative results are presented.

Cell Growth and Apoptosis Assays—A [^{3}H]thymidine uptake assay was performed in Hep3B and HepG2 cells after siRNA treatments as described previously to measure cell proliferation (37).

HepG2 cells cultured on chamber slides were dosed with either control or with human regucalcin siRNA in the presence or absence of ascorbic acid at 0.2 mM final concentration or of *N*-acetylcysteine (NAC) at 20 mM final concentration in 10× phosphate-buffered saline as reported elsewhere (36, 38). 48 h after treatment, slides were fixed in a 3:1 solution of methanol/glacial acetic acid for 5 min and allowed to air-dry. The detection of apoptotic nuclei was determined by terminal TUNEL staining using the ApopTag peroxidase kit (Intergen Co., Purchase, NY) as described previously (39). The numbers of positive cells per field were counted, for a total of five fields per condition. Assays were done in triplicates, and statistical assessment for significance was performed by the Student's *t* test, and a *p* value of less than 0.05 was considered significant.

Serum Ascorbate Estimation—Sera from age- and sex-matched WT and β-catenin KO mice ($n = 3$) were utilized to determine ascorbate levels with the ascorbic acid assay kit II (Bio-Vision, Mountain View, CA) according to the manufacturer's instructions.

RESULTS

Regucalcin Protein and mRNA Expression Correlates with β-Catenin Expression—Livers from 1-, 3-, and 6-month-old WT controls and KO mice were used for isolation of mRNA, protein, and for IHC. Gene array analysis of β-catenin knockout mice generated using three different Cre lines reveals a severalfold decrease in gene expression for regucalcin in β-catenin KOs as compared with their wild-type counterparts (Table 2) (20, 23, 40). Semi-quantitative RT-PCR confirms a decrease in regucalcin mRNA in the KO livers as compared with the WT (Fig. 1*A*). Deletion of β-catenin also leads to a decreased expression of regucalcin protein as seen at 3 and 6 months (Fig. 1*B*) and coincides with loxP recombination driven by albumin-cre recombinase (41).

TABLE 2
Absolute expression of regucalcin and L-gulonolactone oxidase in β-catenin knock-out mice generated using three different Cre lines

Conditional β-catenin KO	Stage (age)	RGN gene expression WT	RGN gene expression KO
Ctnnb1$^{loxp/loxp}$; FoxA3-Cre	E16	5362	289
Ctnnb1$^{loxp/loxp}$; Alb-Cre	3 months	25,218	4418
Ctnnb1$^{loxp/loxp}$; Afp-Alb-Cre	3 months	41,038	1366
Conditional β-catenin KO	**Stage (age)**	**L-Gulonolactone oxidase expression WT**	**L-Gulonolactone oxidase expression KO**
Ctnnb1$^{loxp/loxp}$; FoxA3-Cre	E16	1214	595
Ctnnb1$^{loxp/loxp}$; Alb-Cre	3 months	4761	336
Ctnnb1$^{loxp/loxp}$; Afp-Alb-Cre	3 months	6698	309

IHC detected regucalcin protein in the nucleus and cytosol of WT hepatocytes (C57BL/6) in all zones, although with a clear accentuation in centrizonal and sometimes midzonal areas (Fig. 1*C*). However, this expression was markedly diminished in the KO livers with only a few cells around the central vein showing leftover positivity for regucalcin (Fig. 1*C*). Conversely, low levels of nuclear and cytoplasmic regucalcin were observed in normal FVB hepatocytes, and where regucalcin was clearly less pronounced than normal WT livers of C57BL/6 mice, it was mostly localized to centrizonal areas only and absent periportally (Fig. 1*D*). However, a noteworthy widening of regucalcin localization was observed around the centrizonal areas in β-catenin overexpressing transgenic livers with continued periportal sparing (Fig. 1*C*). Thus regucalcin expression mimicked β-catenin levels in murine livers.

Regucalcin Levels Correlate with β-Catenin Target GS in the Rat Partial Hepatectomy Model—Previously, our laboratory has shown temporal changes in β-catenin expression during liver regeneration in rats after PHx (29). A transient increase in β-catenin protein was accompanied by its nuclear translocation for up to 48 h during rat liver regeneration. We used this model of liver regeneration to determine whether regucalcin expression correlated with levels of GS, a known target of β-catenin (42). Fig. 2*A* shows an increase in regucalcin protein expression in the rat as early as 1 h following partial hepatectomy. Regucalcin then decreases at 12 h but rises again at day 1 post-PHx, suggesting a cyclic regulation of expression. This pattern coincides with GS expression during liver regeneration.

Human Hepatocellular Carcinomas Have Increased Levels of Regucalcin Compared with Normal Liver—Next, we examined human hepatocellular carcinomas for regucalcin expression in relation to β-catenin status. Two different groups used in the study are described under "Experimental Procedures." Group 1 HCC samples with known activating mutations in the β-catenin gene (21) showed a 40–1000-fold increase in regucalcin gene expression as compared with the normal livers shown in a representative real time PCR analysis (Fig. 2*B*).

Next, paraffin tissues from group 2 HCC patients, where the β-catenin mutational status was unknown, were assessed for β-catenin, GS, and regucalcin expression by IHC. Based on the

