# Expression of Wilms' Tumor Suppressor in the Liver With Cirrhosis: Relation to Hepatocyte Nuclear Factor 4 and Hepatocellular Function

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**The Wilms' tumor suppressor WT1 is a transcriptional regulator present in the fetal but not in the mature liver. Its expression and functional role in liver diseases remains unexplored. In this study, we analyzed WT1 expression by reverse-transcription polymerase chain reaction (RT-PCR) and by immunohistochemistry in normal and diseased livers. In addition, we performed *in vitro* studies in isolated rat hepatocytes to investigate WT1 regulation and function. We detected WT1 messenger RNA (mRNA) in 18% of normal livers, 17% of chronic hepatitis with minimal fibrosis, 49% of chronic hepatitis with bridging fibrosis, and 71% of cirrhotic livers. In cirrhosis, WT1 immunoreactivity was localized to the nucleus of hepatocytes. WT1 mRNA abundance correlated inversely with prothrombin time ($P = .04$) and directly with serum bilirubin ($P = .002$) and with the MELD score ($P = .001$) of disease severity. In rats, WT1 expression was present in fetal hepatocytes and in the cirrhotic liver but not in normal hepatic tissue. *In vitro* studies showed that isolated primary hepatocytes express WT1 when stimulated with transforming growth factor β (TGF-β) or when the cells undergo dedifferentiation in culture. Moreover, we found that WT1 down-regulates hepatocyte nuclear factor 4 (HNF-4), a factor that is essential to maintain liver function and metabolic regulation in the mature organ. Hepatic expression of HNF-4 was impaired in advanced human cirrhosis and negatively correlated with WT1 mRNA levels ($P = .001$). In conclusion, we show that WT1 is induced by TGF-β and down-regulates HNF-4 in liver cells. WT1 is reexpressed in the cirrhotic liver in relation to disease progression and may play a role in the development of hepatic insufficiency in cirrhosis.** (HEPATOLOGY 2003;38:148-157.)

The presence of portal hypertension and the development of hepatic insufficiency determine the evolution and prognosis of cirrhosis. Although there is abundant information pertaining to the cellular and molecular basis of fibrogenesis, the factors and mechanisms leading to the decay of liver function are not well understood. In cirrhotic patients, hepatic insufficiency is frequently associated with reduced liver weight,[1] but the breakdown of hepatic functions cannot be merely ascribed to a diminished parenchymal mass. A functional defect of existing hepatocytes rather than a reduced number of liver cells appears to be the main cause of hepatocellular failure in cirrhosis. In fact, recent data have shown that the failing cirrhotic liver is characterized by the presence of a high proportion of hepatocytes that do not express gene products that typify the mature hepatocyte phenotype.[2] The molecular changes that promote the loss of differentiated hepatocellular functions in cirrhosis still remain undefined.

The Wilms' tumor suppressor WT1 is a developmentally regulated transcriptional factor that plays a critical role in organ-specific differentiation (reviewed in Englert,[3] Menke et al.,[4] Lee and Haber,[5] and Scharnhorst et al.[6]). The WT1 gene encodes a 3.5-kb transcript, which

*Abbreviations: mRNA, messenger RNA; PCR, polymerase chain reaction; HNF-4, hepatocyte nuclear factor 4; SAM, S-adenosylmethionine; TGF-β, transforming growth factor β; CMV, cytomegalovirus; MELD, Model for End-Stage Liver Disease; MAT2A, methionine adenosyltransferase 2A.*

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**Table 1. Primers Used in This Study**

| Gene | Sense Primer (5′-3′) | Antisense Primer (5′-3′) |
|---|---|---|
| β-Actin (human) | AGCCTCGCCTTTGCCGA | CTGGTGCCTGGGGCG |
| β-Actin (rat) | CAACCTCCTTGCAGCTC | CTGGTGCCTAGGGCG |
| Aldolase B | CAGAGCATTGTTGCCAATG | GAGAGGCCATCAAGCCCTTG |
| HNF-4α | CTGCACCCTCACCTGATGC | GGCTGGGGGATGGCAGAG |
| MAT2A | ATGCTGTCCTTGATGCAC | GCGTAACCAAGGCAATG |
| Rat seroalbumin | ATGAAGTGGGTAACC | TGTCAGACTCTGTGC |
| Vimentin | TCCTACCGCAGGATGTTCGG | GCACCTTGTCGATGTAGTTGG |
| WT1 | | |
| TD (touchdown) (exon 8-exon 10) | GTGAGAAACCATACCAGTGTG | GTGCAAGGAGGTATGTACAT |
| Multiplex (exon 2-exon 10-2) | ATGAGGATCCCATGGGCCAGCA | GCGCCAGCTGGAGTTTGGTC |
| Real time PCR | | |
| exon 1-exon 4 (human) | AGCCCGCTATTCGCAATCAGG | GTGGCTCCTAAGTTCATCTG |
| exon 4-exon 7 (human) | GGAATCAGATGAACTTAGGAG | CGTTTCTCACTGGTCTCAGATGCCG |
| exon 4-exon 7 (rat) | GGAACCAGATGAACCTCGGAG | CGCTTCTCACTGGTTTCAGATGCTG |
| exon 8-exon 10-2 (human) | GTGAGAAACCATACCAGTGTG | GCGCCAGCTGGAGTTTGGTC |
| Phosphothiorated oligonucleotides | CAGCAAATGGGCTCCGAC | GTCGGAGCCCATTTGCTG |

can undergo alternative splicing, resulting in 4 isoforms differing by the presence or absence of exon 5 (plus or minus exon 5 isoforms) or 3 amino acids encoded by the 3′ end of exon 9 (plus or minus KTS isoforms). WT1 possesses an N-terminal transactivational domain and a carboxy terminus containing 4 contiguous Cys2-His2 zinc fingers mediating sequence-specific DNA binding. WT1 is a multifaceted gene that can transcriptionally regulate a diversity of growth and differentiation-related genes. It can also bind messenger RNA (mRNA) and has been proposed to participate in the splicing apparatus.[7-9] WT1 expression is both developmental and tissue restricted, being detected in fetal liver[4,6] but not in normal adult liver.[10,11] In the present work, we have investigated the expression of WT1 in hepatic tissue of patients with liver diseases and the relationship of this factor to hepatocellular function.

