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BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Convergence of Wnt Signaling on the HNF4α-Driven Transcription in Controlling Liver Zonation

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BACKGROUND & AIMS: In each hepatocyte, the specific repertoire of gene expression is influenced by its exact location along the portocentrovenular axis of the hepatic lobule and provides a reason for the liver functions compartmentalization defined "metabolic zonation." So far, few molecular players controlling genetic programs of periportal (PP) and perivenular (PV) hepatocytes have been identified; the elucidation of zonation mechanisms remains a challenge for experimental hepatology. Recently, a key role in induction and maintenance of the hepatocyte heterogeneity has been ascribed to Wnt/β-catenin pathway. We sought to clarify how this wide-ranging stimulus integrates with hepatocyte specificity. **METHODS:** Reverse transcriptase polymerase chain reaction (RT-PCR) allowed the transcriptional profiling of hepatocytes derived from in vitro differentiation of liver stem cells. The GSK3β inhibitor 6-bromoindirubin-3′-oxime (BIO) was used for β-catenin stabilization. Co-immunoprecipitations were used to study biochemical protein interactions while ChIP assays allowed the in vivo inspection of PV and PP genes regulatory regions. **RESULTS:** We found that spontaneous differentiation of liver stem cells gives rise to PP hepatocytes that, after Wnt pathway activation, switch into PV hepatocytes. Next, we showed that the Wnt downstream player LEF1 interacts with the liver-enriched transcriptional factor HNF4α. Finally, we unveiled that the BIO induced activation of PV genes correlates with LEF1 binding to both its own and HNF4α consensus, and the repression of PP genes correlates with HNF4α displacement from its own consensus. **CONCLUSION: Our data show a direct and hitherto unknown convergence of the canonical Wnt signaling on the HNF4α-driven transcription providing evidences of a mechanism controlling liver zonated gene expression.**

Most of the main metabolic functions of the liver are not uniformly distributed over the hepatic lobule, but follow gradients of enzymatic activities along the centrolobular/portal axis. In fact, adult hepatocytes undergo a postdifferentiation patterning resulting in a zonal heterogeneity of gene expression and functions defined as "metabolic zonation." Specific enzymatic/metabolic activities, namely, carbohydrate metabolism, ammonia detoxification, bile formation/transport/secretion, and drug biotransformation, are confined to the perivenular (PV, near the centrolobular vein) or periportal (PP, near the portal vein) zones of the hepatic lobule.[1] The elucidation of the mechanisms responsible for induction and maintenance of the hepatocyte heterogeneity remains a challenge in experimental hepatology.

Intriguingly, inversion of the blood flow direction changes the enzymatic gradients and, consequently, the zonation of some, but not all of the liver metabolisms, thus revealing the influence exerted by the oxygen and circulating molecules on this phenomenon.[2] For the bloodstream-independent gradients, cell–cell and cell–extracellular matrix interactions and para-

Abbreviations used in this paper: BIO, 6-bromoindirubin-3′-oxime; CPS, carbamoyl-phosphate synthetase; ChiP, chromatin immunoprecipitation; Cyp1a1, cytochrome 1a1; Gls2, glutaminase 2; GLT-1, glutamate transporter 1; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PP, periportal; PV, perivenular; Rex3, reduced in expression 3; RLSC, resident liver stem cell.

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crine signalings have been suggested as instructive stimuli.[3]

Recently, concerning soluble factors, a key role of the Wnt/β-catenin pathway has been unveiled.[4] Within the hepatic lobuli, Wnt signaling has been proposed to originate from endothelial cells of the central vein and to follow a stable gradient that decreases toward the PV–PP axis.[5] The Wnt pathway, triggered by a large family of cytokines, influences several processes such as embryonic development, planar cell polarity, cell fate, and proliferation.[6] In the absence of Wnts, β-catenin is phosphorylated by a "destruction complex" constituted by APC, axins, GSK3β, and CK1, then ubiquitinated and proteasome degraded. On the contrary, Wnt/β-catenin signaling, activated by binding of soluble Wnts to the Frizzled receptors or by oncogenic mutations,[7,8] results in the stabilization and translocation β-catenin into the nucleus where, in conjunction with members of LEF1/TCF transcription factor family, activates the transcription of several target genes.[9] In the liver, Benhamouche et al[4] observed a mutually exclusive localization of activated β-catenin and the negative regulator APC in the PV and in PP hepatocytes, respectively. Moreover, these authors demonstrated that genetic manipulation of APC expression and adenoviral delivery of the extracellular antagonist of Wnts DKK were allowed to switch the phenotype from PP into PV and vice versa.[4]

A second key element in controlling hepatic zonation was identified in the transcriptional factor HNF4α: Stanulovic et al[10] have recently shown that this orphan nuclear receptor regulates the zonated expression of some genes, including Cyp7, UDP-glucuronyltransferase, and apolipoprotein E.[10] Their analysis of HNF4α knockout mice revealed in PV hepatocytes a maintenance of PV genes expression and in PP hepatocytes the inhibition of a PP gene (PEPCK) coupled to the activation of PV genes. These observations led to the conclusion that HNF4α exerts a dual role of activator of PP genes and inhibitor of PV genes in PP hepatocytes.

In this work, we gathered evidence demonstrating a direct and hitherto unknown convergence of the canonical Wnt signaling pathway and HNF4α in controlling the hepatocyte heterogeneity. We have taken advantage of resident liver stem cells (RLSCs) that we recently isolated and described as able to spontaneously differentiate into hepatocytes and cholangiocytes and, when cultured in appropriate conditions, into mesenchymal and neuroectodermal cell lineages.[11]

We first observed that hepatocytes derived from in vitro differentiation of RLSCs (RLSCdH, from RLSC-derived hepatocytes) bear a PP transcriptional profile that is coordinately switched into the PV phenotype by the GSK3β inhibitor 6-bromoindirubin-3′-oxime (BIO). Next, co-immunoprecipitation experiments demonstrated, for the first time, an interaction between the downstream Wnt transcriptional factor LEF1 and HNF4α. Furthermore, we analyzed 5 HNF4α target genes that are inversely modulated by BIO: the PV genes glutamine synthetase (GS) and cytochrome 1a1 (Cyp1a1), the PP genes glutaminase 2 (Gls2) and H19 fetal liver mRNA (H19) and transthyretin (TTR), whose expression was not proven to be zonal in vivo but strongly BIO inhibited in our system. Inspection of regulatory regions by chromatin immunoprecipitation (ChIP) assays demonstrated that Wnt activation correlates with (i) LEF1 recruitment to the HNF4α DNA binding site and PV genes transcriptional activation and (ii) HNF4α displacement from its consensus site and PP genes transcriptional repression.

Our data indicate that HNF4α and Wnt signaling pathway are active members of the same machinery that controls the transcription of differentially zonated HNF4α-dependent genes.

Methods

Cell Lines and Culture Conditions

Nontumorigenic, stable RLSC lines, obtained from murine wild-type liver explants at various stages of development, were previously described.[11] These cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 50 ng/mL epidermal growth factor, 30 ng/mL insulin-like growth factor-II (PeproTech Inc, Rocky Hill, NJ), 10 μg/mL insulin (Roche, Mannheim, Germany), 2 mmol/L l-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY), using collagen I (Transduction Laboratories, Lexington, UK) coated dishes (Falcon–BD, Franklin Lakes, NJ). For BIO treatments, normal medium was supplemented with this inhibitor (Calbiochem, San Diego, CA) at 2.5 μmol/L final concentration for the indicated times.