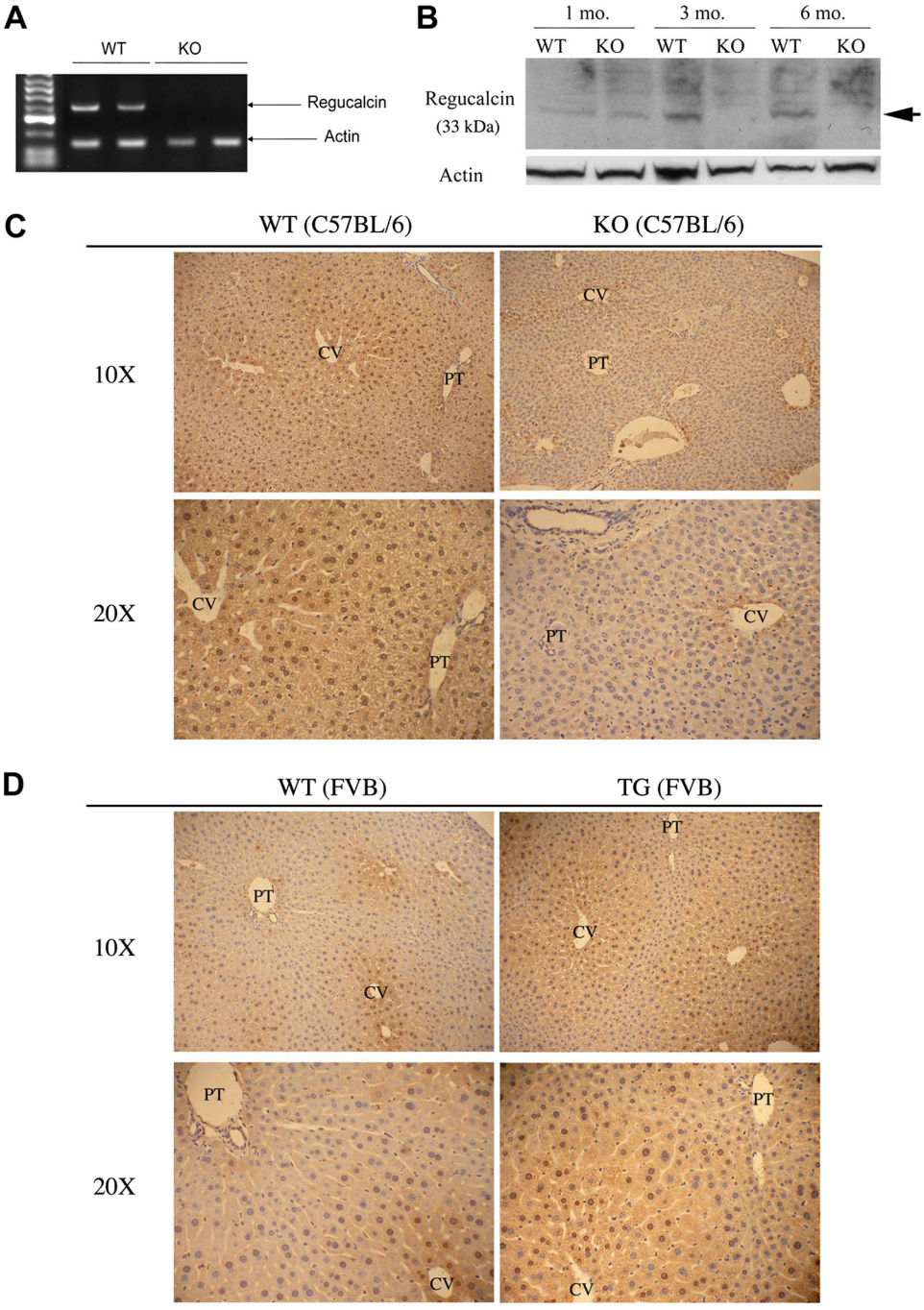


FIGURE 1. **Regucalcin expression is regulated by β-catenin in the liver.** *A,* semiquantitative RT-PCR using mRNA from WT and KO livers at 4–7 months of age shows regucalcin expression in WT livers only. *B,* Western blot using whole-cell lysates from WT and KO livers show regucalcin is present in all WT samples but is decreased at 3 and 6 months in the KO mice. *C,* representative immunohistochemistry for regucalcin on 3-month-old WT and KO livers show cytoplasmic and nuclear expression of regucalcin protein in WT hepatocytes; in contrast, regucalcin expression was dramatically decreased in KO livers. *PT*, portal triad; *CV,* central vein. *D,* immunohistochemistry for regucalcin on 3-month-old WT and stable-β-catenin overexpressing transgenic (*TG*) mice at (*bottom four panels*) centrizonal regucalcin staining in WT FVB livers, whereas the transgenic livers show widespread nuclear and cytoplasmic regucalcin.

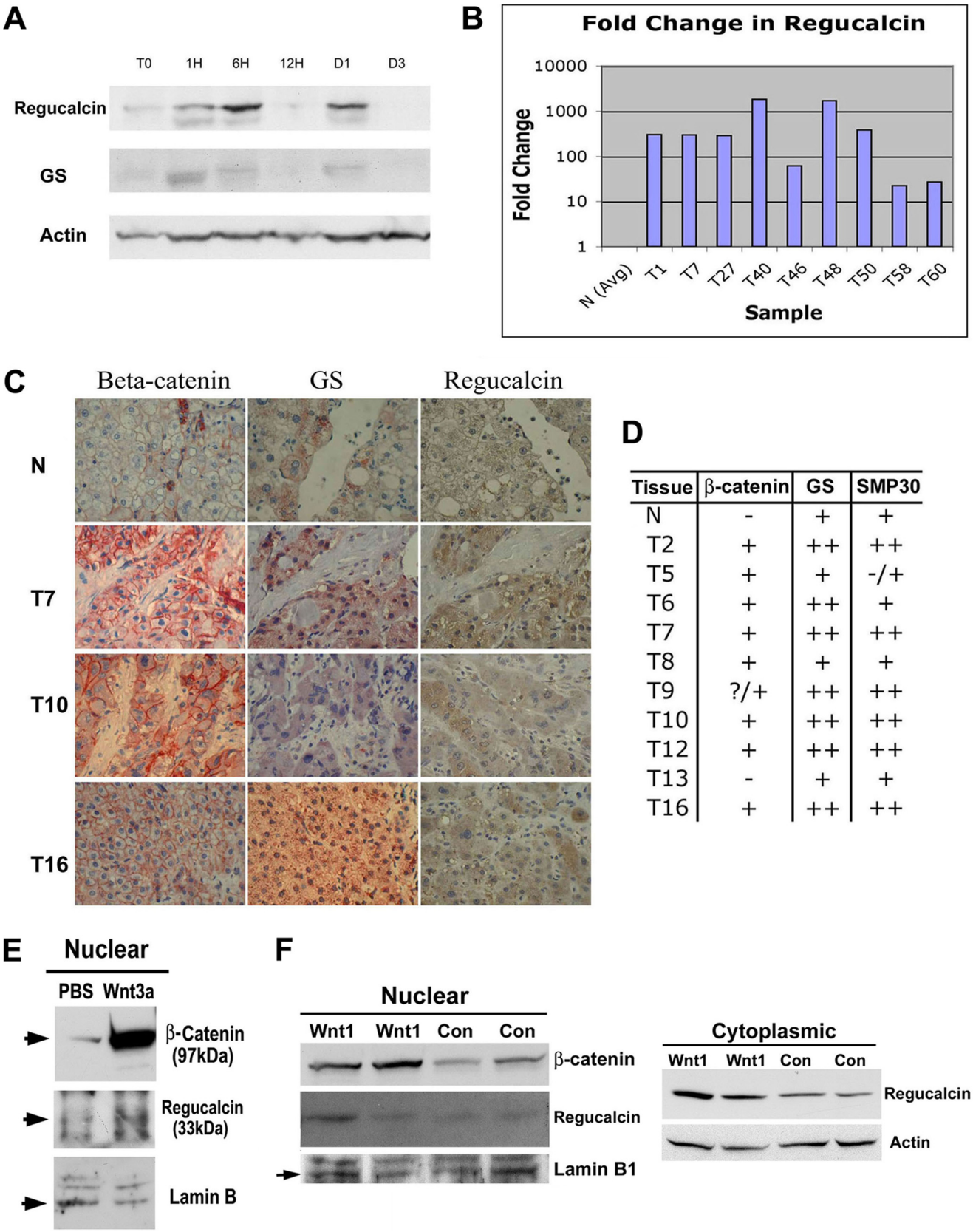
IHC, normal human liver received a score of − for β-catenin in the nucleus and cytosol and + for GS and regucalcin. As shown in representative HCC samples, increased cytoplasmic and nuclear β-catenin coincided with elevated GS and regucalcin staining in adjacent sections (Fig. 2*C*). Overall, 9:10 HCC tissues exhibited some nuclear/cytoplasmic β-catenin in addition to its membranous localization, and 7:9 of these samples showed increased regucalcin, of which 6 showed a coexisting increase in GS (Fig. 2*D*). A small subset (tumor numbers 5 and 8) showed no evidence of increase in GS or regucalcin despite exhibiting intense nuclear β-catenin staining.