## Patients and Methods

***Patients.*** We obtained specimens of liver tissue from 4 groups of subjects: (1) control individuals (n = 28; 19 male; mean age, 50.8; range, 18-73 years) with normal or minimal changes in the liver. We collected tissue samples at surgery (17 cases) of digestive tumors or from percutaneous liver biopsy performed because of small changes in liver function tests (10 cases) or to confirm complete response of chronic hepatitis C to interferon therapy (1 case); (2) chronic hepatitis C with no or only portal fibrosis (score[12] F0-F1) (n = 24; 17 male; mean age, 39.2; range, 19-61 years); (3) chronic hepatitis C with bridging fibrosis (score F3) (n = 39; 25 male; mean age, 50.6; range, 23-69 years); and (4) cirrhosis (n = 73; 50 male; mean age, 56; range, 20-77 years). In 46 patients of the last group, we obtained the sample at the time of liver transplantation and in 3 cases at operation for resection of hepatocellular carcinoma. Cirrhosis was due to hepatitis C virus infection in 44 cases, alcohol abuse in 18 cases, hepatitis B virus infection in 6 cases, hemochromatosis in 4, and cryptogenic in 1 case. Associated hepatocellular carcinoma was present in 13 cirrhotic patients.

***RNA Isolation and Reverse-Transcription Polymerase Chain Reaction.*** We extracted total RNA using TRI Reagent (Sigma, St. Louis, MO). We treated 2 μg of RNA with DNaseI (Gibco-BRL, Paisley, United Kingdom) prior to reverse transcription with M-MLV Reverse Transcriptase (Gibco-BRL) in the presence of RNaseOUT (Gibco-BRL) and used one tenth of the cDNA preparation for each polymerase chain reaction (PCR). We designed all primers to distinguish between genomic and cDNA amplification (Table 1) and sequenced all PCR products to confirm the specificity. We included in the study only the samples with a comparable amplification of β-actin mRNA.

***Real-Time Quantitative PCR.*** We undertook quantitative real-time RT-PCR using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) and the LC-DNA Master SYBR Green I mix in 1:200 dilution of cDNA. To monitor the specificity, we analyzed the final PCR products by melting curves and electrophoresis. We calculated the level of expression of the different genes by plotting the threshold cycle on the standard curve obtained after amplification of serial dilution of a plasmid containing the corresponding full length cDNA. Results were normalized according to β-actin quantitation in the same sample. Only those human samples with a high yield of RNA were used for quantitation of WT1 and hepatocyte nuclear factor 4 (HNF-4) mRNA.

***Immunohistochemistry.*** We dewaxed the paraffin-embedded sections in xylene and rehydrated them in decreasing grades of ethanol. Antigenic unmasking was accomplished by boiling in 10 mmol/L citrate buffer, pH 7. We blocked all preparations with 10% of goat normal serum and 0.3% of oxygen peroxide, and the samples from cultured hepatocytes were additionally blocked with the avidin-biotin blocking kit (Vector Labs., Burlingame, CA). We applied the primary antibody anti-WT1 C-19 (Santa Cruz, Santa Cruz, CA) or preimmune rabbit IgG (Santa Cruz) or phosphate-buffered saline (as negative controls) overnight, followed by the secondary biotinylated anti-rabbit antibody (Vector Labs.) and the streptavidin-biotinylated-HRP (Amersham, Buckinghamshire, United Kingdom) for 45 minutes each. We used DAB (Dako, Carpinteria, California) to visualize the peroxidase activity. Samples were counterstained in Mayer's hematoxylin and mounted in Depex. The specificity of the signal obtained with the anti-WT1 antibody was further supported by the following observations: PLC and HepG2 cell lines that were positive for WT1 expression, as assayed by RT-PCR, also proved positive by immunocytochemistry using this anti-WT1 antibody. When WT1 expression was negative by RT-PCR, such are the cases of Hep3B and HuH7 cells; immunostaining using this antibody was also negative. Upon transfection of HuH7 cells with a WT1-expressing vector, a specific signal was detected when stained with this anti-WT1 antibody. Additionally, immunostaining of rat fetal kidney with this anti-WT1 antibody gave a strong signal in the podocyte layer of developing glomeruli, the cell type that expresses highest levels of WT1 protein in this organ during development.

***Animals.*** We performed all experimental procedures in conformity with our institution's guidelines for the use of laboratory animals. Cirrhosis was induced with $CCl_4$ as previously described.[13]

***Isolation and Culture of Rat Hepatocytes.*** We isolated and cultured adult rat hepatocytes as described previously.[14] After 3 hours of culture, we again fed the hepatocytes with medium containing 0.5% fetal calf serum in the presence or absence of 4 mmol/L S-adenosylmethionine (SAM). Treatment of hepatocytes with transforming growth factor $\beta$ (TGF-$\beta$) was performed by addition of 5 ng/mL of human recombinant TGF-$\beta$1 (Roche Diagnostics) 24 hours after seeding.

Hepatocytes from 20-day-old fetal rats (Wistar) were isolated by collagenase disruption as previously described ($2.5 \times 10^6$ cells/fetus).[15] The cells were plated on noncoated plastic dishes in arginine-free medium 199 (a selective medium for liver parenchymal cell survival and growth), supplemented with ornithine (200 $\mu$mol/L), fetal bovine serum (10%), penicillin (120 $\mu$g/mL), and streptomycin (100 $\mu$g/mL). The medium was changed 4 hours after seeding.

***Transient Transfection of Rat Hepatocytes.*** We seeded hepatocytes 24 hours before transfection with the Tfx-50 reagent (Promega, Madison, WI) according to the manufacturer's instructions, and we transfected the cells in duplicate with an equimolar mixture of pCB6 plasmids encoding the 4 isoforms of WT1 (characterized by the presence or absence of the splice inserts, exon 5 and KTS) kindly provided by Dr. Jochemsen (Leiden University Medical Center, Leiden, The Netherlands). Transfection efficiency of the equimolar mixture of the 4 WT1 isoforms was monitored by RT-PCR analysis using specific primers that discriminated these isoforms. The relative expression of the corresponding mRNA was similar in all experiments. In other experiments, we transfected hepatocytes with 1 mmol/L of either sense or antisense phosphothiorated WT1 oligonucleotides (Table 1).

***CAT Assay.*** We transfected HuH7 cells in quadruplicate using Tfx-50 (Promega) with the pCAT Enhancer plasmid and the pCAT Dual WT1 promoter plasmid encompassing 1.2 kb of the 5′ region of human WT1 gene[16] (kindly provided by Dr. Fraizer from the MD Anderson Cancer Center in Houston, Texas). We measured the CAT activity using the CAT ELISA (Roche) after 18 hours of treatment with 5 ng/mL of TGF-$\beta$. To ascertain the specific effect of TGF-$\beta$ on WT1 promoter transactivation, hepatocytes were also transfected with other constructs in which the reporter gene was under the control of either rat methionine adenosyltransferase 1A[14] (*MAT1A*) (1.4 kb of the 5′ region of this gene) or cytomegalovirus (CMV) promoters.