Sequence Analysis

Regulatory sequences of murine GS, Cyp1a1, β-actin, TTR, H19, and Gls2 were obtained from EnsEMBL and analyzed for transcription factor binding sites with MatInspector (release 7.7; Genomatix Software, Genomatix, Munchen, Germany), a program using the vertebrate matrix library and optimized thresholds.

Immunofluorescence

For indirect immunofluorescence analysis, cells were grown on collagen I–coated dishes, fixed and treated as described previously.[12] The antibodies were used at the following dilutions: mouse monoclonal anti–zonula occludens 1 antibody (Zymed Laboratories, South San Francisco, CA) 1/50; mouse monoclo-

nal anti–E-cadherin (E-cad) antibody (Transduction Laboratories; BD Biosciences Pharmingen, Palo Alto, CA) 1/50; goat polyclonal anti-HNF4α (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) 1/50. Secondary antibodies (anti goat Alexa-Fluor 488 and anti-mouse Alexa-Fluor 594, diluted 1:1,000) were from Molecular Probes (Eugene, OR). Preparations were examined with a Zeiss Axiophot microscope.

Immunoprecipitations and Western Blottings

Cells were lysed on ice in 50 mmol/L Tris HCl pH 8.0, 150 mmol/L NaCl, 5 mmol/L EGTA pH 8.0, 50 mmol/L NaF pH 8.0, 10% glycerol, 1.5 mmol/L $MgCl_2$, and 1% Triton X-100 containing freshly added protease inhibitors cocktail tablets (Complete EDTA free; Roche, Monza, IT). Lysates were clarified by centrifugation at 4°C; protein concentrations were determined by Bio–Rad protein assay reagent (Bio–Rad Laboratories, Hercules, CA) and confirmed by Coomassie staining.

For immunoprecipitations, aliquots of lysates (1 mg of proteins) were incubated with 5 μg of anti-HNF4α rabbit polyclonal antibody (H-171; Santa Cruz Biotechnology) or with 5 μg of anti-LEF1 rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) or with 5 μg anti–β-catenin mouse monoclonal antibody (BD Transduction Laboratories) overnight at 4°C. Immunocomplexes were collected on protein A-sepharose beads (GE Healthcare, Fairfield, CT). The beads were washed 3 times with 1% NP-40 lysis buffer then boiled for 3 minutes in SDS sample buffer. For Western blot analysis, proteins from cell lysates were resolved on 10% SDS-PAGE and transferred to Pure Nitrocellulose membrane (Bio–Rad Laboratories). Blots were blocked in TBS-T (TBS plus 0.05% Tween-20) containing 5% nonfat, dried milk and probed with anti-HNF4α (C-19) goat polyclonal antibody (Santa Cruz Biotechnology) or anti-LEF1 (C-19) goat polyclonal antibody (Santa Cruz Biotechnology) or anti–β-catenin mouse monoclonal antibody (BD Transduction Laboratories). Immunocomplexes were detected with horseradish peroxidase-conjugated species-specific secondary antiserum (Bio–Rad Laboratories) followed by enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL).

RNA Extraction, Reverse Transcription Polymerase Chain Reaction (PCR), and Real-Time Quantitative PCR

Total RNA was extracted from cultured cells using an RNA extraction kit (NucleoSpin RNA II, Machery, Nagel, Germany) according to the manufacturer's instructions. Single-stranded cDNA was obtained by reverse transcription of 1 μg of total RNA using MMLV reverse-transcriptase (Promega, Milan, Italy). cDNA was amplified by PCR using GoTaq enzyme (Promega, Milan, Italy). RT-qPCRs were performed using BioRad-iQ-iCycler with SYBR-green fluorophore; the reactions were carried out using iQ SYBR Green Supermix (Bio–Rad Laboratories); 40 ng of cDNA was used as template and cycling parameters were 95°C for 3 minutes, followed by 45 cycles of 95°C for 30 seconds, 60°C for 1 minute, 72°C for 30 seconds, and 60°C + 0.5°C for 10 minutes. Fluorescence intensities were analyzed using the manufacturer's software and relative amounts were obtained using the $2^{-\Delta\Delta Ct}$ method and normalized for the β-actin. Primers for RT-PCR and RT-qPCR and relative sequence Genbank accession numbers are listed in Table 1.

Transfections and Luciferase Assay

To analyze the HNF4α promoter activity, RLSCdH cells were co-transfected by Lipofectamine 2000 (Invitrogen, San Diego, CA) with 0.5 μg of the pGL3 luciferase vector, containing the proximal region of the murine HNF4α P1 promoter (−867 to +43) fused to the firefly luciferase reporter gene, together with a β-galactosidase expression vector. All transfections were performed in triplicate. The total amount of transfected DNA was standardized with the corresponding empty vector. Cells were either left untreated or treated with BIO for 24 hours. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI) 48 hours after transfection, according to the manufacturer's instructions and normalized for β-galactosidase activity.

ChIP Assay

ChIP assays were performed basically as previously described.[13] After cross-linking with 1% formaldehyde, cultured cells were scraped off in ice-cold phosphate-buffered saline plus phenylmethylsulfonyl fluoride (PMSF), centrifuged at 1000 rpm for 5 minutes at 4°C and pellets resuspended in Swelling Buffer (25 mol/L HEPES pH 7.8; 1.5 mmol/L $MgCl_2$; 10 mmol/L KCl; 0.1% mmol/L dithiothreitol; 0.5 mmol/L PMSF) plus a protease inhibitor mixture and incubated in ice for 10 minutes. The pellets, bounced 10–20 times up–down and centrifuged at 14,000 rpm for 15 minutes, were resuspended in 6 mL Sonication Buffer (50 mmol/L HEPES pH 7.9; 140 mmol/L NaCl; 1 mmol/L EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS; 0.5 mmol/L PMSF) supplemented with a protease inhibitor cocktail. Cell extracts were then sonicated to yield chromatin fragments of 100–300 bp. After centrifugation for 15 minutes at 14,000 rpm, supernatants were collected and precleared with protein A-sepharose previously blocked with 1 μg/mL of salmon sperm DNA and 1 μg/mL of bovine serum albumin overnight with rocking at 4°C. The samples

