Inhibition of Poly Adenosine Diphosphate-Ribose Polymerase Decreases Hepatocellular Carcinoma Growth by Modulation of Tumor-Related Gene Expression

Rosa Quiles-Perez,[1,2*] José Antonio Muñoz-Gámez,[1,2*] Ángeles Ruiz-Extremera,[1,2] Francisco O'Valle,[3] Laura Sanjuán-Nuñez,[1] Ana Belén Martín-Álvarez,[1] David Martín-Oliva,[4] Trinidad Caballero,[2,3] Paloma Muñoz de Rueda,[1,2] Josefa León,[1,2] Raúl Gonzalez,[5] Jordi Muntané,[5] Francisco Javier Oliver,[6*] and Javier Salmerón[1,2*]

Hepatocellular carcinoma (HCC) is associated with a poor prognosis due to a lack of effective treatment options. In HCC a significant role is played by DNA damage and the inflammatory response. Poly (ADP-ribose) polymerase-1 (PARP-1) is an important protein that regulates both these mechanisms. The objective of this study was to examine the effect of pharmacology PARP-1 inhibition on the reduction of tumor volume of HCC xenograft and on the hepatocarcinogenesis induced by diethyl-nitrosamine (DEN). Pharmacologic PARP-1 inhibition with DPQ greatly reduces tumor xenograft volume with regard to a nontreated xenograft (394 mm^3 versus 2,942 mm^3, $P < 0.05$). This observation was paralleled by reductions in xenograft mitosis ($P = 0.02$) and tumor vasculogenesis ($P = 0.007$, confirmed by *in vitro* angiogenesis study), as well as by an increase in the number of apoptotic cells in DPQ-treated mice ($P = 0.04$). A substantial difference in key tumor-related gene expression (transformed 3T3 cell double minute 2 [*MDM2*], *FLT1* [vascular endothelial growth factor receptor-1, *VEGFR1*], epidermal growth factor receptor [*EPAS1*]/hypoxia-inducible factor 2 [*HIF2A*], *EGLN1* [*PHD2*], epidermal growth factor receptor [*EGFR*], *MYC, JUND, SPP1* [*OPN*], hepatocyte growth factor [HGF]) was found between the control tumor xenografts and the PARP inhibitor-treated xenografts (data confirmed in HCC cell lines using PARP inhibitors and PARP-1 small interfering RNA [siRNA]). Furthermore, the results obtained in mice treated with DEN to induce hepatocarcinogenesis showed, after treatment with a PARP inhibitor (DPQ), a significant reduction both in preneoplastic foci and in the expression of preneoplastic markers and proinflammatory genes (*Gstm3, Vegf, Spp1 [Opn], IL6, IL1b,* and *Tnf*), bromodeoxyuridine incorporation, and NF-κB activation in the initial steps of carcinogenesis ($P < 0.05$). *Conclusion:* This study shows that PARP inhibition is capable of controlling HCC growth and preventing tumor vasculogenesis by regulating the activation of different genes involved in tumor progression. (HEPATOLOGY 2010; 51:255-266.)

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of death from cancer in the world, accounting for an estimated 500,000 deaths annually.[1] The prognosis of HCC patients is generally very poor. Most studies have shown a 5-year survival rate of less than 5% in symptomatic patients. Because current therapies are rarely able to achieve complete tumor abla-

Abbreviations: ADAM12, ADAM metallopeptidase domain 12; ADORA3, adenosine A3 receptor; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone; EGFR, epidermal growth factor receptor; ENG/END, endoglin; EPAS1/HIF2A, endothelial PAS domain protein 1, hypoxia-inducible factor 2; Gstm3 (murine), glutathione S-transferase m3; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HIF1A, hypoxia-inducible factor 1; MDM2, transformed 3T3 cell double minute 2, p53 binding protein; NF-κB, nuclear factor-kappa B; PAR, poly (ADP-ribose); PARP-1, poly (ADP-ribose) polymerase 1; PCR, polymerase chain reaction; SPP1/OPN, osteopontin; VEGFR1, vascular endothelial growth factor receptor-1.

From the [1]San Cecilio University Hospital, Granada, Spain; [2]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd); [3]Department of Pathology, [4]Department of Cell Biology, University of Granada, Granada, Spain; [5]Liver Research Unit, Reina Sofia University Hospital, Córdoba, Spain; [6]Instituto de Parasitología y Biomedicina Lopez-Neyra, CSIC, Granada, Spain.

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**These authors contributed equally to this study.*

tion, it is necessary to study any new therapeutic strategy that constitutes a promising alternative.

Poly [ADP-ribose] polymerase 1 (PARP-1) is a highly conserved DNA-binding protein, the most abundant member of the PARP family, which is activated by binding to DNA breaks. A recent description has been made of DNA-independent PARP-1 activation by the extracellular signal-related kinase (ERK) signaling pathway involved in cellular growth, proliferation, and differentiation.[2] Poly (ADP-ribosyl)ation is involved in many molecular and cellular processes, including DNA damage detection and repair, posttranslational modification of proteins by poly ADP-ribosylation, transcription, and cell death. Three modes of PARP-1 transcription regulatory activity have been reported: (1) histone-modifying enzymatic activity, which regulates chromatin structure, promoting the dissociation of nucleosomes and the decondensation of chromatin; (2) transcriptional coregulator activity, which modulates the activity of several transcription factors (nuclear factor-kappa B [NF-κB], JUN, YY1, HIF1A, POU2F1 [OCT1], and FOXO1 among others); and (3) epigenetic activity, regulating genomic methylation patterns.[3] Our understanding of the role and involvement of PARP-1 in many biological mechanisms, in both health and disease, and, specifically, its role in carcinogenesis, has steadily increased in recent years.[4] In a previous report we showed that both PARP-inhibited and *parp-1*-knockout (KO) mice are protected against chemically induced skin carcinogenesis.[5] PARP inhibition has also been shown to reduce different forms of hepatic injury induced by peroxynitrite and heat stroke.[6,7] Other studies have shown that breast cancer cell lines, defective in homologous recombination, are acutely sensitive to PARP inhibitors, presumably because the resultant collapsed replication forks are no longer repaired.[8]