***Statistical Analysis.*** Comparison of the distribution of WT1 expression was based on Fisher exact test and $\chi^2$ test as appropriate. Data for each continuous variable were examined with the Shapiro-Wilk W test to determine whether assumptions of normality were valid. Normally distributed data were compared among groups using an independent Student's *t* test and 1-way analysis of variance. Data not normally distributed were compared using the Kruskal-Wallis and the Mann-Whitney tests. Correlation was assessed by Spearman's or Pearson's correlation coefficients. All the tests were 2-sided, and the significance level was 5%. Descriptive data for continuous variables are reported as means $\pm$ SD or as medians and interquartile range.

## Results

***WT1 Expression in Normal Liver Tissue and in Chronic Liver Diseases.*** Using touchdown PCR, WT1 mRNA was detected in only 18% of control livers and

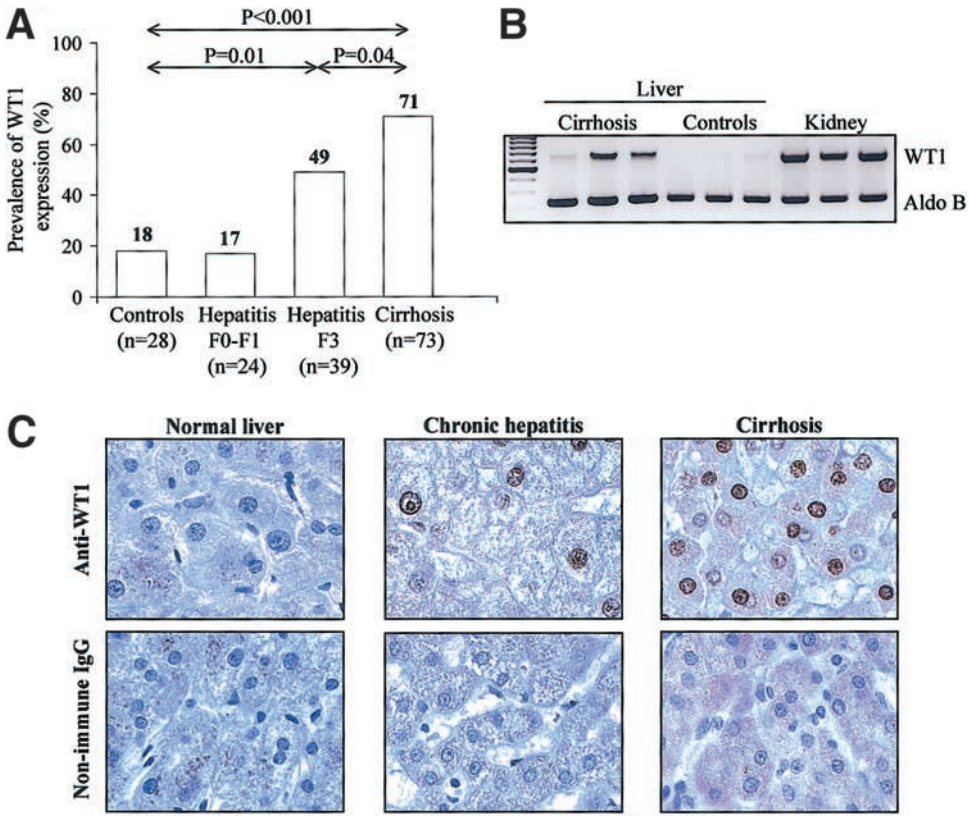


Fig. 1. Expression of WT1 in the liver of patients and controls. (A) Prevalence of WT1 mRNA (as determined by touchdown RT-PCR) in normal liver tissue (controls), patients with chronic hepatitis with fibrosis stage 0-1 (F0-F1), chronic hepatitis fibrosis stage 3 (F3), and cirrhosis. (B) An example of multiplex PCR of WT1 and aldolase B (Aldo B) in control and cirrhotic livers and in 3 independent samples of normal kidneys (a tissue that normally expresses WT1). (C) Immunohistochemical detection of WT1 protein in normal liver and liver from patients with chronic hepatitis and cirrhosis.

17% of chronic hepatitis with fibrosis stage F0-F1. In contrast, 49% of patients with chronic hepatitis with fibrosis stage 3 ($P = .01$ vs. controls) and 71% of cirrhotic patients ($P < .001$ vs. controls) showed WT1 expression (Fig. 1A). These results were confirmed by multiplex PCR with simultaneous amplification of WT1 and aldolase B (Fig. 1B). In these studies, we used human kidney (a tissue that normally expresses WT1) as a positive control. WT1 mRNA was not detected in peripheral mononuclear cells from any of the patients studied.

We performed 2 independent PCRs to amplify ±exon 5 and ±KTS isoforms. The two exon 5 isoforms were found in 40% of WT1-positive control livers (2 out of 5) and in 85% of WT1-positive cirrhotic livers (22 out of 26) ($P = .062$). In the remaining WT1-positive samples, only +exon 5 could be amplified. With respect to ±KTS isoforms, the two variants were present in all the samples tested.

Immunohistochemical studies showed no WT1 immunoreactivity in normal liver tissue. By contrast, in the cirrhotic liver, many hepatocytes showed intense WT1 staining. An intermediate level of expression was found in scattered hepatocytes in samples with chronic hepatitis. WT1 immunoreactivity localized at the nuclei of hepatocytes, showing a distinct nucleolar accumulation in some cells (Fig. 1C).

***WT1 mRNA Levels in Cirrhosis: Relationship to Hepatocellular Function.*** In 42 samples (19 control and 23 cirrhosis), we determined WT1 mRNA levels by 3 different real-time PCR reactions (Table 1). Similar results were obtained in the 3 assays, and those corresponding to exon 4-exon 7 PCR are shown in Fig. 2. We found that none of the control livers that tested negatively using touchdown PCR was positive by real-time PCR, whereas 7 out of 9 cirrhotic livers with undetectable WT1 mRNA by the first method were positive by real-time PCR. This indicates that WT1 mRNA can be found in almost all cirrhotic livers when sensitive PCR methods are used.

The values of WT1 mRNA were significantly higher in cirrhosis than in controls (73.1 WT1 copies per $10^6$ copies of $\beta$-actin; range, 16-160.9 vs. 0.00; range, 0.00-0.00, respectively; $P < .001$) and in patients with cirrhosis Child-Pugh B or C (149.95; range, 72.69-317.59) than in Child-Pugh A (14.31; range, 0.7-32.67; $P = .001$) (Fig. 2A). We found that the level of WT1 mRNA correlated with total bilirubin ($r = 0.62$, $P = .002$) and inversely with prothrombin time ($r = -0.45$, $P = .04$). A significant correlation ($r = 0.64$, $P < .001$) was found with the Model for End-Stage Liver Disease (MELD)[17] score (Fig. 2B), indicating a direct relationship between WT1 expression and disease severity.