Table 1. Genbank Accession Numbers and Primers

Accession number	Gene	Oligos RT-PCR	Position from TSS
NM_008261	HNF-4α	For 5′-ACACGTCCCCATCTGAAG-3′ Rev 5′-CTTCCTTCTTCATGCCAG-3′	+233/+250 +485/+502
NM_009654	Albumin	For 5′-GTGACAAATCCCTTCACACTC-3′ Rev 5′-GTCCTCAACAAAATCAGCAGC-3′	+303/+323 +1034/+1054
NM_013697	TTR	For 5′-CTGGACTGGTATTTGTGTCT-3′ Rev 5′-TTGGCTGTGAAAACCACATC-3′	+61/+80 +360/+379
NR_001592	H19	For 5′-CTGCTGCTCTCTGGATCCTC-3′ Rev 5′-GTGGGTGGGTGCTATGAGTC-3′	+1688/+1707 +2076/+2095
NM_001033264	Gls2	For 5′-AACCCAGTGGTCTGCGCTAT-3′ Rev 5′-ACAATGGCACCAGCATTGAC-3′	+770/+789 +835/+854
NM_009052	Rex3	For 5′-AGGAGGAAGAGCGGAGCA-3′ Rev 5′-AAGCTGGTAACAGGGAGAGATC-3′	+159/+176 +727/+748
NM_001080809	Cps1	For 5′-AGCCAAGGAGCCATTGAAAA-3′ Rev 5′-ATTGAATGGCCCAGAGATGG-3′	+3745/+3764 +3803/+3822
NM_007393	β-actin	For 5′-ATGGATGACGATATCGCTGCG-3′ Rev 5′-ATCTTCATGAGGTAGTCTGTCAGG-3′	+80/+100 +631/+654
NM_008261	HNF4α	For 5′-ATCTTCTTTGATCCAGATGCCA-3′ Rev 5′-GTTGATGTAATCCTCCAGGC-3′	+994/+1015 +1070/+1089
NM_013697	TTR	For 5′-GTCCTCTGATGGTCAAAGTC-3′ Rev 5′-CTCCTTCTACAAACTTCTCATCTG-3′	+115/+134 +265/+288
NM_001033264	Gls2	For 5′-AACCCAGTGGTCTGCGCTAT-3′ Rev 5′-ACAATGGCACCAGCATTGAC-3′	+770/+789 +835/+854
NM_009052	Rex3	For 5′-AGGAGGAAGAGCGGAGCA-3′ Rev 5′-AAGCTGGTAACAGGGAGAGATC-3′	+159/+176 +727/+748
NM_007482	Arginase	For 5′-AAGCTGGTCTGCCTGGAAAAA-3′ Rev 5′-CTGGTTGTCAGGGGAGTGTT-3′	+200/+219 +490/+509
NR_001592	H19	For 5′-CTGCTGCTCTCTGGATCCTC-3′ Rev 5′-GTGGGTGGGTGCTATGAGTC-3′	+1688/+1707 +2076/+2095
NM_009992	CyP1A1	For 5′-CCGGCATTCATCCTTCGT-3′ Rev 5′-GCCATTCAGACTTGTATCTCTTGT-3′	+1320/+1337 +1363/+1386
NM_008131	GS	For 5′-CGGCCACCGCTCTGAA-3′ Rev 5′-ACATTTGCTTGATGCCTTTGTTC-3′	+102/+117 +155/+177
NM_001077514	GLT-1	For 5′-ATGTCCACGACCATCATTGC-3′ Rev 5′-ACCTCGTCGTTCTTCTTCCC-3′	+373/+392 +466/+485
NM_009803	CAR	For 5′-GCTGCCTAAGGGAAACAGGA-3′ Rev 5′-AGCAAACGGACAGATGGGAC-3′	+187/+206 +371/+390
NM_016978	OAT	For 5′-GCCCTTTCTGGCGGTTTATAC-3′ Rev 5′-TGGTTTAATGGTCAGCATTATCTCA-3′	+1017/+1037 +1064/+1088
NM_007393	β-actin	For 5′-ACCACACCTTCTACAATGAG-3′ Rev 5′-AGGTCTCAAACATGATCTGG-3′	+339/+358 +439/+458
ENSMUSG00000032315	CyP1A1	For 5′-CTATGAACTGCCAGAGCACA-3′ Rev 5′-TACTGGACCTCACTGAGACT-3′ (Consensus HNF4α)	+36494/+36513 +36587/+36606
	CyP1A1	For 5′-CTGGCTGCAGCATTGGATGA-3′ Rev 5′-TGTGCTCTGGCAGTTCATAG-3′ (Consensus LEF1)	+36344/+36363 +36494/+36513
	CyP1A1	For 5′-CCACGCCAACATCTACCTCA-3′ Rev 5′-AGTCCCTTCATGTGCTCTGG- 3′ (Interconsensus region)	+36473/+36492 +36504/+36520

Table 1. Continued

Accession number	Gene	Oligos RT-PCR	Position from TSS
ENSMUSG00000026473	GS	For 5′-GCAAGCCAGTTAAGGAGGGA-3′ Rev 5′-CTCCCGTAGCCCTCGAATAG-3′ (Consensus HNF4α)	−2774/−2755 −2483/−2463
	GS	For 5′-ATCGTGTTCACTGCTCCCAT-3′ Rev 5′-GGTGTAGACCAGCAACAA-3′ (Consensus LEF1)	−2351/−2331 −2159/−2142
	GS	For 5′-GCAAGCCAGTTAAGGAGGGA-3′ Rev 5′-GGTGTAGACTCAGCAACAA-3′ (Interconsensus region)	−2774/−2574 −2160/−2142
ENSMUSG00000000031	H19	For 5′-CGTGACGACTCACCCAGAAAA-3′ Rev 5′-CGGAGTATGAACAGCCTACG-3′ (Consensus HNF4α)	−580/−560 −456/−437
	H19	For 5′-CTCTCCACGCTGTGCAGATT-3′ Rev 5′-CCGCCTATAACCGATTCTGT-3′ (Consensus LEF1)	−2252/−2233 −2020/−2001
ENSMUSG00000061808	TTR	For 5′-CAACACTGTCAGACTCAAAG-3′ Rev 5′-CTGCCAAGCTGACTCCAAAC-3′ (Consensus HNF4α)	−301/−282 −80/−62
	TTR	For 5′-GGTCATCAGTAGTTTTCCAT-3′ Rev 5′-TAGTTCTGACCAACTACCAA-3′ (Consensus LEF1)	−1821/−1802 −1651/−1632
ENSMUSG00000044005	Gls2	For 5′-CAGATCTCATTACAGATGAT-3′ Rev 5′-GACAGTGTACTTACATACATT-3′ (Consensus HNF4α)	−2560/−2541 −2433/−2413
	Gls2	For 5′-CTGAGTACTACGGCAACAAG-3′ Rev 5′-Rev 5′-TCATTGCTAGGCGTCATGGA-3′ (Consensus LEF1)	−988/−969 −680/−661
ENSMUSG00000029580	β-actin	For 5′TTACGCCTAGCGTGTAGACTCT-3′ Rev 5′AATACTGTGTACTCTCAAGATGGA-3′	+219/+241 +378/+402

were centrifuged and the supernatants (precleared soluble chromatin) divided into 1-mL aliquots in Eppendorf tubes for IP. From each supernatant, 1/20th of the amount used for IP were utilized as input sample. Immunoprecipitation was performed adding 5 μg of anti-HNF4α rabbit polyclonal antibody (H-171; Santa Cruz Biotechnology) or anti-LEF1 rabbit polyclonal antibody (Cell Signaling Technology), or without specific antibody as control, rotating samples for 2 hours at 4°C, adding protein A-sepharose beads to collect the antibody/protein/DNA complexes and finally incubating overnight at 4°C by constant rotation. The beads were centrifuged at 6,000 rpm for 3 minutes and washed twice with 1 mL of sonication buffer, twice with 1 mL of wash buffer A (50 nmol/L HEPES pH 7.9; 500 mmol/L NaCl; 1 mmol/L EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS; 0.5 mmol/L PMSF) supplemented with protease inhibitor cocktail, twice with 1 mL of wash buffer B (20 mmol/L Tris pH 8.0; 1 mmol/L EDTA; 250 mmol/L LiCl; 0.5% NP-40; 0.5% Na-deoxycholate; 0.5 mmol/L PMSF, protease inhibitors), and twice with 1 mL of Tris-EDTA buffer. Precipitates were then extracted twice with 200 μL of elution buffer (50 mmol/L $NaHCO_3$; 1% SDS; 1 nmol/L EDTA; 50 mmol/L Tris pH 8.0) incubating at 65°C for 10 minutes and centrifuging at 14,000 rpm for 1 minute. Both elutions were combined, supplemented with 21 μL of NaCl 4 mol/L, and incubated overnight at 65°C to reverse formaldehyde cross-linking. Samples, previously incubated at 37°C for 1 hour with 10 μg of RNAse, were then treated with 20 μg of proteinase K for 1 hour at 51°C plus 4 μL of EDTA 0.5 mol/L. Chromatin was extracted with phenol/chloroform/isoamyl alcohol, precipitated at −20°C overnight with ethanol in the presence of tRNA as carrier, and finally resuspended in 100 μL 10 mmol/L Tris (pH 7.5). For PCR amplification, 1 μL of each ChIP reaction and 1 μL of a 1:20 dilution of starting chromatin