In the present study we show that the inhibition of PARP activity prevents both the tumor growth of a xenograft derived from HCC cells (HepG2) and also the hepatocarcinogenesis induced by the hepatotoxic agent diethyl-nitrosamine (DEN), due to its ability to modulate both the activity of key transcription factors and the transcription of oncogenes, growth factor, proangiogenic molecules, etc., involved in tumor promotion/progression, angiogenesis, and metastasis (such as JUND, MDM2, NF-κB, HGF, FLT1 [VEGFR1], EGFR, and EPAS1 [HIF2A]). Our data indicate that PARP inhibition may enable the design of specific, more effective, and less toxic therapies for HCC cancer.

Materials and Methods

Cell Lines and Drugs. Three well-characterized human liver cancer cell lines (HepG2, PLC-PRF-5, and Hep3B) and one human liver nontumoral cell line (WRL-68) were maintained as an adherent monolayer in Roswell Park Memorial Institute medium 1640 (RPMI-1640; GIBCO-BRL, HepG2 and Hep3B) and Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, PLC-PRF-5 and WRL-68) supplemented with 10% fetal bovine serum (JRH Biosciences), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma) and incubated at 37°C in 5% CO_2. For the stable depletion of PARP-1 in WRL-68 and PLC-PRF-5 cells, a DNA vector-based RNA interference (RNAi) approach was used. The sequence of DNA vector has been described by other authors (siP912), (further data are shown in the Supporting Materials and Methods and Supporting Fig. S1). DPQ (3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone, Alexis Biochemicals), and ANI (4-amino-1,8-naphthalimide, Alexis Biochemicals) were used for the inhibition of the catalytic activity of PARP. DEN (Sigma) was used as the hepatotoxic agent inducing HCC.

Subcutaneous Xenograft of HepG2 Cells in Nude Mice. All animal care and experimentation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Academy of Sciences. Twenty male nude mice (Athymic NCr-Nude, Taconic, NY), aged 5 weeks (weighing 18-22 g) were randomly divided into two groups (control and treated groups). HepG2 cells were harvested and mixed with isotonic solution (100 μL per mouse) and then inoculated into one flank of each of the 20 nude mice (8×10^6 of HCC cells). When the tumors had reached a volume of about 500 mm^3 (30 days after the inoculation), half of the mice ($n = 10$) were given an intraperitoneal injection of DPQ (15 mg/kg, treated group), and the others ($n = 10$) were treated with the vehicle (100 μL isotonic solution, control group) for 4 days. The tumor dimensions were measured every 2 days (seven measurements) using a digital caliper and the tumor volume was calculated using the formula: $V = \pi/6 \times$ larger diameter $\times$ (smaller diameter).[2] The mice were killed 2 weeks after the last DPQ injection and the

Address reprint requests to: Dr. Jose A. Munoz, Laboratory of Medical Research, San Cecilio University Hospital. Avda de Madrid s/n, 18012, Granada, Spain. E-mail: josea.munoz.exts@juntadeandalucia.es or jamg@ugr.es; fax: 34-958-023434.

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xenografts were then removed and the histological techniques for conventional morphology were applied following published techniques.[10] Xenograft sections were stained with hematoxylin/eosin (H&E) and with the monoclonal antibodies: Ki-67 (clone SP6, Master Diagnóstica, Granada, Spain) to identify proliferating cells, *Ulex europaeus* lectin to identify endothelial cells (Master Diagnostica), and the ApopTag Peroxidase In Situ Oligo Ligation (ISOL) Kit (S7200, Chemicon International, Germany) to detect apoptosis. A millimeter scale in the eyepiece was used to count the positive nuclei and vessels per mm^2. The results are expressed as number of positive nuclei or vessels per mm^2.

RNA Amplification, Analysis of Complementary DNA (cDNA) Microarray, and Quantitative Real-Time Polymerase Chain Reaction (PCR). Data regarding RNA extraction and purification, and cDNA synthesis can be found elsewhere.[5] Gene expression assessments were performed in mouse xenograft samples using the Affymetrix Human 133 Plus 2.0 microarray chip containing 54,675 gene transcripts and were conducted following procedures described in the Affymetrix GeneChip Technical Manual. The chip was scanned and the signal intensity evaluated as described.[11] The genes selected for quantitative real-time PCR confirmation were from major pathways identified by the microarray analysis. For the gene expression studies in tumor induction experiments, we selected several genes involved in inflammation and tumor progression in hepato-carcinogenesis (*Tnf, IL6, IL1b, Gstm3, Vegf* and *Spp1 [Opn])*. Real-time PCR was performed with SYBR green technology using the iQ SYBR Green supermix kit (Bio-Rad Laboratories) and following the manufacturer's protocol. All values were normalized for the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (xenografts) and *18S* (tumor induction experiments) expression levels. All samples were repeated in triplicate and mean expression values were used. The sequences of primers used for these studies are shown in Supporting Table S1.

Colony-Forming Assays (CFA). Semiconfluent culture flasks were trypsinized and the appropriate cell number seeded in six-well culture flasks. One day later the cells were either exposed to DPQ at the indicated concentrations (PARP-1 proficiency cells), or in the case of PARP-1-depleted cells (siP912 vector, WRL-68 C1, and PLC-PRF-5 C6 clones), not treated. After 14 days, adherent colonies were rinsed in phosphate-buffered saline (PBS), fixed in 100% methanol for 5 minutes, and stained with 1% crystal violet. Aggregates of more than 50 cells were counted as a colony and the data pooled from three independent experiments were presented as relative clonogenicity with respect to the corresponding nontreated controls.