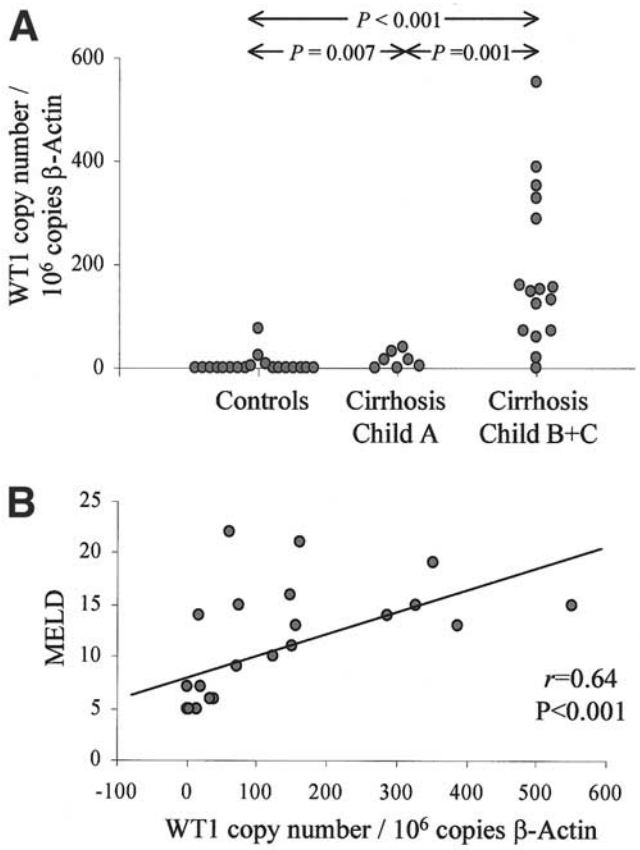


Fig. 2. Quantitation of WT1 mRNA levels by real time PCR in the liver of cirrhotic patients and liver function. (A) WT1 mRNA values in control livers and cirrhosis Child-Pugh A, B, or C. (B) Correlation between WT1 mRNA values and MELD score in cirrhosis.

***WT1 in the Cirrhotic Liver of Rats.*** The transcriptional activation of WT1 that we observed in the human fibrotic liver was paralleled by our findings in rats with experimental cirrhosis. Utilizing multiplex PCR we found that, although WT1 mRNA was undetectable in the normal rat liver, a clear amplification band was obtained in $CCl_4$-induced cirrhosis and in fetal rat hepatocytes (Fig. 3A). In these studies, we used rat kidney (a tissue that normally expresses WT1) as a positive control. In addition, real-time PCR (with exon 4-exon 7 primers) demonstrated that the content of WT1 mRNA in liver tissue was significantly higher in cirrhotic animals than in controls (25.18 ± 11.46 vs. 2.69 ± 2.62, respectively, $P < .001$) (Fig. 3B).

***Induction of WT1 by TGF-β.*** TGF-β is an essential factor in liver fibrogenesis,[18] and it is also a negative signal for hepatocellular differentiation.[19] Thus, we investigated whether this cytokine could influence WT1 expression in hepatocytes. We found that incubation of primary rat hepatocytes with TGF-β for 24 hours caused a significant increase in the levels of WT1 mRNA (Fig. 4A and B). This effect was associated with induction of vimentin, a marker of hepatocellular dedifferentiation (Fig. 4A). As shown in Fig. 4C, TGF-β–stimulated WT1 expression is completely abolished by the transcription inhibitor actinomycin D or by the translation inhibitor cycloheximide, indicating that TGF-β induces WT1 by a transcriptional mechanism requiring protein synthesis. Although some of the biologic actions of TGF-β are mediated by increasing the overall oxidative state of treated cells and enhancing the production of $H_2O_2$,[20] this effect does not seem to mediate WT1 induction because neither catalase nor desferrioxamine could abrogate the expression of WT1 after stimulation with TGF-β (Fig. 4D).

To analyze the effect of TGF-β on WT1 transcription, we performed CAT assays in HuH7 cells (that do not express WT1) transfected with a plasmid containing the reporter gene under the control of the dual WT1 promoter.[16] This construct encompasses 1.2 kb of the human WT1 promoter and includes the minimal promoter region capable of initiating transcription, as well as potential regulatory elements. We found that, at 18 hours after transfection, CAT activity was more than 3 times higher in the presence of TGF-β than in its absence (Fig. 4E), indicating that TGF-β enhances WT1 transcription by activation of WT1 promoter. When hepatocytes were transfected with reporter plasmids under the control of MAT1A[14] or CMV promoters—genes that do not respond to TGF-β—no stimulatory effect was observed after TGF-β treatment (Fig. 4E), thus supporting the specificity of the effect observed on WT1 promoter.

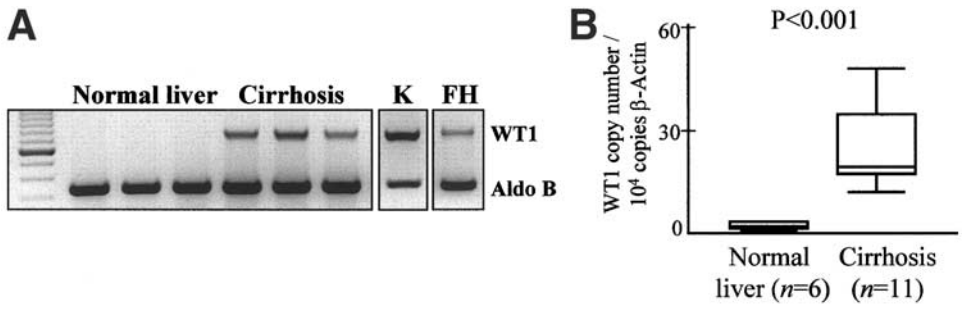


Fig. 3. WT1 mRNA in the liver of normal and cirrhotic rats. (A) Multiplex PCR amplification of WT1 and aldolase B (Aldo B) in the liver of normal rats, cirrhotic rats, and isolated fetal hepatocytes (FH). Rat kidney (K) was used as positive control. (B) Quantitative real-time PCR for WT1 mRNA in the liver of normal and cirrhotic rats.