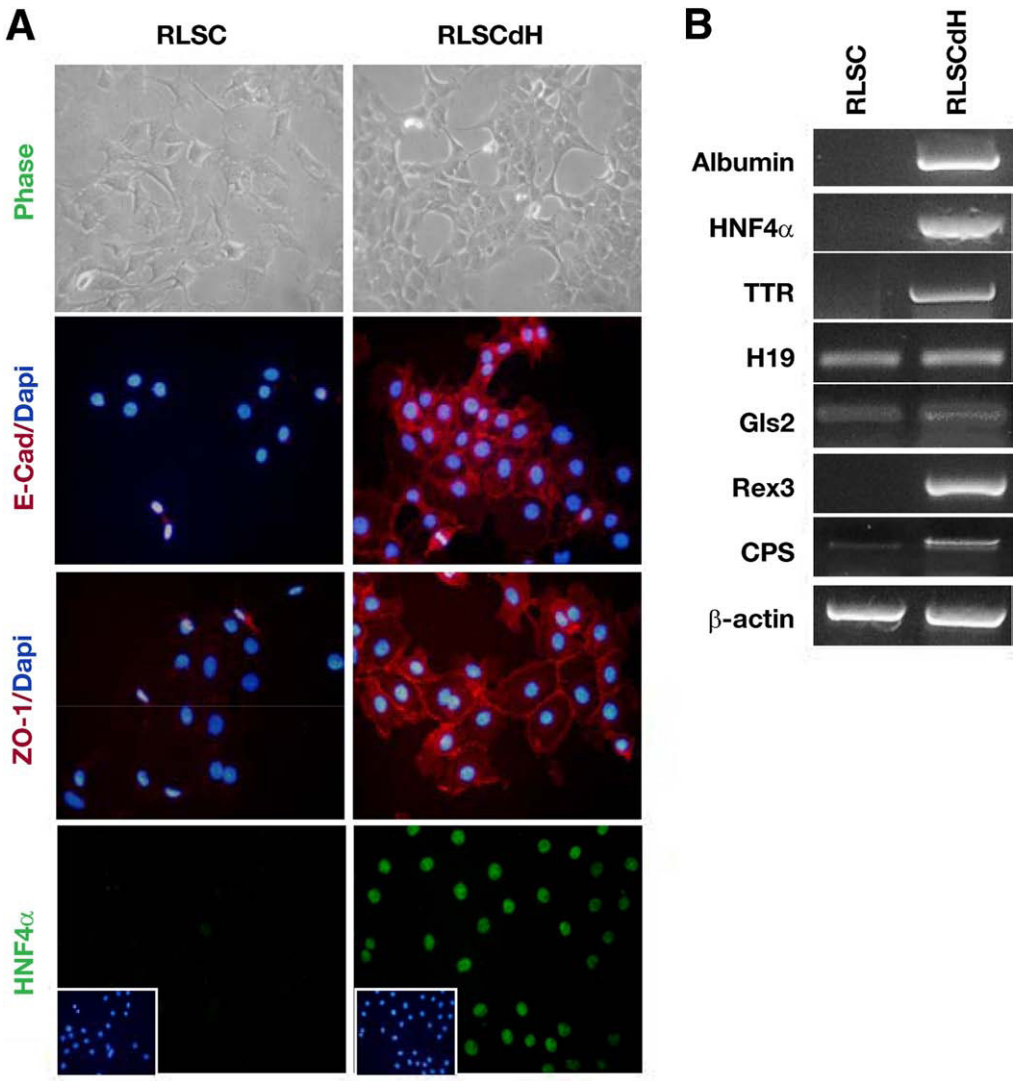

Figure 1. RLSC spontaneously differentiate in PP hepatocytes. (*A*) Phase-contrast micrographs and immunofluorescence analysis for the polarization markers E-cad and zonula occludens 1 and for HNF4α of RLSC and their counterpart differentiated into hepatocytes (RLSCdH). (*B*) Comparative RT-PCR analysis between RLSCs and RLSCdH cell lines for the indicated liver specific transcriptional factors and hepatic products; β-actin amplification was used for template normalization.

DNA (Input) were used as templates and 35 PCR cycles were performed by using the primers listed in Table 1.

Results

Hepatocytes Derived From RLSCs Show a PP Phenotype

We first analyzed the RLSC spontaneous differentiation program. Within a few weeks of culture, these cells, characterized by a flattened morphology and growing in a scattered fashion, spontaneously acquired a cuboidal and tightly packed hepatocyte-like morphology. These differentiated cells, here indicated as RLSC-derived hepatocytes (RLSCdH), expressed the polarity markers zonula occludens 1 and E-cad as well as the liver enriched transcriptional factor HNF4α (Figure 1*A*).

Next, we analyzed whether RLSCdH recapitulate one of the mutually exclusive PP or PV molecular phenotypes. Notably, RT-PCR analysis demonstrated that RLSCdH express, together with albumin and TTR, several PP markers, such as H19[14], Gls2[4], reduced in expression 3 (Rex3[14]) and carbamoyl-phosphate synthetase 1 (CPS[4]), but not the PV markers Cyp1a1,[14] GS[14], glutamate transporter 1 (GLT-1[4]), constitutive androstane receptor (CAR[14]), and ornithine amino transferase (OAT[4]) (Figures 1*B* and 2*A*).

Thus, the in vitro spontaneous differentiation of RLSC into RLSCdH results in the acquisition of a PP phenotype.

Inhibition of GSK3β Activity in RLSCdH Induces a Reversible Switch From PP to PV Phenotype

Although animal models allowed the demonstration that Wnt centroportal gradient correlates with PV hepatocyte markers expression, the study of conversion of distal PP hepatocytes into PV as well as the negative influence of β-catenin–mediated canonical Wnt signaling on PP phenotype cannot be easily assessed in vivo.