Wound-Healing Experiment. A wound-healing experiment was performed to test the effect of the PARP inhibitor on the motility of hepatocarcinoma cells. This assay measured the cell's capacity to fill an empty area of culture flask created by scratching a confluent monolayer of cells. The cells were grown to confluence on six-well cell culture dishes and then either treated with ANI (10 μM) for 3 hours (PARP-1 PLC-PRF-5 proficiency cells) or, in the case of PARP-1-depleted cells (siP912 vector, PLC-PRF-5 C6 clone), not treated. A scratch was made through the cell layer using a pipette tip. After washing with PBS, serum-free medium (to prevent cell proliferation) containing ANI (PARP inhibitor, 10 μM) was added. Photographs of the wounded area were taken immediately after the scratch was made. Cell movements into the wounded area were analyzed 24 hours later. The area without cells was measured by means of Leica Qwin software and the percentage of recovery was calculated.

Matrigel Angiogenesis Assay In Vitro. Matrigel-coated plates (BD Biosciences) were used. Primary human umbilical vein endothelial cells (HUVEC) (25,000 cells/well on a 24-well plate) were plated and incubated at 37°C in a 5% CO_2 environment, in MRECE-HVC01 medium supplemented with endothelial growth medium (Advancell, Barcelona, Spain) and treated with diverse concentrations of DPQ. The development of capillary structures and tubular networks was analyzed after 18 hours under a Leica inverted microscope.

Tumor Induction Experiments on Mice. Thirty C57BL/6 mice aged 2 weeks (weighing 15-20 g) were randomly divided into three groups (control [n = 10], DEN [n = 10], and DEN + DPQ group [n = 10]) and given an intraperitoneal injection of DEN (Sigma), 5 mg/kg body weight in saline for liver carcinogenesis. One week later, the DEN + DPQ group was treated weekly with an intraperitoneal injection of DPQ (PARP-1 inhibitor), at a dose of 15 mg/kg body weight in saline. The control group was treated in parallel with saline. The experiment was concluded at week 12 and the animals' livers were removed and the histological techniques for conventional morphology were applied following published techniques.[10]

Electrophoretic Mobility Shift Assay (EMSA). Band shift assays were used to detect NF-κB-binding activity in whole livers of the DEN model; the methodology has been reported by Munoz-Gamez et al.[12] NF-κB consensus oligonucleotides 5′-AGTTGAGGGGACTTTCCC-AGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ (Promega, Madison, WI) were used. The antibody supershift assays for the identification of NF-κB complex were performed by incubation of the mixture with 1 μg/mL of affinity-purified polyclonal antibodies against p50, p52, and p65 sub-

units (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour on ice before adding ^{32}P-labeled probe. A competitive assay was also carried out by incubating the mixture with cold probe (100-fold excess) for 1 hour on ice before^{32} P-labeled probe addition.

Hepatocyte Incorporation of Bromodeoxyuridine (BrdU). Proliferation, measured by means of BrdU incorporation, was developed according to published techniques.[10] For quantification, random fields per section were documented by confocal microscopy (Leica SP2 Confocal Microscope).

Statistical Analysis. Data are expressed as the means ± SEM. A *P*-value of less than 0.05 was considered statistically significant. For the data shown in Fig. 2,the values of the average number of mitosis/apoptosis/vessels per mm^2 were fitted using the Mann-Whitney *U* test. For statistical analysis of other experiments the unpaired Student's *t* test was used.

Results

Inhibition of PARP Activity Suppressed HepG2 Xenograft Tumor Growth In Vivo. To test the effect of the inhibition of the catalytic activity of PARP on HepG2 xenograft tumor growth, NCr-Nude mice were treated with intraperitoneal injections of DPQ (PARP inhibitor). The DPQ administration (15 mg DPQ/kg body weight) blocked the PARP activation in the xenografts (Supporting Fig. S2) and was well tolerated by tumor-bearing mice; there was no significant weight loss and the DPQ was not toxic *per se* to the mice (no evidence of DPQ-induced toxicity was detected at liver histological examination and the transaminase levels in serum were similar prior to DPQ administration and after the PARP-1 inhibitor treatment, Supporting Table S4). The PARP inhibition significantly suppressed tumor growth in comparison to control mice (Fig. 1). In the DPQ group the mean tumor volume was 564 mm^3 at the beginning of treatment versus 394 mm^3 at the end of the experiment (n = 10), whereas in the control mice the volume of the xenografts had increased by the end of the experiment (445 mm^3 versus 2,942 mm^3, $P < 0.05$, n = 10, Fig. 1A). Of particular interest is the significant difference with regard to the final xenograft volume between the control group and the DPQ-treated group (2,942 mm^3 versus 394 mm^3, $P < 0.05$, Fig. 1A,B). Furthermore, complete tumor response was evident in three animals after DPQ treatment (30%).