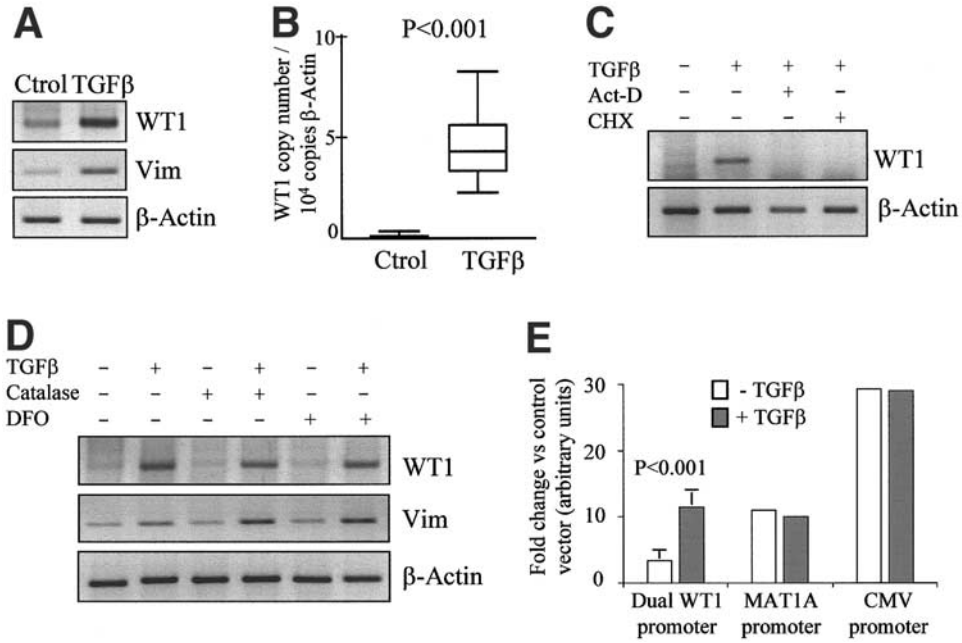


Fig. 4. Induction of WT1 by TGF-$\beta$. (A and B) Rat hepatocytes were cultured for 24 hours and then maintained in culture for an additional 24 hours in the absence (Ctrol) or in the presence of TGF-$\beta$. The Fig. (A) shows induction of WT1 and vimentin expression (Vim) by TGF-$\beta$. Induction of WT1 mRNA is demonstrated by touchdown PCR with exon 8-exon 10 primers (A) and by quantitative real-time PCR with exon 4-exon 7 primers (B). The graph represents the results of 3 independent experiments performed in triplicate. (C) Effect of actinomycin-D (Act-D) (5 $\mu$g/mL) and cycloheximide (CHX) (10 $\mu$g/mL) in the induction of WT1 by TGF-$\beta$ (hepatocytes were cultured for 24 hours and then treated as indicated for 16 hours with TGF-$\beta$ in the absence or presence of Act-D or CHX). (D) Effect of catalase (1,000 U/mL) and desferrioxamine (1 mmol/L) in the induction of WT1 by TGF-$\beta$ (hepatocytes were cultured for 24 hours and then treated for 24 hours with TGF-$\beta$ in the absence or presence of catalase or desferrioxamine). (E) HuH7 cells were transfected with a construct harboring the dual WT1 promoter coupled to a CAT reporter gene and were treated with TGF-$\beta$. CAT activity of control and treated cells is represented as fold change with respect to the activity of pCAT-enhancer control plasmid. Transfections with plasmids harboring the reporter gene under the control of MAT1A or CMV promoters were performed to control for the specificity of WT1 response to TGF-$\beta$ treatment. Figure represents the mean of 3 independent experiments in quadruplicate.

***Expression of WT1 and Hepatocyte Dedifferentiation.*** Cultured hepatocytes undergo a time-dependent process of dedifferentiation with decreased levels of HNF-4 and serum albumin mRNAs and up-regulation of vimentin expression (Fig. 5A). We observed that these changes take place in parallel with an activation of WT1 expression. Thus, WT1 mRNA was undetectable immediately after plating and increased progressively with time in culture (Fig. 5B). Similarly, immunocytochemical analysis showed no WT1 protein in hepatocytes cultured for 6 hours, whereas WT1 immunoreactivity could be clearly seen at 24 hours in the cytoplasm, at 72 hours in both cytoplasm and nucleoli, and after 96 hours only in nucleoli (Fig. 5C). Interestingly, when cells were incubated from the onset of culture ($t = 0$) in the presence of SAM, a molecule known to maintain the differentiated phenotype of hepatocytes in culture,[14] WT1 induction was prohibited, and the fall of albumin expression was reduced (Fig. 5D). These effects of SAM were also observed after 96 hours of treatment, the last time point tested (data not shown).

Given that WT1 induction appeared to be associated with acquisition of a dedifferentiated hepatocellular phenotype, we analyzed whether WT1 could influence the expression of HNF-4 and methionine adenosyltransferase 2A (MAT2A) because the former is a key factor in maintaining metabolic functions in the mature parenchymal liver cells and the latter is induced upon dedifferentiation of hepatocytes.[14] We found that transfection of primary hepatocytes with an equimolar mixture of the 4 WT1 isoforms induced a decrease of HNF-4 mRNA at 48 hours (12,127 $\pm$ 2,806 copies of HNF-4/$10^3$ copies of $\beta$-actin in control cells, with a 31.25% reduction upon WT1 transfection) while simultaneously stimulating the expression of MAT2A (136 $\pm$ 40 copies of MAT2A/$10^3$ copies of $\beta$-actin in control cells, with a 43.75% induction upon WT1 transfection) (Fig. 6A). In confirmation of these findings, we observed up-regulation of HNF-4 and down-regulation of MAT2A in TGF-$\beta$–treated primary hepatocytes when WT1 transcription was inhibited using anti-sense oligos (Fig. 6B).

***HNF-4 Expression in Normal and Cirrhotic Human Liver.*** As mentioned above, HNF-4 plays a central role in the preservation of liver functions. This fact, together with our present observations on the potential role of WT1 on HNF-4 gene expression down-regulation, led