Exogenous Wnt Stimulation Induces Regucalcin Expression in Vitro and in Vivo—Next, we examined if exogenous Wnt stimulation had an effect on regucalcin protein expression. Rat primary hepatocytes were cultured in the presence of recombinant Wnt3a protein as described under "Experimental Procedures." As expected, there was a significant increase in nuclear β-catenin in response to Wnt3a treatment. There was a corresponding increase in nuclear regucalcin levels after 24 h of Wnt3a treatment of the rat hepatocytes (Fig. 2*E*).

We have previously employed hydrodynamic delivery of naked DNA as a means to induce expression of a gene in the liver (23). Here we utilized this approach to induce Wnt1 expression in the liver after partial hepatectomy, as outlined under "Experimental Procedures." An increase in the hepatic nuclear β-catenin level was observed in the Wnt1 and not pcDNA3-injected mice (Fig. 2*F*). A modest increase in hepatic nuclear and cytosolic expression of regucalcin was concomitantly observed in the Wnt1-injected group only (Fig. 2*F*). Thus, Wnt/β-catenin activation induces regucalcin expression *in vitro* and *in vivo* in both rat and mouse hepatocytes.

Expression and Association of Regucalcin and β-Catenin in Human Hepatoma Cell Lines—We next examined the human hepatoma cell lines Hep3B, which contains the wild-type β-catenin gene, and HepG2, which contains the β-catenin gene with exon-3 deletion rendering a nondegradable protein, for expression and functional analysis of regucalcin (5). Western blots demonstrate higher levels of regucalcin protein in HepG2 cells as compared with Hep3B cells (Fig. 3*A*). This result was also confirmed by real time PCR, which showed higher expression of regucalcin in HepG2 cells than Hep3B cells (Fig. 3*B*). Finally, immunofluorescence studies were concordant with

the above results, with HepG2 cells displaying higher levels of regucalcin in the cytoplasm and nucleus than Hep3B cells (Fig. 3*C*). Interestingly, when cells were stained with both regucalcin and β-catenin, there appeared to be areas of overlapping signal in both cell lines; however, this colocalization was greater in HepG2 than the Hep3B cells (Fig. 3*C*).



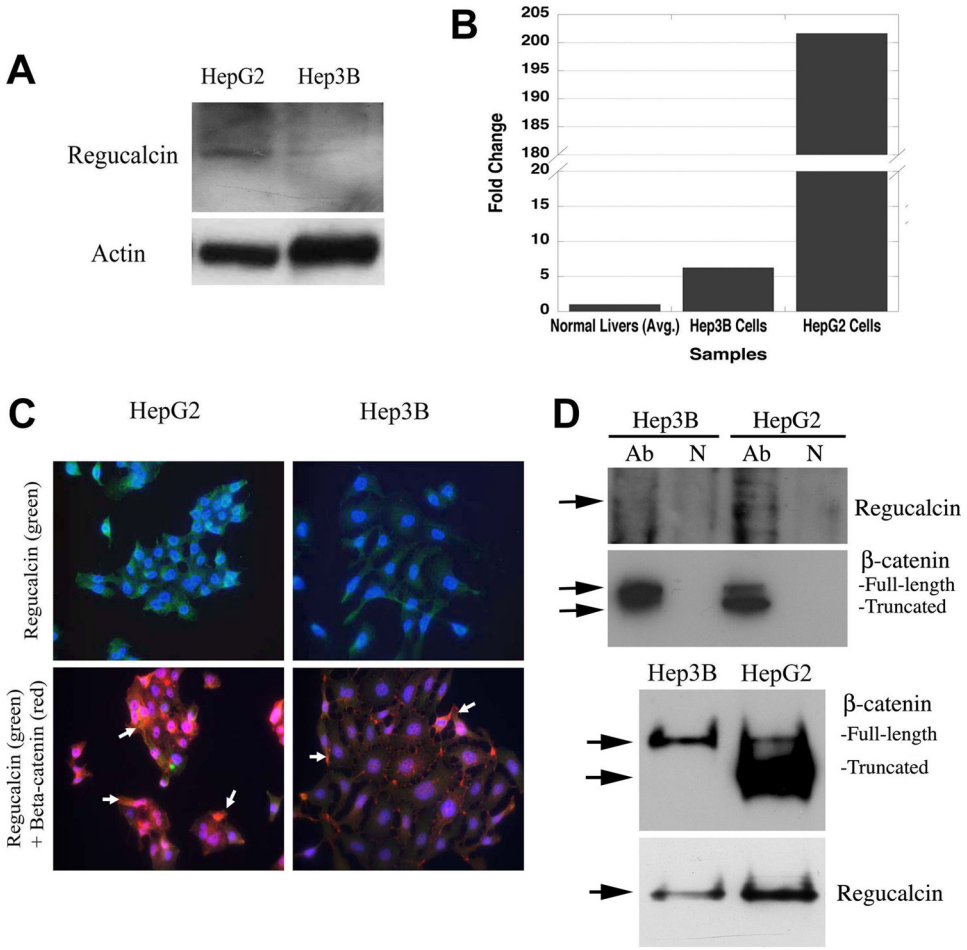


FIGURE 3. **Regucalcin, which is expressed in both Hep3B cells and HepG2 cells, also associates with β-catenin in the cell.** *A,* Western blot of proteins harvested from HepG2 and Hep3B human hepatoma cell lines shows that HepG2 cells express higher levels of regucalcin than Hep3B cells. Western blot for actin serves as the loading control. *B,* real time PCR analysis of mRNA harvested from HepG2 and Hep3B human hepatoma cell lines. Regucalcin mRNA expression was 6-fold higher in Hep3B cells but more than 200-fold higher in HepG2 cells as compared with average of normal human livers. Regucalcin mRNA expression was standardized to actin expression. *C,* immunofluorescence on cultured HepG2 and Hep3B cells demonstrates higher regucalcin (*green*) and β-catenin (*red*) in HepG2 cells than Hep3B cells. Also, as seen in *bottom panels*, colocalization (*white arrows*) of β-catenin and regucalcin was evident especially in HepG2 cells. Images were taken at ×600 magnification. *D,* HepG2 and Hep3B cell lysates immunoprecipitated with β-catenin antibody and probed for regucalcin (*top*) show association of the two proteins. Nonspecific (*N*) IgG did not pull down regucalcin. Similar lysates immunoprecipitated with regucalcin (SMP30) antibody (*Ab*) and probed for β-catenin also show association of full-length β-catenin with regucalcin in both cell types and truncated β-catenin-regucalcin association in HepG2 cells. Successful pulldown of β-catenin and regucalcin by their antibodies is verified in respective *lower panels*.

To determine whether this colocalization of β-catenin and regucalcin in HepG2 represented a physical association between the two proteins, we immunoprecipitated protein lysates from both HepG2 and Hep3B cells with regucalcin/SMP30 antibody and probed the blots for β-catenin and vice versa. Fig. 3*D* shows coprecipitation of β-catenin and regucalcin in both HepG2 and Hep3B cells, although HepG2 cells showed a stronger association. Also, following immunoprecipitation utilizing HepG2 cells, it was apparent that regucalcin associates with both the truncated (predominant species) and full-length form of β-catenin (minor species). Lysates incubated with IgG instead of β-catenin or regucalcin antibodies did not pull down protein in either preparation (Fig. 3*D* and data not shown). Thus, these data demonstrate physical interactions between β-catenin and regucalcin proteins.