DPQ Reduces Mitosis and Vessel Formation Rate and Increases the Number of Cells in Apoptosis. Histological examination showed that the HepG2 xenografts of the control group presented increased cell proliferation in comparison to the DPQ-treated group xenografts

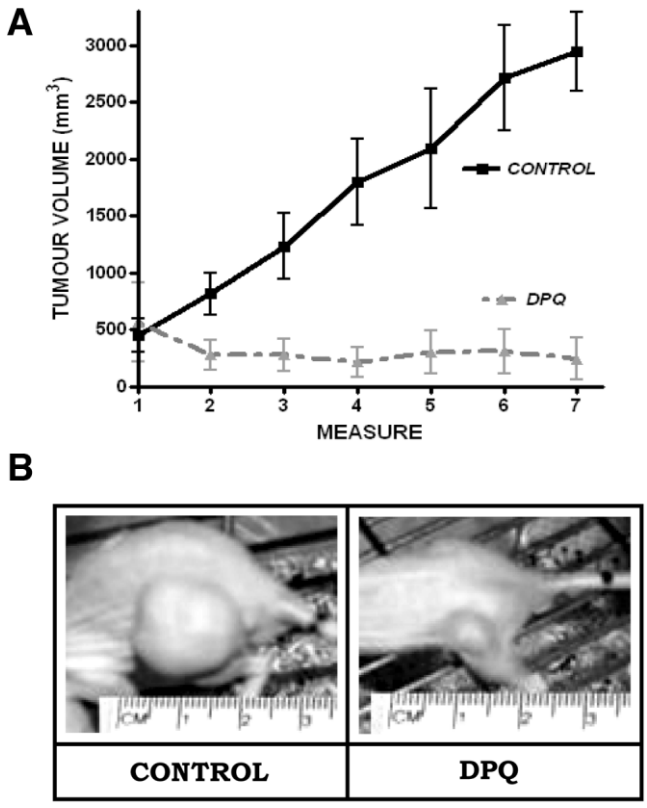

Fig. 1. Antitumoral activity of DPQ in xenografts of the human HCC cell line. (A) Tumor volume in xenografts treated with DPQ. The PARP inhibition significantly reduced the xenograft volume at the end of the experiment (394 mm^3 versus 2,942 mm^3, 20 samples, control n = 10 and DPQ n = 10, $P < 0.05$). (B) Examples of xenograft size in DPQ group (right) and control mice (left) at the end of the experiment.

(72.4 versus 29.4 cells in mitosis per mm^2, $P = 0.02$; and 5,755 versus 2,836 Ki-67-staining cells per mm^2, $P = 0.002$, Fig. 2A). Regarding the apoptotic rate, a striking difference was found between the control and the treated group xenografts (12.5 versus 28.1 cells in apoptosis per mm^2, $P = 0.04$; Fig. 2B). This restricted apoptotic process and accelerated mitotic activity in the control group xenografts may explain the spectacular differences observed in the tumor xenograft volume at the end of the treatment (Fig. 1). The vessel density count in the DPQ-treated xenografts showed an important decrease (81.2 in DPQ-treated mice versus 200.6 vessels/mm^2 in the control group, $P = 0.007$, Fig. 2C), suggesting that differences in tumor vascularity may account for the decreased xenograft size after treatment with the PARP inhibitor.

Analysis of Differential Gene Expression. Transcription regulation by means of PARP-1 has been widely described and this PARP-1 ability is known to be involved in the development of different pathologies, including carcinogenesis.[3] To test the effect of PARP inhibition on gene expression, cDNA microarray technology was applied to the