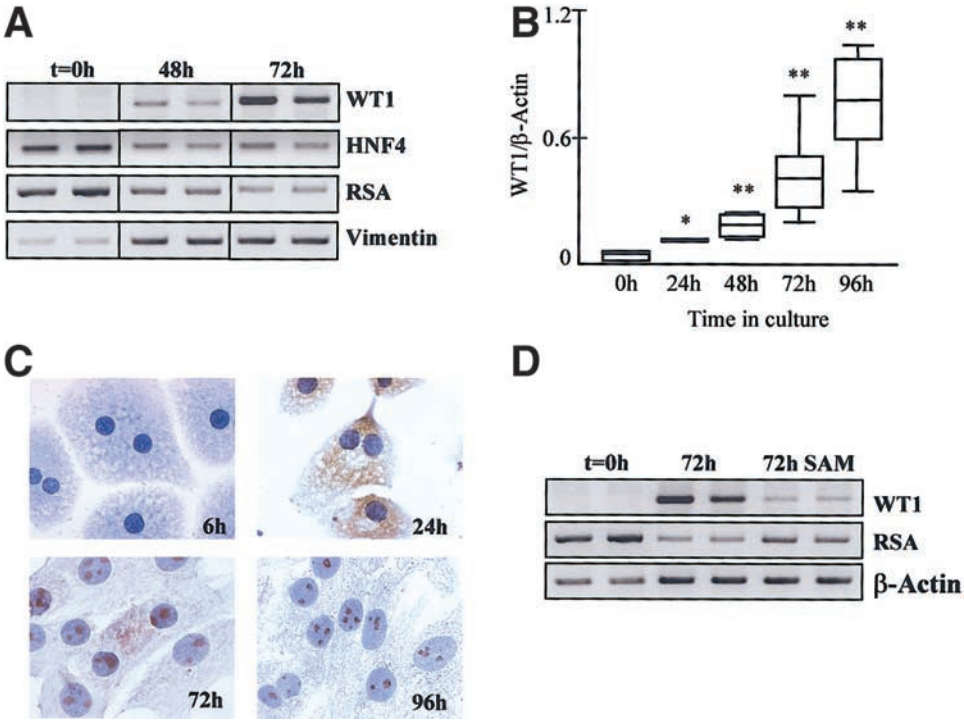


Fig. 5. WT1 expression in cultured primary rat hepatocytes: relationship to hepatocellular differentiation. (A) RT-PCR amplification bands for WT1, HNF-4, rat serum albumin (RSA), and vimentin in cultured hepatocytes at different times of culture (0, 48, and 72 hours). (B) WT1 mRNA levels by real-time PCR in hepatocytes at different times of culture. *$P < .05$, **$P < .001$ for comparison with basal values. Results of 4 independent experiments in duplicate are represented. (C) Immunohistochemical detection of WT1 in cultured hepatocytes at different times of culture. (D) Effect of SAM on the induction of WT1. RT-PCR amplification bands for WT1, rat serum albumin (RSA), and $\beta$-actin in hepatocytes cultured in the presence or absence of SAM (4 mmol/L) for 72 hours.

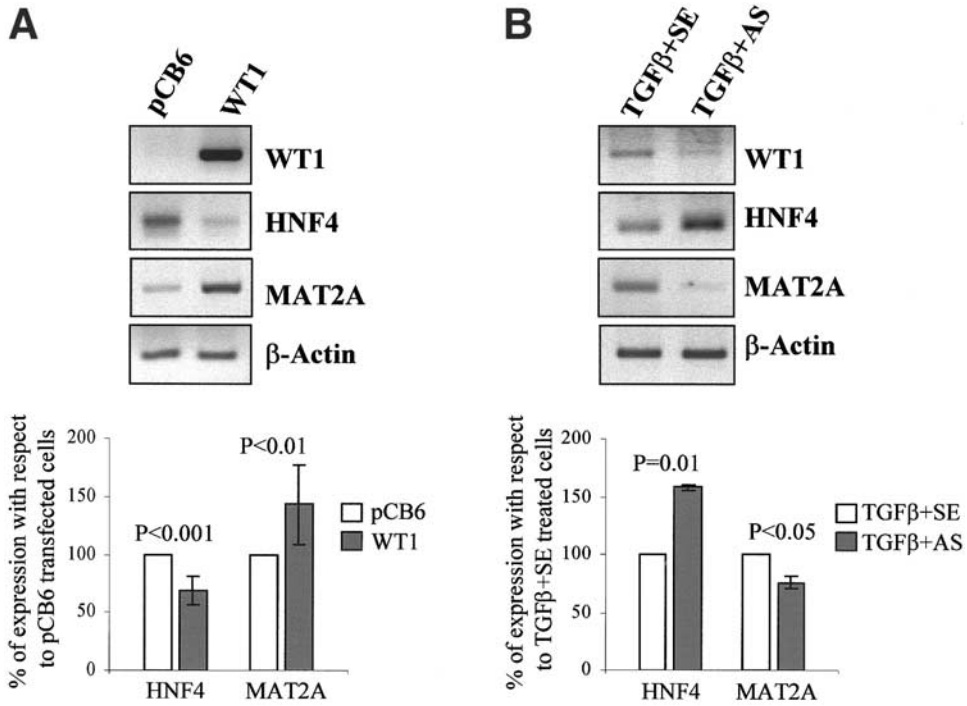


Fig. 6. Effect of WT1 on HNF-4 and MAT2A expression. (A) HNF-4 and MAT2A mRNA amplification bands in rat hepatocytes 48 hours after transfection with an equimolecular mixture of the 4 WT1 isoforms (WT1) or empty pCB6 plasmids (pCB6). A representative example and the results of 3 independent experiments are shown. (B) HFN4 and MAT2A mRNA amplification bands in TGF-$\beta$-treated rat hepatocytes transfected with sense or antisense WT1 oligonucleotides. Hepatocytes were cultured for 24 hours and then transfected with either sense or antisense WT1 oligonucleotides and treated with TGF-$\beta$ (added to the medium at the time of transfection) for another 24 hours. A representative example and the results of 3 independent experiments are shown. In both A and B, bars represent the percentage change in the number of copies of HNF-4 or MAT2A per $10^3$ copies of $\beta$-actin with respect to cells transfected with empty plasmid or sense oligonucleotide, with this value being taken as 100%.

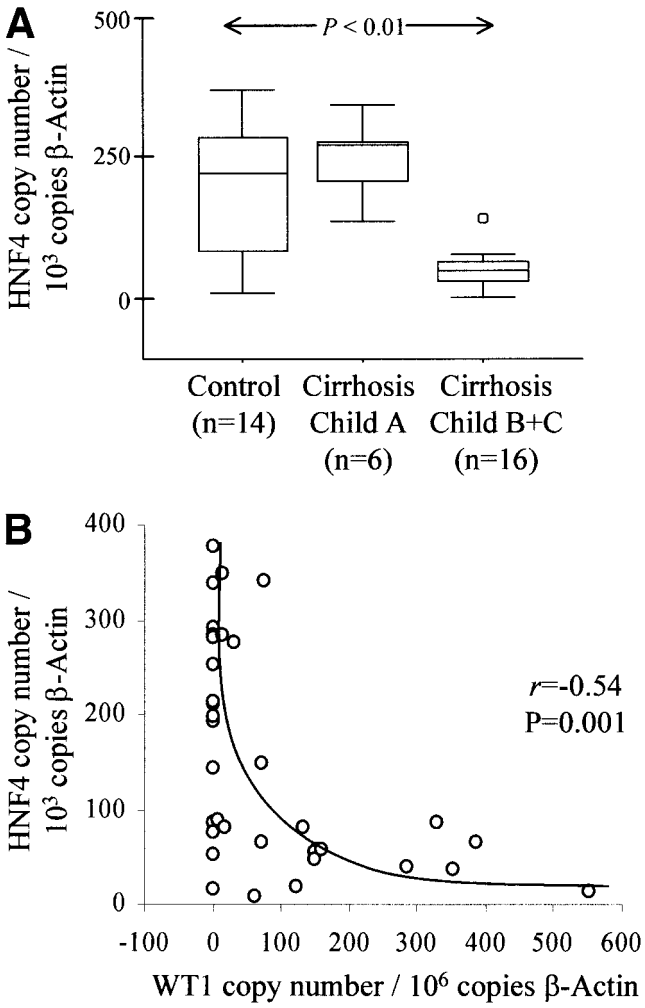


Fig. 7. Expression of HNF-4 in the liver of patients and controls. (A) HNF-4 mRNA levels were quantitated by real-time PCR in control livers and cirrhosis Child-Pugh A, B, or C. (B) Correlation between WT1 and HNF-4 mRNA levels in controls and cirrhosis.