Critical TCF-4-binding Site in the Regucalcin Promoter Region Mediates β-Catenin Responsiveness—To address β-catenin-dependent expression of regucalcin, we searched the human regucalcin promoter region 864 bp upstream to 102 bp downstream of the transcription start site (*H. sapiens* regucalcin mRNA, NM_004683) in *H. sapiens* chromosome X (NC_000023) for DNA-binding sites. The choice of these regions is based on previous observations that transcription factors frequently bind to proximal promoter sequences. Four putative TCF-4-binding sites with close homology to the TCF core consensus sequence (5′-(A/T)(A/T)CAAAG-3′) were identified in this region (Fig. 4*A*) (43–45).

To identify the TCF-4-binding site in the regucalcin promoter that is necessary for β-catenin activation, we cotransfected HepG2 cells with plasmids containing restriction fragments isolated from the 5′-flanking region of the human regucalcin gene and a luciferase reporter construct (Fig. 4*A*). As mentioned previously,

FIGURE 2. **Regucalcin protein expression correlates with β-catenin activation during liver regeneration in HCC and after exogenous Wnt administration.** *A,* Western blot with lysates from rat livers harvested at various time points after partial hepatectomy display concomitant increase in regucalcin and known β-catenin target-GS expression at 1 and 6 h and day 1 (*D1*) during regeneration. *B,* real time PCR analysis of mRNA harvested from human HCCs with known β-catenin mutations (group 1) shows manyfold increase in regucalcin mRNA expression, which was plotted for each tumor. Regucalcin mRNA expression was standardized to actin expression in each sample and normalized to average signal in four normal livers. *C,* immunohistochemistry for β-catenin, regucalcin, and GS on representative human HCCs where the mutational status of β-catenin is unknown (group 2). Areas of increased β-catenin staining in the nucleus and cytoplasm often correlated to areas of high regucalcin and GS expression. *D,* arbitrary scoring of relative amounts of β-catenin, regucalcin, and GS in human HCCs with unknown mutational status of β-catenin (group 2). Each tumor sample was given arbitrary immunohistochemical scores of + or − for the presence or absence of nuclear and/or cytoplasmic β-catenin in hepatocytes. Regucalcin and GS samples were scored based on a comparison with the normal liver tissue; an increase above normal scored ++ or a level of expression similar to normal +. Most biopsies that showed nuclear/cytoplasmic β-catenin also showed elevated levels of regucalcin and/or GS. *E,* Western blot analysis utilizing nuclear fractions from rat hepatocytes cultured in the presence of Wnt3a or phosphate-buffered saline (*PBS*) for 24 h shows an increase in β-catenin and regucalcin expression in Wnt3a-treated cultures only. Lamin B1 represents the loading control. *F,* Western blot utilizing nuclear and cytoplasmic extracts from livers of two representative Wnt1- or pcDNA3 (*Con*)-injected mice show an increase in nuclear β-catenin and nuclear and cytoplasmic regucalcin expression in the experimental group.

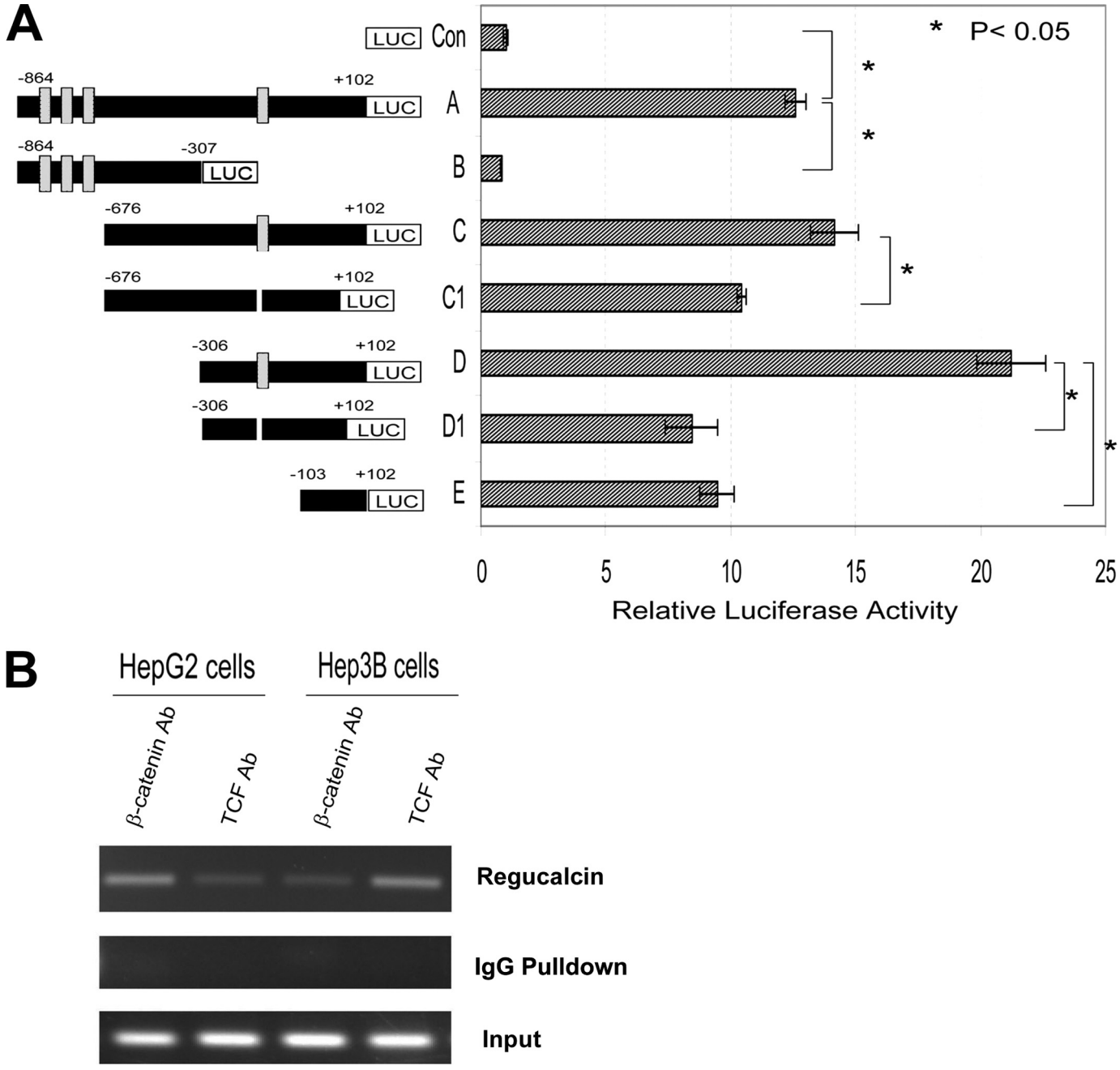


FIGURE 4. **Regucalcin promoter region contains a proximal TCF-4/β-catenin-binding site, which is essential for full transcriptional activation.** *A, left panel* depicts various luciferase (*LUC*) reporter constructs used for the study. The *gray boxes* represent TCF-4-binding sites. The *right panel* represents the relative luciferase activity after transfection into HepG2 cells that harbor constitutively active β-catenin. Deletion of the distal three TCF-4-binding sites (*C* and *D*) does not affect luciferase reporter activity (compared with *A*, which contains all 4 TCF-4 sites). Removal of the proximal TCF-4-binding site and minimal promoter abolishes basal transcription (*B*); however, if the fourth site is removed by enzymatic digest (*C1* and *D1*), luciferase activity is decreased significantly (compare with *C* and *D*). Removal of the proximal TCF-4-binding site while leaving the basal promoter intact (*E*) also decreases luciferase activity. *B,* representative PCR gel of ChIP assay shows binding of β-catenin and TCF-4 to the regucalcin promoter in HepG2 and Hep3B cells as compared with absent binding in IgG pulldown only. Immunoprecipitation was carried out using an antibody (*Ab*) to β-catenin and TCF4. The PCR primers were located in the −306 to −139 to region of the regucalcin promoter in reference to the transcription start site.