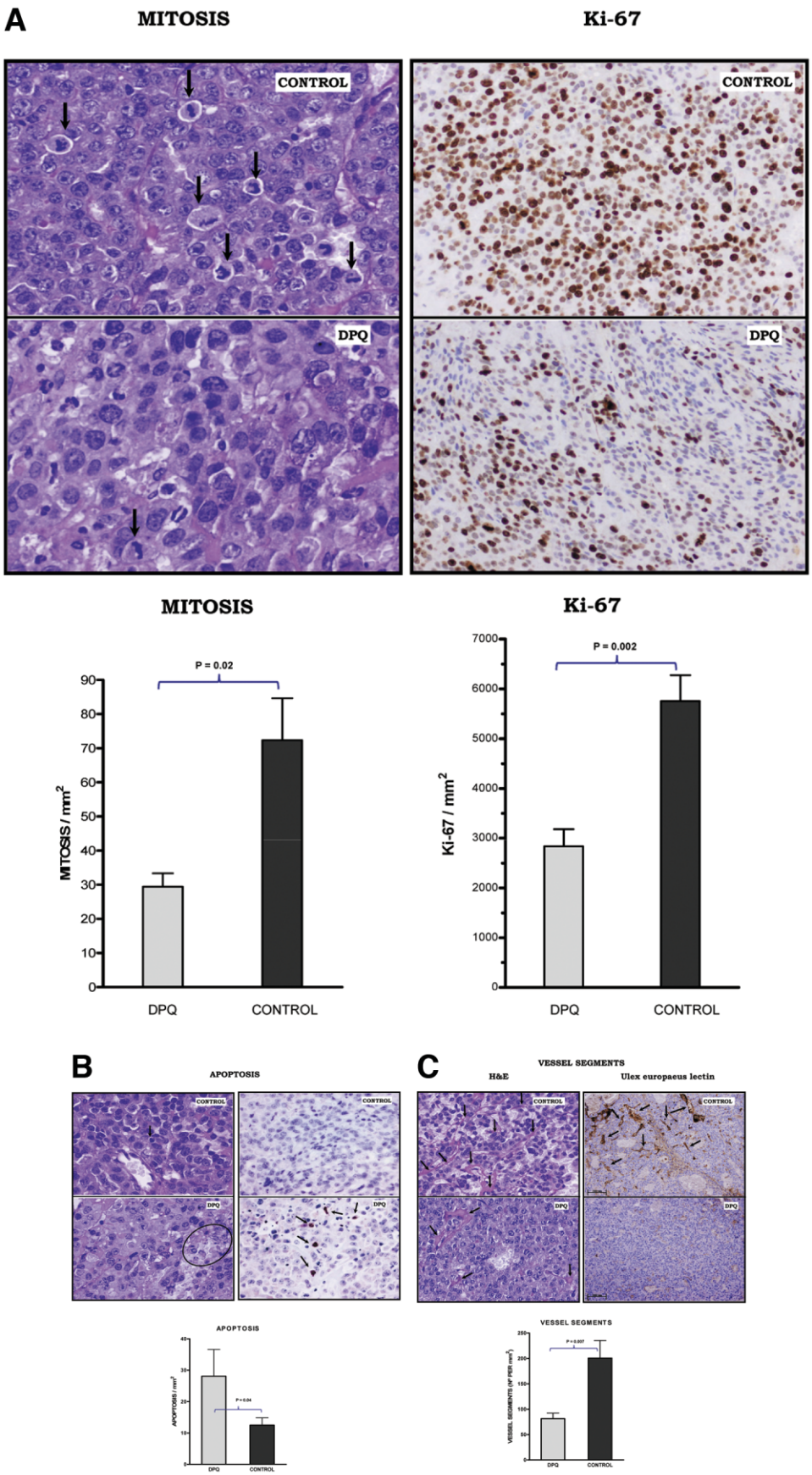

Fig. 2. Morphological analyses of tumoral xenografts. (A) Histological examination and diagnosis of cell proliferation (mitosis H&E and Ki-67), (B) apoptosis (apoptosis H&E and ApopTag Peroxidase In Situ Oligo Ligation), and (C) angiogenesis (measured as blood vessels per mm^2, H&E and *Ulex europaeus* lectin) in tumor sections of DPQ-treated and control tumor xenografts (20 samples, control n = 10 and DPQ n = 10). In control mice, results show a higher rate of cell proliferation and blood vessels and a lower number of apoptotic cells than for the DPQ-treated xenograft.

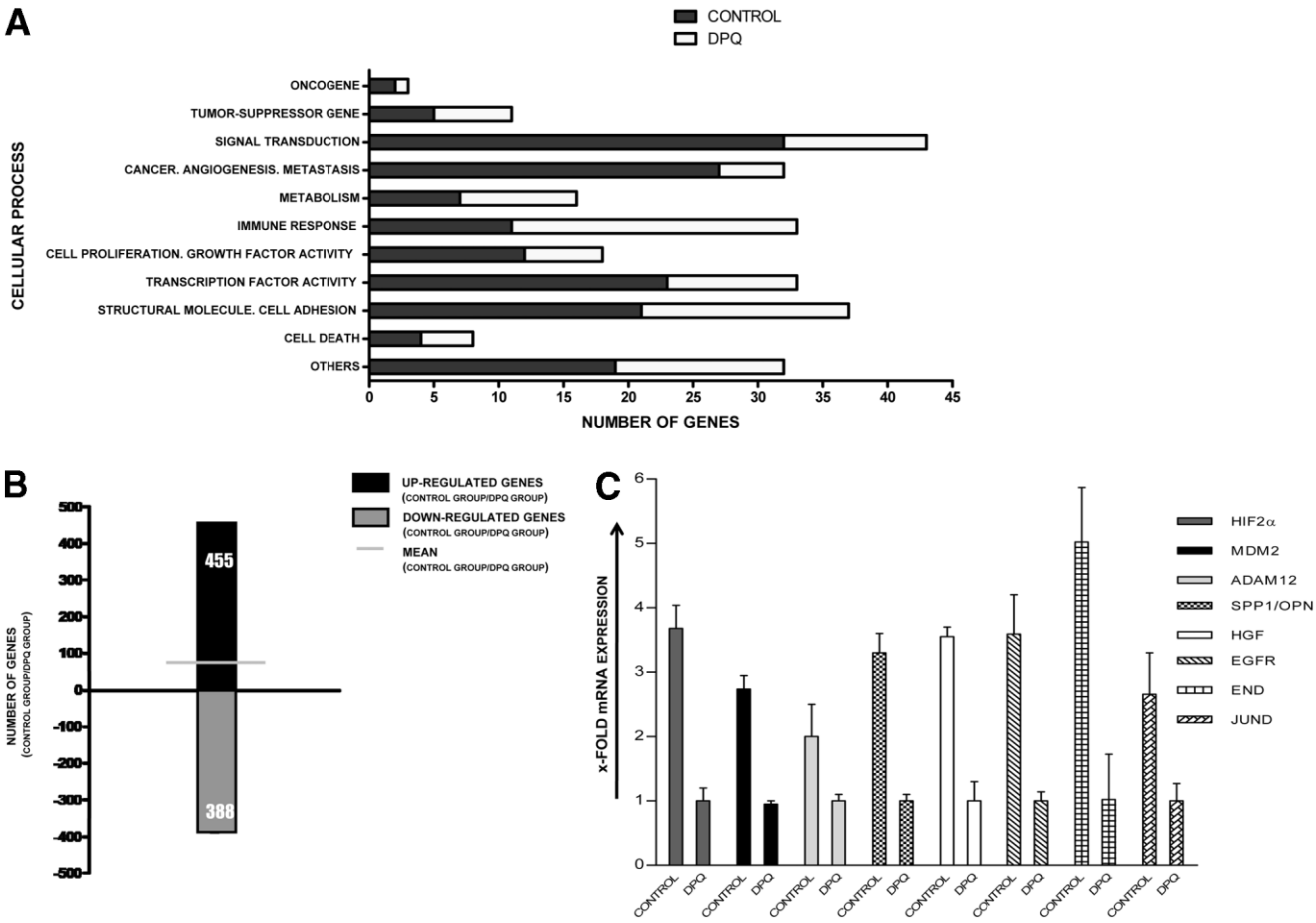

Fig. 3. Gene expression analyses. Analysis of the mRNAs, which are deregulated (up-regulated or down-regulated) at least two-fold in the control xenograft versus DPQ-treated tumors, revealed the following: (A) the results shown are grouped by the corresponding gene ontology biological process. Of considerable interest is the fact that PARP inhibition produces a different expression pattern of the genes involved in different cellular functions. (B) PARP inhibition reduces overall gene expression. (C) qRT-PCR confirmation. The results were normalized to the expression of *GAPDH* for all of the samples. The sequences of primers used for these studies are shown in Supporting Table S1. The results indicate that expression of these genes was greatly influenced by PARP inhibition during xenograft development.