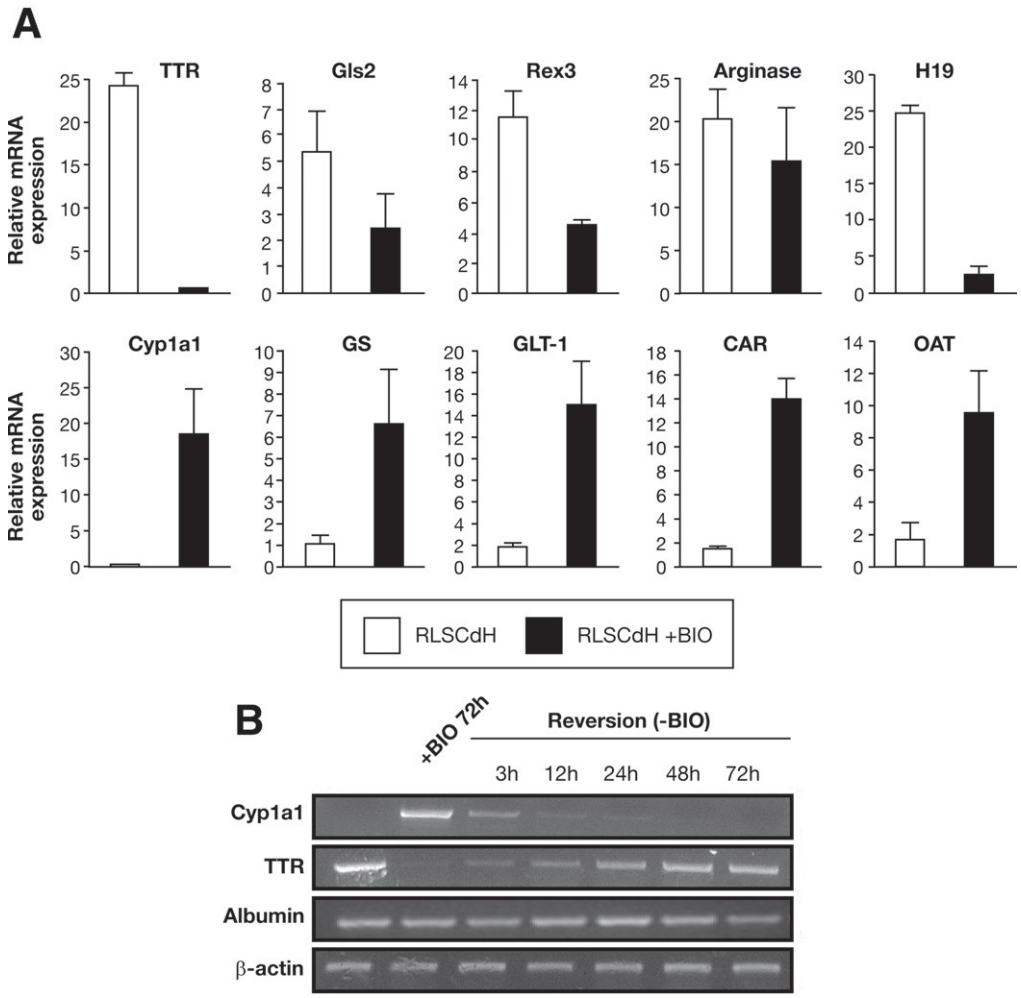

Figure 2. Inhibition of GSK3β activity in RLSCdH changes the phenotype from PP to PV in a reversible manner. (*A*) qRT-PCR analysis for the indicated genes on RLSCdH treated or not with BIO for 72 hours. The real-time PCR results were calculated by the $\Delta\Delta$CT Method. (*B*) Semi-quantitative RT-PCR analysis for TTR and Cyp1a1 mRNAs from RLSCdH untreated (−), treated with BIO for 72 hours and treated with BIO for 72 hours and then cultured for the indicated times in absence of the inhibitor (Reversion, -BIO); β-actin and the nonresponsive albumin amplifications were used for template normalization.

We analyzed the RLSCdH transcriptional PP and PV profiles upon treatment with the indirubin BIO, a widely employed pharmacologic inhibitor of GSK-3β function that mimics Wnt canonical signaling activation, inducing efficient β-catenin stabilization.[15,16]

Interestingly, RLSCdH, which are responsive to BIO as assessed by a TopFlash luciferase reporter assay (data not shown), modified their PP phenotype in response to the GSK3β inhibition. Figure 2*A* shows the results of a qRT-PCR analysis performed on cells treated with BIO for 72 hours. In this condition, PP markers such as Gls2, Rex3, arginase and H19, as well as TTR, were found to be down-regulated and the PV markers Cyp1a1, GS, GLT-1, CAR and OAT resulted significantly induced. Thus, pharmacologic Wnt pathway activation (i) interferes with PP and (ii) induces PV gene expression. Notably, the BIO effect was reversible, as demonstrated by semiquantitative RT-PCR analysis of BIO-treated cells at different time points after withdrawal of this inhibitor; the BIO-inhibited TTR expression was progressively restored, whereas the BIO-induced Cyp1a1 expression was repressed in a few hours (Figure 2*B*).

HNF4α Interacts With the Wnt Canonical Signaling Pathway Effector LEF1

A recent study by Stanulovic et al[10] revealed that HNF4α deficiency causes expression of PV genes in PP hepatocytes. Because 7 out of 10 genes we proved to be BIO-responsive in RLSCdH have been described as HNF4α-dependent (ie, TTR, H19, Arg1[19]; GS, OAT[10]; Cyp1a1,[17] CAR), we asked whether Wnt-mediated canonical signaling pathway converges on HNF4α transcriptional activity in controlling hepatic zonation.

First, we considered the possibility that GSK3β inactivation modulates HNF4α isoform-specific mRNA steady-state levels. Although we found that both HNF4α1 and HNF4α7 are expressed in RLSCdH (data not shown), both qRT-PCR analysis of endogenous HNF4α (Figure 3*A*) and luciferase assay using an HNF4α promoter construct (Figure 3*B*) demonstrated that HNF4α transcription is not affected by BIO treatment. Furthermore,