us to measure HNF-4 mRNA values in cirrhotic livers. Real-time PCR analysis showed that the levels of HNF-4 mRNA were significantly reduced in patients with cirrhosis Child-Pugh B or C as compared with control livers ($P < .01$) (Fig. 7A). Expression levels of HNF-4 in the liver of patients with cirrhosis Child-Pugh A were not distinguishable from control livers (Fig. 7A), indicating that loss of HNF-4 expression was associated with the progression of the disease. In fact HNF-4 mRNA values correlated inversely and significantly with the MELD score of disease severity ($r = -0.60$, $P = .003$). Interestingly, we found that the level of WT1 gene expression inversely correlated with that of HNF-4 in the liver of control and cirrhotic patients ($r = -0.54$, $P = .001$) (Fig. 7B). As shown in Fig. 7B, all patients (10 out of 10) with more than 100 copies of WT1 mRNA/$10^6$ copies $\beta$-actin in the liver manifested reduced hepatic expression of HNF-4 (<100 copies/$10^3$ copies $\beta$-actin mRNA). However, there were 4 control livers and 2 cirrhotic livers that, although showing no expression of WT1, expressed reduced levels of HNF-4 (Fig. 7B). The biologic significance of low HNF-4 expression in some noncirrhotic livers is not known and is currently under investigation in our laboratory.

## Discussion

Hepatocytes are highly differentiated cells that fulfill a great number of biosynthetic, metabolic, detoxifying, and secretory activities. Accomplishment of all these functions depends on the coordinated expression of a vast array of genes determining the mature hepatocyte phenotype. This specific pattern of gene expression is altered in cirrhosis. Thus, it has been shown that, although 60% of hepatocytes express factor VII mRNA in normal hepatic tissue, less than 4% of liver cells are positive for this transcript in advanced cirrhosis.[2] The cellular and molecular mechanisms responsible for these transcriptional changes remain poorly understood. Our findings point to WT1 as a potential factor implicated in the impairment of hepatocellular function that occurs during the progression of cirrhosis.

WT1 has been shown to be involved in cell differentiation processes, although its effects may differ according to the cell system analyzed.[21-26] In hepatocytes, the presence of WT1 appears to denote functional immaturity in that we observed that this transcriptional regulator is expressed in the fetal hepatocyte but not in the mature parenchymal cell. Supporting this idea, WT1 immunoreactivity was found in hepatocyte precursors but not in fully differentiated parenchymal cells in a model of liver regeneration from undifferentiated progenitor cells.[27]

The negative role of WT1 on hepatocellular differentiation is supported by our *in vitro* findings showing the following: (1) WT1 accumulates in cultured hepatocytes in parallel with diminished expression of serum albumin and HNF-4 and up-regulation of vimentin; (2) WT1 is down-regulated in cultured hepatocytes by SAM, a compound known to maintain the differentiated hepatocyte phenotype in culture; (3) overexpression of WT1 induces down-regulation of HNF-4 and up-regulation of MAT2A, a marker of hepatocyte dedifferentiation; and (4) silencing of WT1 results in up-regulation of HNF-4 and down-regulation of MAT2A.

The influence of WT1 on HNF-4, a previously unrecognized phenomenon, is of particular importance because of the critical role of HNF-4 not only in hepatocyte differentiation during mammalian liver development but also because this factor is essential to maintain liver function and metabolic regulation in the mature organ.[28] This

paper shows, to our knowledge for the first time, that HNF-4 expression is impaired in livers with advanced cirrhosis (Child-Pugh score B and C). These cases are precisely those in which WT1 is strongly stimulated. Down-regulation of HNF-4 gene expression may be relevant to understand the mechanisms involved in the loss of hepatocellular function in the diseased liver, given the central role played by this transcription factor in the maintenance of liver-specific gene expression.[29,30] As mentioned previously, hepatic WT1 mRNA levels correlate inversely both with HNF-4 expression and with parameters of liver function such as prothrombin time and serum bilirubin. Moreover, WT1 mRNA values correlate directly with the severity of liver disease as estimated by the MELD score. Taken together, our *in vitro* and *in vivo* data point to the concept that WT1 is a developmental-restricted factor, which is reexpressed in cirrhosis, that may negatively affect hepatocyte differentiation and function possibly by influencing HNF-4 expression. Thus, WT1 may be implicated in the progression of cirrhosis to advanced stages of hepatic insufficiency.

Interestingly, in cirrhosis, WT1 immunoreactivity localizes to the nuclei of hepatocytes, showing a distinct nucleolar accumulation in some cells. However, in cultured liver cells, WT1 is initially seen in the cytoplasm and later on in nucleoli. In addition to ribosomal biogenesis, nucleoli also seem to be involved in the transport and turnover of specific mRNA.[31] These data are compatible with previous studies showing that WT1 can localize in the cytoplasm or in the nucleus depending on its phosphorylation status.[32] Thus, the distribution of WT1 in hepatocyte nuclei appears to reflect the ability of WT1 to bind not only to DNA but also to RNA,[7-9] suggesting its participation in the organization of subnuclear architecture and regulation of transcription.

The association between WT1 and the presence of liver fibrosis both in rats and in man led us to investigate whether TGF-$\beta$, a key profibrogenic cytokine, could activate WT1 gene expression. Our data show that TGF-$\beta$ enhances WT1 transcription by stimulating WT1 promoter. This novel effect of TGF-$\beta$ may be implicated in its activity as a negative signal for hepatocyte differentiation.[19] In addition to TGF-$\beta$, other factors could participate in the induction of WT1 in cirrhosis. Hypoxia, which occurs in the cirrhotic liver by diverse mechanisms,[33] has been shown to activate WT1 in the ischemic adult heart.[34] Because WT1 is induced by profibrogenic stimuli, its stimulation may be a link between fibrosis progression and impairment of hepatocellular function in cirrhosis. In summary, WT1 is a TGF-$\beta$–inducible gene expressed in fibrotic livers that may play a role in the evolution of cirrhosis to advanced stages of hepatic insufficiency.