untreated control group and for the DPQ-treated group xenografts. The differentially expressed genes found belonged to a variety of functional categories, in accordance with the descriptions made by Gene Ontology Biological Process (http://www.ebi.ac.uk/GOA/) (Fig. 3A). It should be emphasized that DPQ treatment produces an important reduction in the expression of genes involved in signal transduction, oncogenes, genes related to tumor development, angiogenesis, and metastasis, as well as in those expressing transcription factors and cell adhesion molecules. On the other hand, treatment with the inhibitor increases the expression of immune response and metabolism genes as well as that of tumor-suppressor genes. These results are consistent with the data from the macroscopic tumor evaluation, shown in Fig. 1, and the microscopic data, shown in Fig. 2. Supporting Tables S2 and S3 show the analysis of different gene classes (grouped in accordance with the Gene Ontology Biological Process), which match the arbitrary criterion of a four-fold change, with a statistical significance of $P < 0.01$ (Supporting Table S2: genes up-regulated in control xenografts versus DPQ tumors; Table S3: genes up-regulated in DPQ tumors versus control xenografts). Of considerable interest is the up-regulation in control tumors of *MYC, JUND, SPP1 (OPN), FLT1 (VEGFR1), EPAS1 (HIF-2A), MDM2, MMP28, HGF, EGFR, ANGPT2 (ANG-2), PDGF* and other genes involved in carcinogenesis, angiogenesis, and inflammation and associated with the development of hepatocellular carcinoma.[13] Furthermore, the number of up-regulated genes was higher in the control xenografts (455 genes) than the up-regulated genes in the DPQ xenografts (388 genes, Fig. 3B). These results suggest that PARP-1 is involved in transcriptional regulation mainly as a coactivator rather than a corepressor.

Quantitative Real-Time PCR (qRT-PCR) Gene Expression. The up-regulation of some of the genes involved in cancer, angiogenesis, and metastasis obtained by