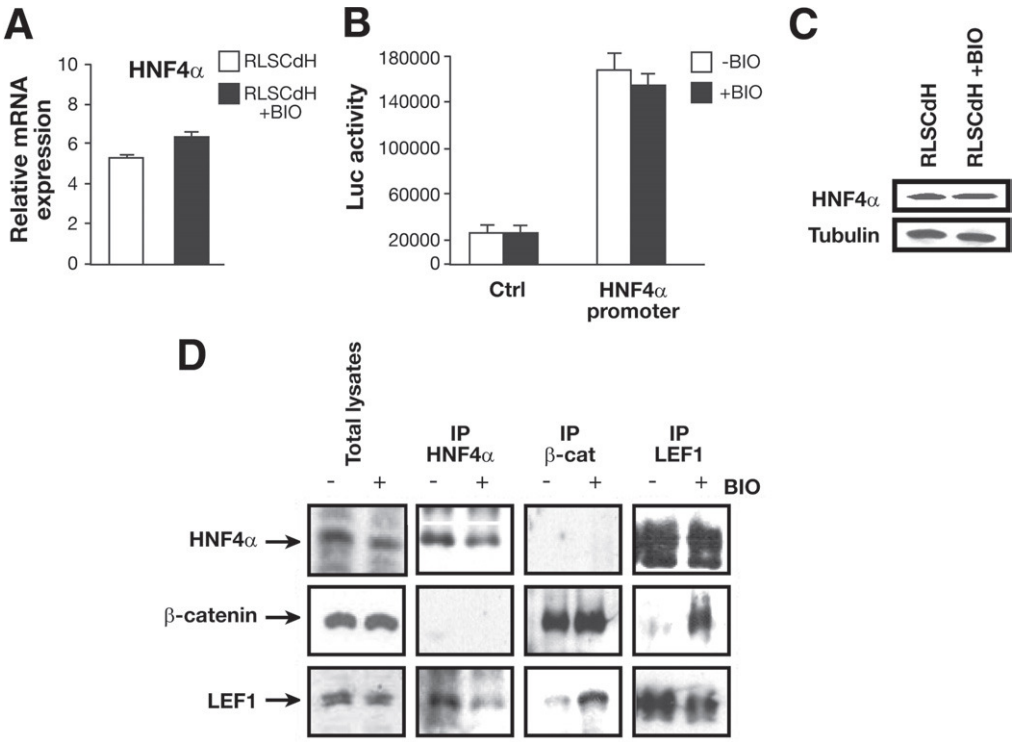

Figure 3. HNF4α is not a Wnt target gene, but interacts with LEF1. qRT-PCR analysis of HNF4α transcripts from RLSCdH treated or not with BIO for 72 hours. The real-time PCR results were calculated by ΔΔCT Method. (*A*) HNF4α promoter Luciferase activity. RLSCdH cotransfected with the reporter construct carrying the proximal −867/+43 region of the HNF4α P1 promoter or with the pGL3basic vector (Ctrl), were treated or not with BIO for 72 hours. Data represent the mean values ± SD of 3 independent experiments. (*B*) Western blot analysis of HNF4α expression in RLSCdH treated or not with BIO for 72 hours. Protein amount was normalized by immunoblotting for tubulin. (*C*) Co-immunoprecipitation analysis of HNF4α, LEF1, and β-catenin. Lysates from RLSCdH cells treated or not with BIO for 72 hours, were subjected to immunoprecipitation with anti-HNF4α, or anti-LEF1, or anti–β-catenin antibodies and protein A sepharose. After SDS/PAGE, blots were probed with the indicated antibodies (see Methods). Aliquots (7%) of the total cell extracts were used for Western blot analysis as a control for expression level.

HNF4α was neither influenced at protein level as revealed by Western blot analysis (Figure 3*C*). Altogether, these data exclude that HNF4α is a Wnt target gene.

Next, we asked whether HNF4α, LEF1, and β-catenin directly cooperate in their functions. To this aim, we performed experiments of co-immunoprecipitations by using total protein lysates from RLSCdH. We found that endogenous proteins HNF4α and LEF1 can be reciprocally immunoabsorbed from common complexes, whereas an interaction between HNF4α and β-catenin was not detected (Figure 3*D*). These results provide the first intriguing evidence of a biochemical interaction between HNF4α and the Wnt canonical pathway effector LEF1, thus supporting the hypothesis of a convergence between these 2 molecular pathways in liver zonation.

Inhibition of GSK3β Activity Modifies HNF4α and LEF1 Recruitment on "Zonated" Genes

The biochemical evidence of the HNF4α/LEF1 interaction prompted us to investigate functional cooperation of these transcriptional factors in the chromatin context by means of ChIP assays. We focused our analysis on GS and Cyp1a1, prototypes of PV genes, on Gls2 and H19, prototypes of PP genes, as well as on TTR; these genes are in fact all modulated by BIO in RLSCdH and described to be HNF4α targets.[10,17–19]

Figure 4 illustrates the results of ChIP assays performed on the PV genes GS and Cyp1a1 regulatory regions, using either anti-LEF1 (Figure 4*A* and *C*) or anti-HNF4α (Figure 4*B* and *D*) antibodies. Interestingly, we found that BIO treatment, which activates transcription of these genes, induces LEF1 recruitment not only on its own binding site but also on HNF4α site; in particular, on the Cyp1a1 gene, both transcription factors were found on HNF4α consensus whereas for the GS gene, their binding seemed to be mutually exclusive. The PCR analysis (Figure 4*C*) of amplicons spanning LEF1 and HNF4 interconsensus regions in the GS gene ruled out the possibility that ChIP with LEF1 antibody pulled down DNA fragments with both LEF1 and HNF4 sites. For Cyp1a1, the results obtained are less interpretable (Figure 4*A*).

On the other hand, regarding TTR and the PP genes Gls2 and H19, the BIO-induced transcriptional repres-

sion correlates with both LEF1 binding and HNF4α displacement from their own consensus sites. In particular, LEF1 binds its own consensus site exclusively upon BIO treatment (Figure 5*A*, *C*, and *E*), whereas HNF4α, which is constitutively bound to DNA, is displaced by GSK3β inhibition (Figure 5*B*, *D*, and *F*). Thus, Gls2, H19, and TTR transcriptional activity correlates directly to the HNF4α binding to its own binding site, whereas LEF1 seems not to be a positive regulator. Figure 6 summarizes all described results.

Altogether these data provide new molecular basis for the Wnt- and HNF4α-mediated transcriptional activation and repression of zone-specific expressed genes.

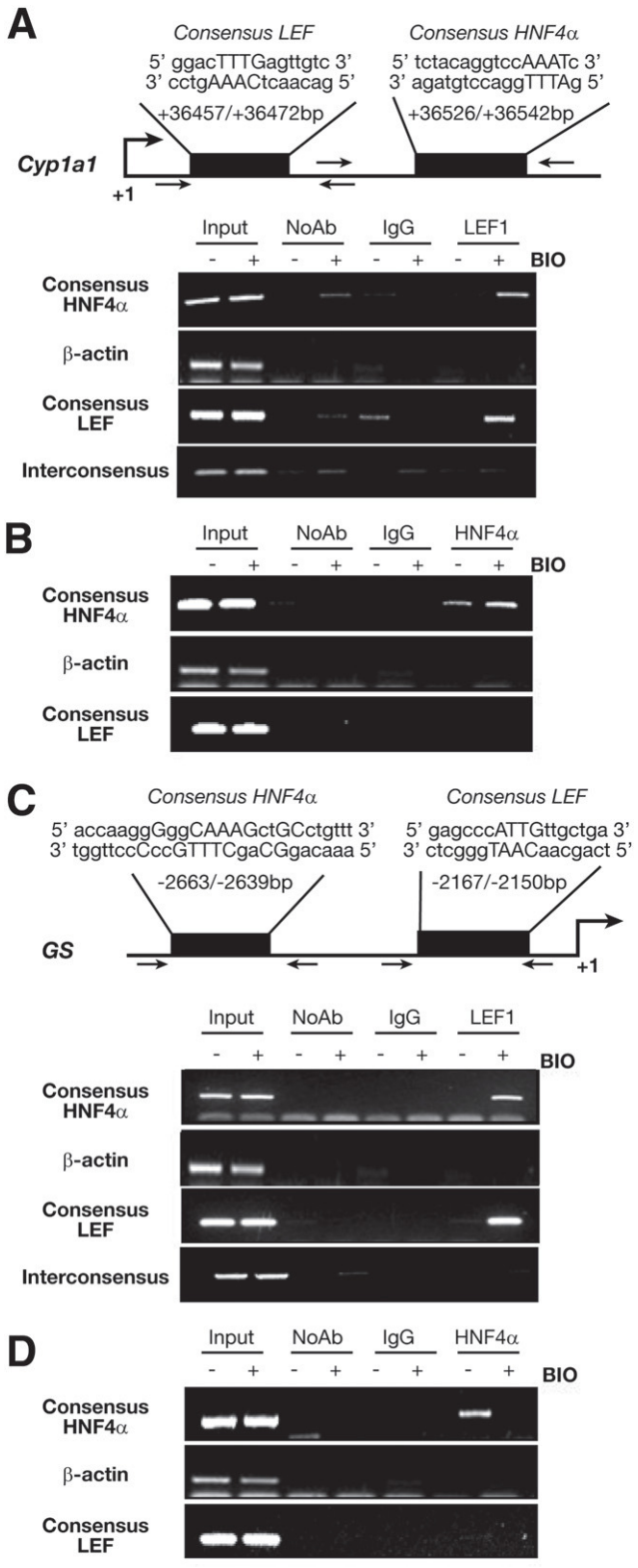

Figure 4. Inhibition of GSK3β activity influences the binding of HNF4α and LEF1 on PV GS and Cyp1a1 genes. ChIP analysis with anti-LEF1 (*A* and *C*) or anti-HNF4α (*B* and *D*) antibodies of chromatin derived from RLSCdH treated or not with BIO. As controls, ChIPs were also performed without antibody or with unrelated IgG. β-Actin gene sequence was amplified as control unrelated DNA. A 1:20 dilution of starting chromatin DNA was used as PCR template for input normalization. The consensus sites for the transcriptional factors LEF1 and HNF4α in the regulatory region of GS and Cyp1a1 are depicted as *black boxes* and their double-strand sequences and positions relative to the transcription start point (+1) are indicated. PCR primers are represented as *dotted arrows* and listed in Table 1.