HepG2 harbors constitutively active β-catenin and confers significantly higher levels of TCF-dependent transcriptional activity than that found in other HCC cell lines (5, 46). As shown in Fig. 4*A*, fragment A (from −864 to +102), which contained all four TCF-4-binding sites, exhibited 6-fold greater promoter activity over base line. Deletion from −307 to the transcription start site (fragment B), which removed the basal promoter region and the proximal TCF-4-binding site, completely abolished promoter activity. Removal of the first three distally located TCF-4-binding sites (fragments C and D) had no effect, and the promoter activity was comparable with fragment A. Therefore, we reasoned that the proximal TCF-4-binding site might be regulating regucalcin expression. To demonstrate this conclusively, we excised the proximal site without disturbing the basal promoter region. Two fragments (C1 and D1) were constructed by excising the TCF-4-binding site proximal to the

transcription start site (located from −163 to −157). This was accomplished with enzymatic digest using SmaI, which removed 109 bp (−180 to −71) from fragments C and D. Removal of this proximal TCF-4-binding site while leaving the basal promoter intact reduces luciferase activity 1.3–2.8-fold (relative to fragments C and D). Fragment E, which contains only the basal promoter region downstream of the proximal TCF-4-binding site, also shows a 1.4-fold reduction in luciferase activity relative to fragment A.

Results of the ChIP assay further confirmed that the regucalcin promoter region contains an essential TCF4/β-catenin-binding site. Chromatin fragments harvested from Hep3B and HepG2 cells were subjected to immunoprecipitation using TCF-4, β-catenin antibodies, or IgG, and the resulting products were amplified using PCR primer-encompassing region from −306 to −82 on the regucalcin promoter. Fig. 4*B* confirms that only TCF and β-catenin and not protein G beads only bind to the regucalcin promoter *in vivo*. Together, these data indicate that β-catenin is a transcriptional regulator of the regucalcin gene through the TCF4 site located between −163 and −157 (CTTTGCA) on the regucalcin promoter.

Regucalcin Exerts a Prominent Effect on Cell Survival—Next, we attempted to address the biological role of regucalcin in liver. Because HepG2 cells exhibit higher levels of regucalcin, we investigated the effect of regucalcin inhibition on cell proliferation and survival in this cell line. HepG2 cells were cultured and treated with control and regucalcin siRNA constructs as outlined under "Experimental Procedures." We found a 50–80% decrease in regucalcin mRNA expression over multiple experiments as shown in a representative real time PCR at 48 h after regucalcin siRNA transfection (Fig. 5*A*). This also coincided with decreased protein (Fig. 5*B*). Next, we tested the impact of regucalcin knockdown on cell proliferation by [^{3}H]thymidine incorporation. No significant differences in DNA synthesis were evident in HepG2 cells after regucalcin knockdown (Fig. 5*C*). We also tested DNA synthesis in Hep3B cells despite lower regucalcin expression. Interestingly and consistently, a marginal but significant reduction in thymidine incorporation was observed following regucalcin siRNA transfection in Hep3B cells (Fig. 5*D*).

We then examined the effect of regucalcin suppression on cell survival in the HepG2 cells by TUNEL analysis. Transfection with regucalcin siRNA and not control siRNA led to the presence of significant apoptotic nuclei in these cells (Fig. 5, *E* and *F*). Thus regucalcin seems to play an important role in cell survival in the hepatoma cells.

β-Catenin Regulates Vitamin C Synthesis through Regulation of Regucalcin Expression—Regucalcin/SMP30 is an essential gluconolactonase in the liver and plays a pivotal role in regulating ascorbate biosynthesis (18). Because SMP30 knock-out mice fed a vitamin C-deficient diet have been shown to develop scurvy because of decreased levels of ascorbate, we investigated whether the β-catenin KO mice, which have decreased expression of regucalcin/SMP30, are also deficient in ascorbate production because these mice were being fed normal mouse chow (Prolab Isopro RMH 3000), which lacks vitamin C. This became more relevant when gene array analysis of β-catenin-deficient livers also identified a significant decrease in the expression of L-gulonolactone oxidase, which is the final and rate-limiting step in the ascorbic acid biosynthesis (Table 2). This decrease was also verified by RT-PCR (Fig. 6*A*). Next, sera from WT and β-catenin KO were tested for ascorbate content ($n = 3$). A 3.5-fold reduction in serum ascorbate levels was observed in β-catenin KO mice as compared with the WT littermates (Fig. 6*B*). Therefore, β-catenin, through its role as a mediator of expression of regucalcin and L-gulonolactone oxidase, plays an important role in regulating ascorbate levels.

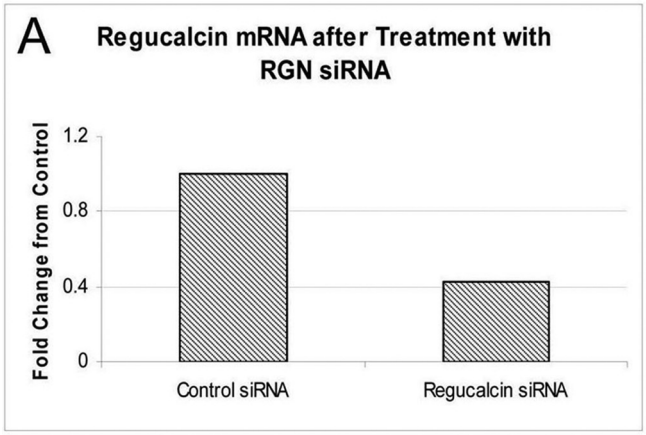
Enhanced Apoptosis of β-Catenin-deficient Hepatocytes Is Rescued by Vitamin C Supplementation—Because we previously reported a basal increase in hepatocyte apoptotic index in the β-catenin-conditional null mice, and even greater increase in apoptosis during liver regeneration, we were interested to investigate if this could be contributed by regucalcin loss (20). We cultured primary hepatocytes from β-catenin conditional null mice for 48 h. Greater than 90% of hepatocytes showed significant loss of viability resulting from apoptosis shown by TUNEL immunohistochemistry (Fig. 6, *C* and *D*). When β-catenin null hepatocytes were cultured in the presence of ascorbate, a dramatic and almost 100% rescue of cell viability was evident as reflected by negligible apoptosis assayed by TUNEL staining (Fig. 6, *C* and *D*).