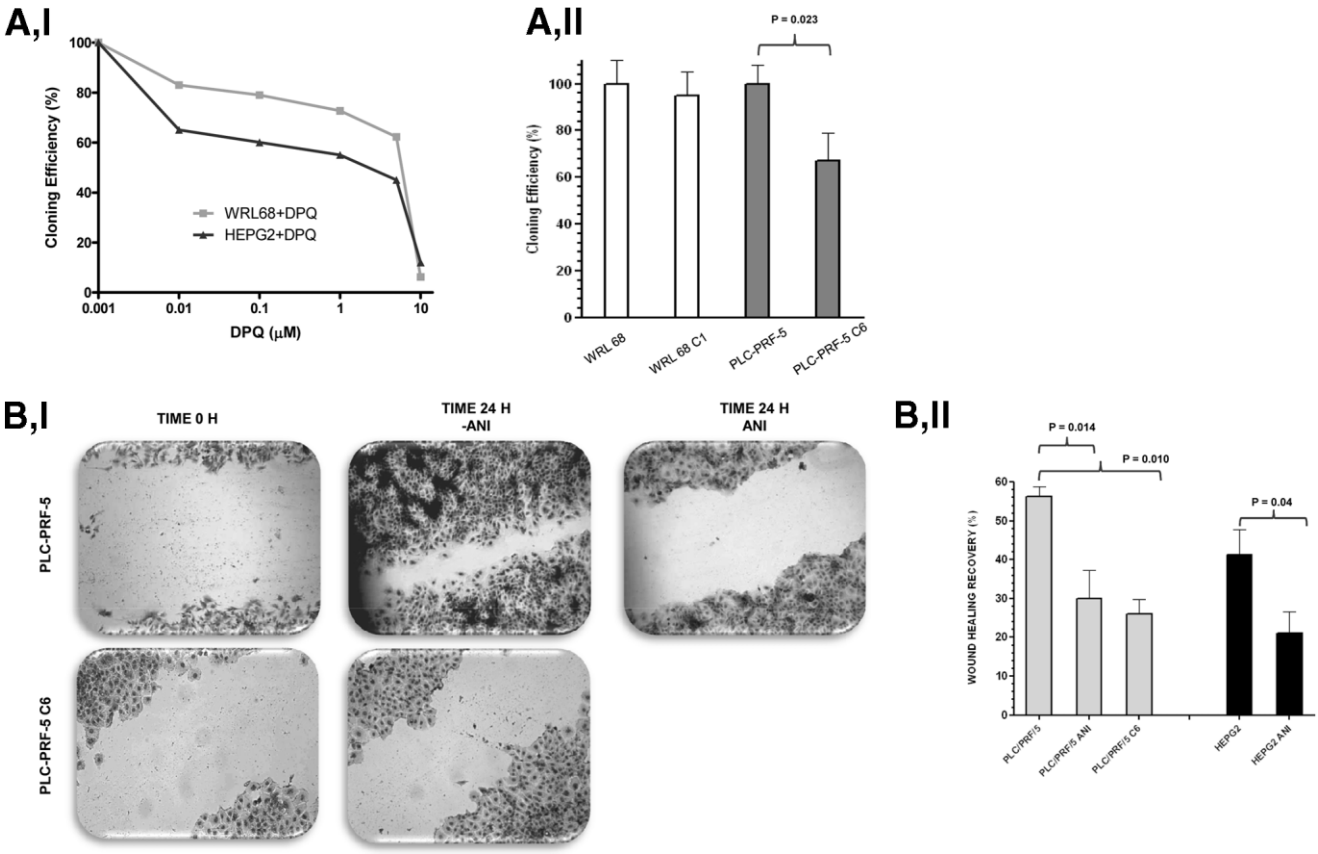

Fig. 4. Effects of PARP inhibition and blocking on human HCC cell lines. (A,I) Effect of DPQ (PARP inhibitor) on nontumoral liver cells (WRL-68) and HCC cells (HepG2). Cell death was monitored by clonogenic survival. (A,II), Effect of PARP-1 depletion on cloning efficiency. (B) Inhibitory effect of PARP-1 depletion and PARP inhibition on the motility of PLC-PRF-5. PARP-1 knockdown PLC-PRF-5 C6 clone were used to study the effect of this protein on cell motility. Photographs of the wounded area (B,I) were taken immediately after the scratch was made; 24 hours later, cell movements within the wounded area were analyzed. Wound-healing recovery quantification in PLC-PRF-5, PLC-PRF-5 C6 clone, and HepG2 cells (for HepG2 cells the photographs are not shown) (B,II). Data represent the mean of at least three independent experiments done in triplicate.

cDNA microarrays (*EPAS1 [HIF-2A], MDM2, ADAM metallopeptidase domain 12 [ADAM12], SPP1 [OPN], HGF, EGFR, ENG [END], JUND*) was confirmed by qRT-PCR (Fig. 3C). The results show that expression of these genes was greatly influenced by PARP inhibition during xenograft development. These results are in agreement with the cDNA microarray study and with the macroscopic tumor data shown in Fig. 1, as well as with the microscopic data shown in Fig. 2.

Antitumoral Effect of PARP Inhibition or Depletion in Cell Lines. We further examined the effect of PARP inhibition or depletion in several liver cell lines. DPQ decreased cell viability in HepG2 in a dose-dependent manner, and this reduction in the colony-forming capacity was far more significant than that induced in the nontumoral WRL-68 cell line (Fig. 4A,I), confirming the results reported previously by Huang et al.[14] In addition, the PARP-1 depletion by means of stable siRNA in the PLC-PRF-5 C6 clone (siP912, Supporting Fig. S1) confirmed the decreased colony-forming capacity in this cell line in contrast to PLC-PRF-5 and nontumoral cells (Fig. 4A,II, WRL-68 and PARP-1 knockdown WRL-68 C1 clone, siP912, Supporting Fig. S1). Furthermore, PARP-1 inhibition or depletion (PLC-PRF-5 C6 clone) delayed the cell motility of HepG2 and PLC-PRF-C5 cells (wound-healing assay, Fig. 4B,I/II). To investigate the ability of the PARP inhibitor to modulate angiogenesis *in vitro*, HUVEC cells were exposed to different doses of DPQ. The results shown in Fig. 5 indicate that DPQ at this dose markedly reduces the formation of vessel-like structures. This effect was not due to a cytotoxic response because cells treated with the same dose of DPQ were viable, data that are in agreement with those given in Fig. 4A,I/II, in which DPQ administration is shown not to affect nontumoral cell viability (Supporting Fig. S3).

We also compared the effect on proliferation, angiogenesis, and survival-related gene expression of pharmacological PARP inhibition and the PARP-1 knockdown (siP912 PARP-1-siRNA, Supporting Fig. S1) in the PLC-PRF-5 HCC cell line (Fig. 6A). PARP inhibition and

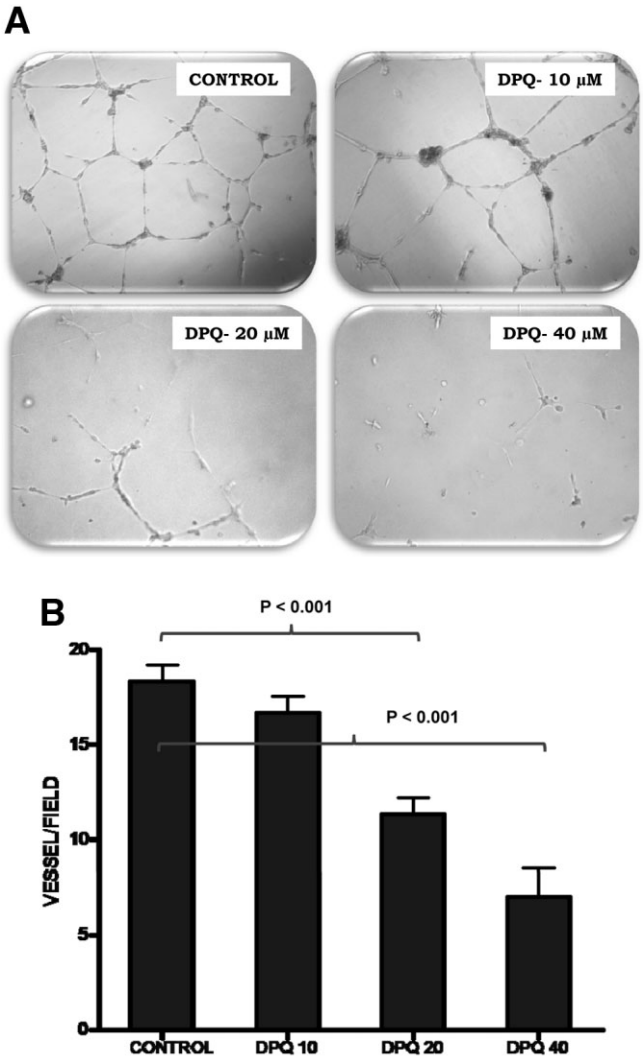

Fig. 5. PARP inhibition decreases the formation of vessel-like structures. Photographs of vessel-like structures of HUVEC cells growing on Matrigel (A). Quantification of vessel-like structures (B). Data represent the mean of at least three independent experiments done in triplicate.

blockage both significantly reduce the expression of *EGFR, MDM2,* and *EPAS1 (HIF2A)*. These results are in agreement with the xenograft cDNA microarray study (Fig. 3A,C). Furthermore, the reduction of gene expression was dependent on the intensity of PARP-1 blockage, confirming the role of PARP-1 in the expression of these genes (Supporting Fig. S4).