Discussion

The specific gene expression repertoire of single hepatocyte, is correlated with its anatomic location in the hepatic lobule and, therefore, with the different demand of liver functions. How the liver-specific gene expression is zonally controlled by the microenvironmental signals is a relevant issue in experimental hepatology.

In vivo evidence arising from genetic manipulation and from liver tumors, although indicating the involvement of specific morphogenetic factors (ie, oxygen, Wnts, and growth factors) and cellular effectors (ie, β-catenin and Ras), suggest that hepatocytes zonation is a complex phenomenon and that it cannot be reconducted to a single specific effector.

The main finding of this work was to unveil that LEF1 interacts with HNF4α and, upon β-catenin stabilization, may be also recruited on HNF4 DNA binding sites, thus providing first evidence that may explain how the Wnt signaling pathway influences the zonal control of liver gene expression.

In the attempt to contribute to the clarification of molecular mechanisms controlling zonation, we took advantage of a cellular model that we recently developed,[11] the RLSC, which spontaneously differentiates into PP hepatocytes (RLSCdH from RLSC-derived hepatocytes). This is a first, provocative result and suggests that PP hepatocytes could represent the initial differentiation step of liver precursors. In line with this possibility are the in vivo observations that (i) the stem cells compartment is located around portal tracts and (ii) during liver growth hepatocytes increase the distance from the portal tract toward central vein, although the

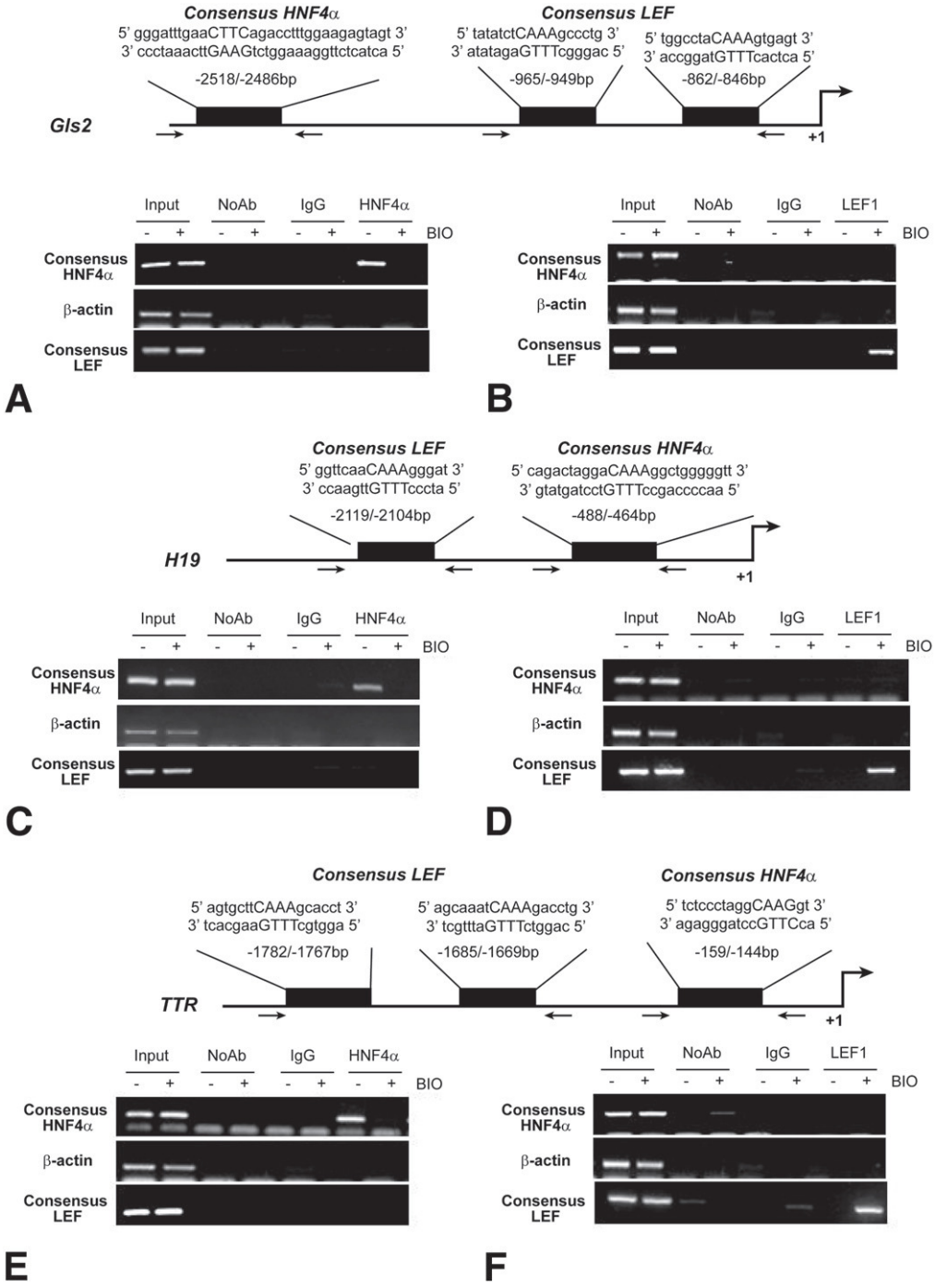

Figure 5. Inhibition of GSK3β activity in RLSCdH influences the recruitment of HNF4α on PP Gls2, H19 genes, and on TTR gene. ChIP analysis with anti-HNF4α (*A*, *C*, *E*) or anti-LEF1 (*B*, *D*, *F*) antibodies of chromatin derived from RLSCdH treated or not with BIO. As controls, ChIPs were also performed with no antibody or with unrelated IgG. β-Actin gene sequence was amplified as unrelated DNA control. A 1:20 dilution of starting chromatin DNA was used as PCR template for input normalization. The consensus sites for the transcriptional factors HNF4α and LEF1in the regulatory regions of Gls2, H19, and TTR are depicted as *black boxes* and their double-strand sequences and positions relative to the transcriptional start point (+1) are indicated. PCR primers are represented as *dotted arrows* and listed in Table 1.

proliferation gradient decreases.[20,21] A second, relevant observation came from the analysis of RLSCdH differentiation pattern after treatment with the GSK3β-inhibitor BIO, which mimics Wnt pathway activation. RLSCdH respond to this chemical with modification of their transcriptional profile, inducing all PV and repressing all PP genes analyzed, coherently recalling what unveiled for PV hepatocytes in vivo. Moreover, we conclude that both regulatory processes, induction and repression of subsets of genes, can be ascribed to the Wnt pathway activity and thus, at least for two of them (Cyp1a1 and TTR), in a notably reversible fashion.