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## References

1. Yanaga K, Honda H, Ikeda Y, Nishizaki AT, Yamamoto K, Sugimachi K. Significance of liver size in hepatic surgery. HPB Surg 1997;10:195-199.
2. Rodriguez-Inigo E, Bartolome J, Quiroga JA, Hedner U, Suarez A, Tomas JF, Manzarbeitia F, et al. Expression of factor VII in the liver of patients with liver disease: correlations with the disease severity and impairment in the hemostasis. Blood Coagul Fibrinolysis 2001;12:1-7.
3. Englert CH. WT1-more than a transcription factor? Trends Biochem Sci 1998;23:389-393.
4. Menke AL, van der Eb AJ, Jochemsen AG. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? Int Rev Cytol 1998;181:151-212.
5. Lee SB, Haber DA. Wilms tumor and the WT1 gene. Exp Cell Res 2001; 264:74-99.
6. Scharnhorst V, van der Eb AJ, Jochemsen AG. WT1 proteins: functions in growth and differentiation. Gene 2001;273:141-161.
7. Caricasole A, Duarte A, Larsson SH, Hastie ND, Little M, Holmes G, Todorov I, et al. RNA binding by the Wilms tumor suppressor zinc finger proteins. Proc Natl Acad Sci U S A 1996;93:7562-7566.
8. Bardeesy N, Pelletier J. Overlapping RNA and DNA binding domains of the WT1 tumor suppressor gene product. Nucleic Acids Res 1998;26: 1784-1792.
9. Larsson SH, Charlieu JP, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, et al. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell 1995; 81:391-401.
10. Johnstone RW, See RH, Sells SF, Wang J, Muthukkumar S, Englert C, Haber DA, et al. A novel repressor, par-4 modulates transcription and growth suppression functions of the Wilms' tumor suppressor WT1. Mol Cell Biol 1996;16:6945-6956.
11. Amin KM, Litzky LA, Smythe WR, Mooney AM, Morris JM, Mews DJ, Pass HI, et al. Wilm's tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma. Am J Pathol 1995;146: 344-356.
12. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, et al. Formulation and application of a numerical score system for assessing histological activity in asymptomatic chronic active hepatitis. HEPATOLOGY 1981;1:431-435.
13. Castilla-Cortazar I, Garcia M, Muguerza B, Quiroga J, Perez R, Santidrian S, Prieto J. Hepatoprotective effects of insulin-like growth factor I in rats with carbon tetrachloride-induced cirrhosis. Gastroenterology 1997;113: 1682-1691.
14. Garcia-Trevijano ER, Latasa MU, Carretero MV, Berasain C, Mato JM, Avila MA. S-adenosylmethionine regulates *MAT1A* and *MAT2A* gene expression in cultured rat hepatocytes: a new role for S-adenosylmethionine in the maintenance of the differentiated status of the liver. FASEB J 2000;14:2511-2518.
15. Sánchez A, Álvarez AM, López Pedrosa JM, Roncero C, Benito M, Fabregat I. Apoptotic response to TGF-$\beta$ in fetal hepatocytes depends upon their state of differentiation. Exp Cell Res 1999;252:281-291.
16. Fraizer GC, Wu YJ, Hewitt SM, Maity T, Ton CC, Huff V, Saunders GF. Transcriptional regulation of the human Wilms' tumor gene (WT1). Cell type-specific enhancer and promiscuous promoter. J Biol Chem 1994;269: 8892-8900.

17. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, et al. A model to predict survival in patients with end-stage liver disease. HEPATOLOGY 2001;33:464-470.
18. Castilla A, Prieto J, Fausto N. Transforming growth factors $\beta$ 1 and $\alpha$ in chronic liver disease. Effects of interferon alfa therapy. N Engl J Med 1991;324:933-940.
19. Spagnoli FM, Cicchini C, Tripodi M, Weiss MC. Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGF($\beta$) is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci 2000;113:3639-3647.
20. Shibanuma M, Kuroki T, Nose K. Release of $H_2O_2$ and phosphorylation of 30 kilodalton proteins as early responses of cell cycle-dependent inhibition of DNA synthesis by transforming growth factor $\beta$1. Cell Growth Differ 1991;2:583-591.
21. Hosono S, Luo X, Hyink DP, Schnapp LM, Wilson PD, Burrow CR, Reddy JC, et al. WT1 expression induces features of renal epithelial differentiation in mesenchymal fibroblasts. Oncogene 1999;18:417-427.
22. Ellisen LW, Carlesso N, Cheng T, Scadden DT, Haber DA. The Wilms' tumor suppressor WT1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells. EMBO J 2001;20:1897-1909.
23. Smith SI, Weil D, Johnson GR, Boyd AW, Li CL. Expression of the Wilms' tumor suppressor gene, WT1, is up-regulated by leukemia inhibitory factor and induces monocytic differentiation in M1 leukemic cells. Blood 1998;91:764-773.
24. Sekiya M, Adachi M, Hinoda Y, Imai K, Yachi A. Down-regulation of Wilms' tumor gene (WT1) during myelomonocytic differentiation in HL60 cells. Blood 1994;83:1876-1882.
25. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, Oji Y, et al. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. Blood 1998;91:2969-2976.
26. Phelan SA, Lindberg C, Call KM. Wilms' tumor gene, WT1, mRNA is down-regulated during induction of erythroid and megakaryocytic differentiation of K562 cells. Cell Growth Differ 1994;5:677-686.
27. Gordon GJ, Coleman WB, Grisham JW. Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol 2000;157:771-786.
28. Li J, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. Genes Dev 2000;14:464-474.
29. Parviz F, Li J, Kaestner KH, Duncan SA. Generation of a conditionally null allele of HNF-4$\alpha$. Genesis 2002;32:130-133.
30. Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, et al. The orphan nuclear receptor HNF4$\alpha$ determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nature Med 2003;9:220-224.
31. Bond VC, Wold B. Nucleolar localization of myc transcripts. Mol Cell Biol 1993;13:3221-3230.
32. Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR. Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. EMBO J 1996;15:5606-5615.
33. Corpechot C, Barbu V, Wendum D, Kinnman N, Rey C, Poupon R, Housset C, et al. Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis. HEPATOLOGY 2001;35:1010-1021.
34. Wagner KD, Wagner N, Bondke A, Nafz B, Flemming B, Theres H, Scholz H. The Wilms' tumor suppressor Wt1 is expressed in the coronary vasculature after myocardial infarction. FASEB J 2002;16:1117-1119.