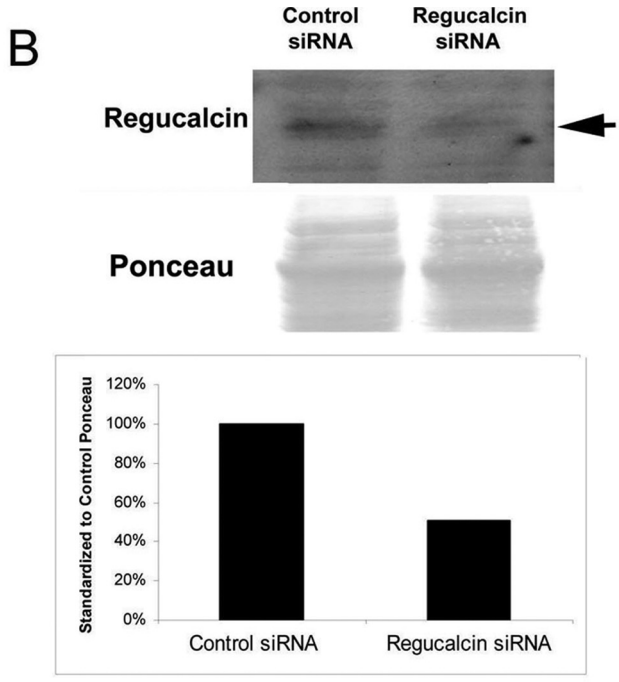
Apoptosis in HepG2 Cells Because of Regucalcin Knockdown Is Rescued by Vitamin C and NAC—Next, we wanted to explore if similar to mouse hepatocytes, apoptosis of HepG2 cells following regucalcin knockdown could be rescued by vitamin C. As expected and shown earlier (Fig. 5, *E* and *F*), a significant increase in apoptosis was evident in HepG2 cells in response to 48 h of regucalcin and not control si-RNA transfection (Fig. 6*E*). However, although the presence of vitamin C in control cultures did not alter the basal apoptosis in HepG2 cells, it led to a significant decrease in apoptosis brought about by regucalcin knockdown (Fig. 6*C*).

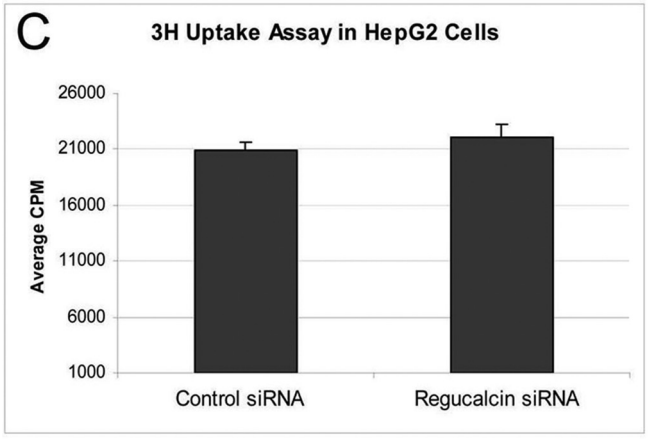
It was interesting to note that ascorbic acid repletion led to the rescue of cell survival following siRNA-mediated regucalcin knockdown. Because human cells lack L-gulonolactone oxidase and are unable to synthesize vitamin C, we wondered if the rescue effect of ascorbate was specific to vitamin C deficiency brought about by regucalcin knockdown or through the general anti-oxidant effect of vitamin C (19). To test this hypothesis, we utilized NAC, another well known antioxidant, to determine its effect on regucalcin-siRNA-mediated apoptosis of HepG2 cells (38). A significant decrease in apoptosis was evident in NAC-treated HepG2 cells secondary to regucalcin knockdown (Fig. 6*F*). These observations suggest that regucalcin might be important in cell survival in humans through regulation of oxidative stress, which might be independent of vitamin C biosynthetic function.

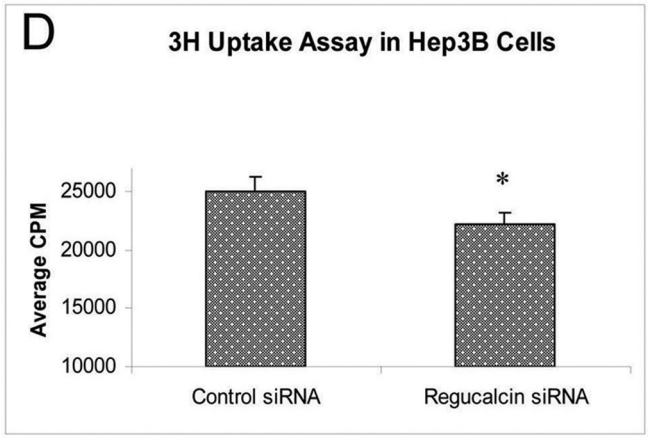
DISCUSSION

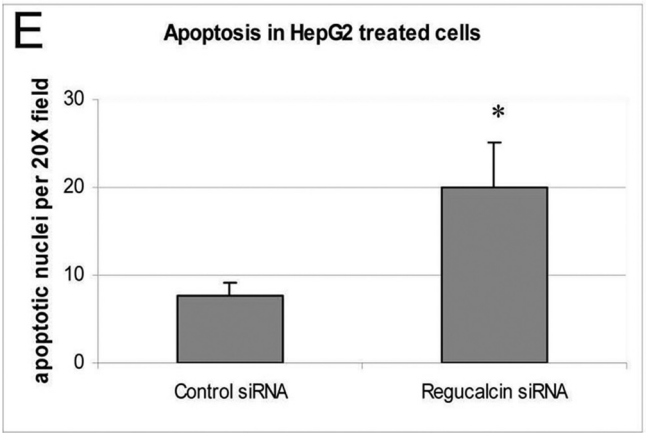
β-Catenin is the crucial downstream effector of the canonical Wnt pathway, and it plays many roles in development and adult tissue homeostasis in various organs. Because it is a transcriptional coactivator, it is critical to identify the target genes that in turn are responsible for the widespread roles of the pathway in many cellular and tissue processes. β-Catenin has critical roles in regulating cell proliferation, differentiation, apopto-











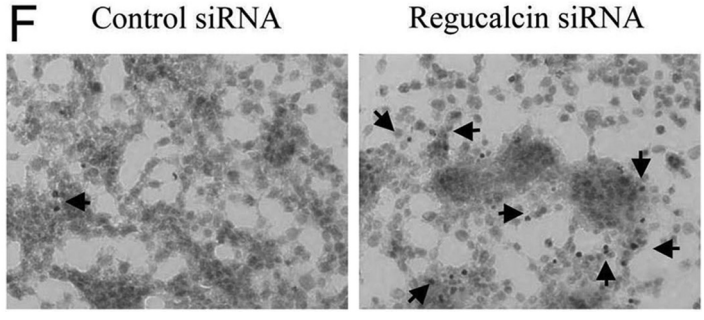


FIGURE 5. **Regucalcin knockdown using siRNA affects survival of hepatoma cells.** *A,* regucalcin siRNA decreases mRNA expression in hepatoma cells as shown in representative real time PCR analysis of mRNA harvested from HepG2 cells. The $\Delta\Delta Ct$ of regucalcin mRNA was plotted for each cell line. In this representative plot, siRNA decreases regucalcin expression in HepG2 cells by 60% as compared with cells transfected with control siRNA. Regucalcin mRNA expression was standardized to actin expression. *B,* comparable decrease in regucalcin protein was also observed as shown in representative Western blot using lysates from Hep3B cells. Ponceau red staining verifies comparable loading in the Western blot. *Bottom panel* is the plot for normalized integrated optical density from densitometric analysis of a representative Western blot. *C,* unremarkable differences in thymidine uptake assay by control or regucalcin siRNA-transfected HepG2 cells. *D,* small but significant ($p < 0.05$) decrease in thymidine uptake by regucalcin-transfected Hep3B cells as compared with control siRNA-transfected cells. *E,* greater than 2-fold increase in the numbers of TUNEL-positive apoptotic nuclei in regucalcin siRNA-transfected HepG2 cells transfected as compared with control siRNA-transfected HepG2 cells. *F,* increased numbers of TUNEL-positive apoptotic nuclei (*arrowhead*) are observed after regucalcin (*right panel*) and not control (*left panel*) siRNA transfection of HepG2 cells by representative immunohistochemistry. Images were taken at ×200 magnification.