A similar, dual role of activator and repressor of zonated genes has been proposed also for HNF4α on the basis of the results obtained with knockout mice. In fact, its genetic depletion resulted in both the activation of PV genes (ie, GS and OAT) and the impairment of the PP gene PEPCK in PP hepatocytes.[10] On the other hand, the quite homogenous expression of HNF4α throughout the hepatic lobule[22] suggests the existence of further elements controlling zonation.

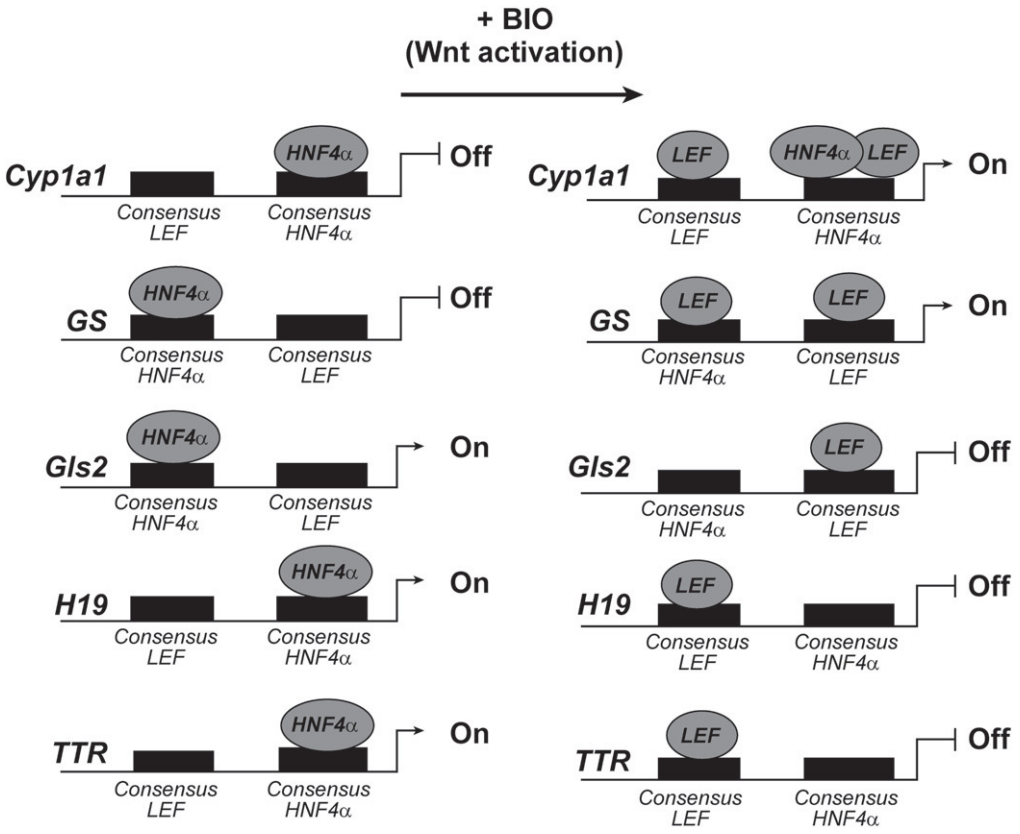

Figure 6. Schematic depiction of LEF1 and HNF4α consensus sites occupancy in RLSCdH in relation with BIO treatment.

Our data indicate Wnt as a regulative element of the differential HNF4α transcriptional activity.

In this work, we sought to explore whether Wnt-mediated canonical signaling converges on HNF4α transcriptional activity. Ruling out the possibility that HNF4α is a Wnt target gene, we surprisingly observed, by means of reciprocal coimmunoprecipitation experiment, a biochemical interaction between the endogenous downstream Wnt transcriptional factor LEF1 and HNF4α. This unexpected biochemical evidence does not provide per se any indication of its occurrence in the context of chromatin or of its functional significance in the process of liver zonation. Therefore, we performed ChIP assays on HNF4α-dependent regulatory regions of "differentially zonated" genes. We focused our analysis on GS and Cyp1a1, prototypes of PV genes, and on Gls2 and H19, prototypes of PP genes as well as on the TTR gene, all modulated by BIO in RLSCdH and described to be HNF4α targets.[10,17–19] The inspection of both HNF4α and LEF1 consensus binding sites of these genes gave us interesting evidences for proposing the schematic model depicted in Figure 6.

Concerning GS regulatory region, we found that HNF4α binds its own consensus site only when the gene is not transcribed; on the contrary, transcription correlates with LEF1 recruitment on its own binding site and, surprisingly, on HNF4α *cis*-element. For Cyp1a1, we found that HNF4α binds its own *cis*-element irrespective of transcriptional activity; gene expression is instead induced when LEF1 is recruited to its own consensus. Also for this gene we gathered evidence suggesting LEF1 binding to HNF4a consensus site, thus paralleling the observation made for GS. However, the closed proximity of the 2 consensus sites on this promoter may result in ChIP amplicons covering both consensus sites. Even if such amplicons are not found in the reciprocal HNF4a antibody ChIP assay, suggesting a chromatin fragmentation appropriate for our analysis, we prefer to leave open this specific matter.

Our data, although obtained analyzing only 2 exemplificative PV genes, suggest a repressive role for HNF4α; this seems to be in line with the previous report of Stanulovic et al,[10] where HNF4α was found to be necessary for GS PP suppression, through the recruitment of HDAC on its regulatory regions.

The unexpected finding of a β-catenin–induced LEF1 recruitment, not only on its consensus site but also on that of HNF4α, suggests that the transcriptional activation of PV genes may require an LEF1-mediated inhibition of HNF4α repressive activity. On the other hand, for the PP genes Gls2 and H19, and for TTR, we found that during their expression HNF4α is bound to its own DNA binding sites, whereas upon activation of Wnt/β-catenin pathway (ie, when transcription is re-

pressed) HNF4α is displaced, concomitant with the recruitment of LEF1 only to its own consensus sites. Although, for these genes, HNF4α clearly seems to explicate a positive role in transcription, further studies are necessary to address the BIO-mediated mechanism(s) of its displacement.

Overall, we provided new evidence of cooperation among a microenvironmental signal and a tissue-specific factor in maintaining the heterogeneity of gene expression within the same histotype.

We propose that both PP and PV hepatocytes actively respond to influences arising from the same effectors, namely HNF4α and Wnt signaling, resulting, within the same cell, in negative and positive transcriptional activity independence on different promoter structures.

This is in line with other, previously identified, tissue-specific mechanisms conferring specificity to Wnt signaling. For example, myoblast differentiation requires the cross-talk between Wnt/LEF1 and BMP-2, whereas the physical interaction between Alx-4 and LEF1 gives rise to Wnt pathway mesenchymal-specific activities.[23,24] Further experiments shall dissect molecular machinery that, most likely through epigenetic modification, mediates the activation and repression of zonated genes in the hepatocyte. Moreover, we believe it would be desirable to extend our observations to hepatocytes isolated from specific zones of liver lobules. Indeed, we believe that certain data indicate that further elements control in vivo zonation, for example (i) the TTR gene, found strongly BIO-inhibited in our system, is not repressed in PV hepatocytes in vivo; and (ii) other genes whose zonality depends on Wnt dynamically respond also to metabolic or hormonal changes (ie, CPS, OAT, and CyPs).[4]

Finally, Wnt activation has also been related to proliferation of hepatic precursors cells (ie, oval cells[25]) that are believed to be located in the PP region where Wnt is low. This suggests that in conditions favoring the precursor compartment expansion Wnt gradient and in turn hepatic zonation may be perturbed as intriguingly it occurs during posthepatectomy regeneration.[26]

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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