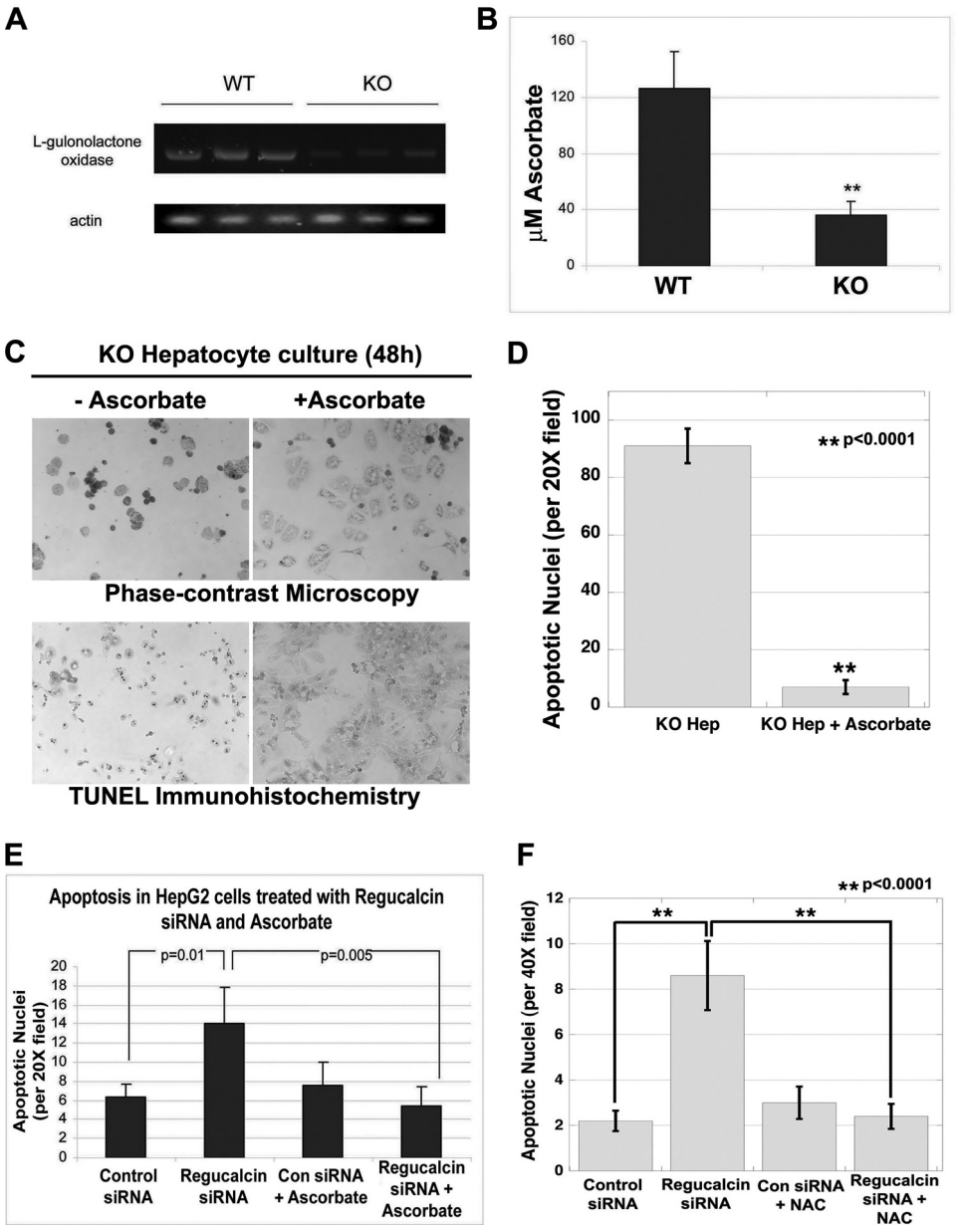


FIGURE 6. **Decreased regucalcin expression in the absence of β-catenin negatively affects ascorbate levels and cell survival.** *A,* RT-PCR identified a dramatic decrease in L-gulonolactone oxidase expression in β-catenin-deficient livers as compared with the WT livers. *B,* greater than 3-fold decrease in serum ascorbate levels were evident in the β-catenin KO mice ($n = 3$) as compared with the WT littermates, and the difference is statistically significant (**, $p < 0.001$). *C,* β-catenin-deficient hepatocytes (*KO*) exhibit loss of viability after 48 h in culture shown by phase contrast microscopy (*upper left panel*) and increased TUNEL-positive nuclei (*bottom left panel*). Inclusion of vitamin C in the culture prevented loss of hepatocyte viability (*upper right*) along with a dramatic decrease in the TUNEL-positive hepatocytes (*lower right*). *D,* quantitative analysis of TUNEL immunohistochemistry identifies an extremely significant decrease in apoptosis in KO hepatocytes in the presence of vitamin C ($p < 0.0001$). *E,* regucalcin siRNA induces significant apoptosis in HepG2 cells over the control siRNA-transfected cultures ($p = 0.01$). Addition of 0.2 mM ascorbic acid significantly reduces apoptosis observed in response to regucalcin knockdown only ($p = 0.005$) and does not impact the basal apoptosis in control siRNA treated HepG2 cells. *F,* similar rescue of apoptosis in HepG2 cells brought about by regucalcin knockdown is observed in the presence of 20 mM NAC ($p = 0.01$).

sis, and adhesion in the liver, which in turn makes it relevant in liver development, regeneration, zonation, metabolism, and cancer (40, 42, 47–49). In this study, we establish regucalcin as a novel target of β-catenin in the liver. Further studies identified an important role of β-catenin in vitamin C biosynthesis and cell survival through the regulation of expression of regucalcin and L-gulonolactone oxidase.

The first evidence of regulation of regucalcin/SMP30 by β-catenin came from the gene array analysis using livers from β-catenin conditional KO and wild-type littermates (20). This was directly investigated in this study, and a decrease in regucalcin correlated with β-catenin loss in KO livers. Similarly, β-catenin transgenic mice, which display elevated levels of β-catenin, also showed elevated levels of regucalcin protein in the nucleus and cytosol of hepatocytes as compared with controls. It is important to highlight the observed difference in regucalcin during steady state in the adult livers between the two wild-type samples that belonged to C57BL/6 and FVB strains. Clearly, a wider regucalcin expression in all zones was observed in C57BL/6 livers, although it was more pronounced around central veins, as compared with the FVB livers, which showed a narrow centrizonal regucalcin expression only. This coincides with β-catenin signaling that is typically limited to centrizonal areas in normal adult livers (47). Strain-specific differences in the basal regucalcin expression add to the category of a growing list of genes and proteins that exhibit such phenomena and might eventually be the basis of diverse responses or phenotypes observed in response to similar stimuli (50).

To further reaffirm the regulation of SMP30 by β-catenin, we examined a series of models that are known to exhibit β-catenin activation. Results of the liver regeneration studies show an increase in regucalcin protein expression as early as 1 h after PHx in the rat. This expression remains increased over time 0 (with the exception of 12 h post-PHx) until approximately day 3. This expression corresponds to that of β-catenin during liver regeneration after partial hepatectomy, where nuclear translocation of β-catenin is observed within 5 min after hepatectomy and is retained in the hepatocyte nucleus until around 48 h (29). Moreover, regucalcin expression mirrored the levels of GS, a known target of β-catenin, during liver regeneration (42). Others have found that liver regucalcin mRNA levels are clearly increased 1–5 days after hepatectomy, in comparison with that

of sham-operated rats (51). Human hepatocellular carcinomas containing activating β-catenin mutations were also investigated for regucalcin expression, which was clearly elevated in these samples along with GS (21). Similarly, those tumors with unknown β-catenin gene mutation status, but exhibiting β-catenin staining in the nucleus and/or cytosol, also showed an increase in both regucalcin and GS expression. Finally, we were interested to examine any impact of exogenous Wnts on regucalcin expression. Indeed, treatment of rat hepatocytes with Wnt3a induced β-catenin nuclear translocation as well as regucalcin expression. In an *in vivo* mice study, we successfully delivered Wnt1 plasmid DNA hydrodynamically through tail vein (23), followed by partial hepatectomy, and harvested livers at 30 h. This led to an increase in nuclear β-catenin and regucalcin expression in the livers of Wnt1-injected and not pcDNA-injected mice. Thus higher regucalcin levels are observed in the event of Wnt/β-catenin activation in mice, rats, and HCC patients.

Previous data on regucalcin in hepatocellular carcinomas have been somewhat sparse. Serum levels of regucalcin are markedly increased 30 days after administration of carbon tetrachloride, suggesting a role for regucalcin as a biomarker for chronic liver injury (52). Decreased regucalcin expression was observed in CuZn superoxide dismutase-deficient mice with fully developed HCCs (53). Another study showed that regucalcin RNA was down-regulated in diethylnitrosamine-generated mouse HCCs *versus* regenerating (−10.7), quiescent (−12.6), and newborn liver (−4.4). In these samples, β-catenin, which was indirectly measured by the detection and measurement of β-catenin putative target genes, was not found to be deregulated (54). Another study of mouse liver carcinogenesis has shown that regucalcin was up-regulated in early mouse liver carcinogenesis induced by oxazepam, which is known to induce β-catenin gene mutations (6, 55). This demonstrates some existing correlation between β-catenin activation and regucalcin expression, but the exact role of regucalcin in HCC remains elusive.

To understand the biological implications of regucalcin in the liver, we utilized siRNA-mediated suppression in liver tumor cells. It was interesting to note that inhibition of regucalcin in Hep3B and HepG2 tumor cells had little or no effect on proliferation. These meek differences might in fact be an indirect result of enhanced cell death. However, we did not find any increase in hepatocyte proliferation following suppression of regucalcin, as reported elsewhere (14, 15). It has been previously suggested that regucalcin may have a role as an inhibitor of proliferation during liver regeneration in rats. Thus the role of regucalcin in cell proliferation will need to be investigated further as a primary or perhaps a secondary event because of cell death.

An important role of regucalcin has been reported in vitamin C biosynthesis (18). Indeed β-catenin KO mice show significantly lower ascorbate levels secondary to decreased expression of both regucalcin and L-gulonolactone oxidase, both critical in vitamin C synthesis in murine hepatocytes (19). It is also relevant to note that β-catenin KO livers show an increase in basal hepatocyte apoptosis, which is aggravated during liver regeneration (20). To demonstrate a direct role of β-catenin in regulating apoptosis through vitamin C homeostasis, we cultured hepatocytes from β-catenin KO mice. These cells displayed massive apoptosis within 48 h of culture. When cultured in the presence of vitamin C, there was a complete rescue of apoptosis of the β-catenin-deficient hepatocytes. This is in agreement with previous studies where regucalcin overexpression has been shown to suppress cell death (17). Our results are also concordant with previous studies where mice containing a germ line null mutation of SMP30 have been shown to be highly susceptible to both tumor necrosis factor-α- and Fas-mediated apoptosis, thereby indicating that SMP30 plays a role in protection from apoptosis and contributes to cell survival (56, 57).

A significant increase in apoptosis was also evident following regucalcin suppression in HepG2 cells, although the increase in apoptosis was nearly not as pronounced as in the murine hepatocytes. Moreover, the apoptosis in regucalcin siRNA-transfected hepatoma cells was rescued by supplementation with ascorbic acid or NAC. Because both molecules possess strong anti-oxidant properties, these results suggest that decreased regucalcin in human hepatoma cells might be inducing cell death through oxidative stress. Indeed the role of SMP30 in regulating oxidative stress has been reported (58). Thus, the role of regucalcin in regulating cell survival might be through regulation of the redox state of the cell and independent of vitamin C biosynthesis in human cells. However, in murine cells, the role of regucalcin in vitamin C biosynthesis may contribute to cell survival through distinct yet unexplored apoptotic pathways, as well as through control of oxidative stress. Indeed in our unpublished data,[3] we also observe a modest increase in basal lipid peroxidation, a marker of oxidative stress, in β-catenin conditional null mice. Also, these studies suggest that one mechanism by which β-catenin might be promoting cell survival is through regulation of regucalcin expression.

Analysis of the promoter region of mouse regucalcin revealed four putative TCF-4/LEF-binding sites. These sites are between 750 and 150 bp upstream of the +1 site and are either very similar or identical to consensus sequence sites for TCF-4 binding identified previously (2, 59, 60). Using multiple deletion mutants isolated from the promoter region of the human regucalcin gene, we identified the specific site at which β-catenin transcriptionally regulates regucalcin expression. The regucalcin promoter is also known to contain an AP-1-binding site (61). One recent study demonstrates that AP-1 and β-catenin can act in synergy to transcriptionally activate β-catenin target genes such as cyclin D1 (62). In previous studies, AP-1 has been shown to mediate regucalcin mRNA expression in hepatocytes by binding to the promoter region of the regucalcin gene and activating transcription (61). The existence of AP-1- and TCF-binding sites in the regucalcin promoter region may suggest a mechanism by which regucalcin can contribute to HCC secondary to β-catenin activation.

It was interesting to note that not only was the expression of regucalcin regulated by β-catenin but that it associated with β-catenin in Hep3B and HepG2 cells, as well as in resting wild-type mouse livers (data not shown). This is not unusual in the

[3] K. N. Nejak-Bowen, G. Zeng, X. Tan, B. Cieply, and S. P. Monga, unpublished data.

Wnt signaling pathway where several key regulators are in fact transcriptional targets of β-catenin/TCF4, including TCF-1 (63), Axin-2 (64), and DKK1 (65). Whether regucalcin itself is able to have an impact on Wnt signaling will need to be investigated further.

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