Finally, the higher PARP-1 expression in tumoral cell lines than in normal liver cells was interesting and provided evidence of the role of PARP-1 in HCC formation and progression (Fig. 6B). This was confirmed in human HCC versus nontumoral tissues (1,828 versus 423 and 424 in numbers of PARP-1 stained cells/mm^2 in HCC, nontumoral, and peritumoral tissues, respectively, and, in accordance with the grade of PARP-1-stained intensity, the data indicated 95.2% versus 17.6% and 13.3% of

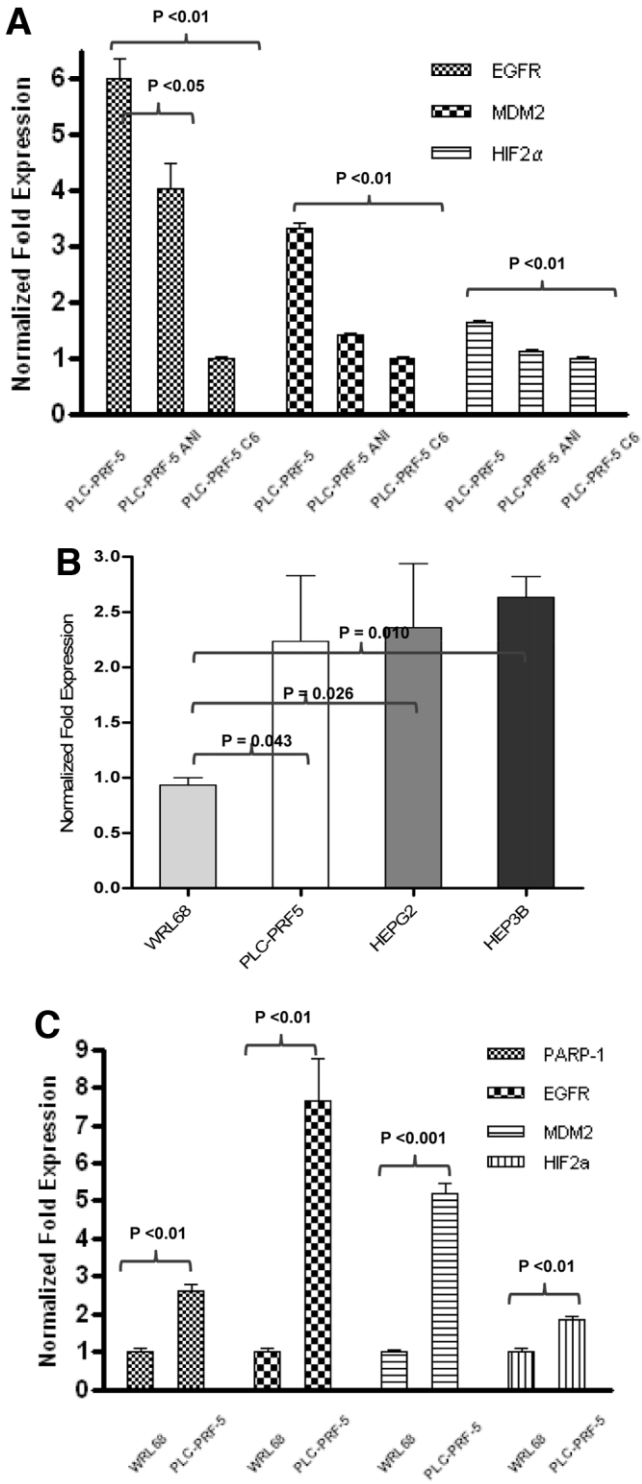

Fig. 6. PARP-1 inhibition by specific siRNA modulates gene expression. (A) The pharmacological PARP inhibition has the same effect as PARP-1 siRNA knockdown (PLC-PRF-5 C6 clone) on gene expression. (B) Increased PARP-1 expression in HCC cell lines determined by quantitative RT-PCR. (C) This increased PARP-1 in HCC is correlated with the expression of tumoral-related genes. Data represent the mean of at least three independent experiments done in triplicate.

high intensity in HCC, nontumoral, and peritumoral tissues, respectively, $P < 0.001$, Supporting Fig. S5). Furthermore, the *EGFR* and *END* expression in the WRL-68 and PLC-PRF-5 cell lines correlated with the PARP-1 levels of these cells, confirming the role of PARP-1 in the expression of these genes (Fig. 6C).

PARP Inhibitor DPQ Protects Against DEN-Induced Preneoplastic HCC Markers. A model of chemically induced carcinogenesis was used to determine whether PARP inhibition might also delay the hepatocarcinogenesis process. No evidence of DPQ-induced toxicity was detected at liver histological examination. Furthermore, the serum levels of liver enzymes were similar prior to DPQ administration and after the PARP-1 inhibitor treatment (Supporting Table S5). Histological examination revealed significant differences in the development of preneoplastic transformation (preneoplastic foci per field, $P < 0.05$) among these animals (DEN mice group versus DEN+DPQ mice group, Fig. 7A). For further support of the previous observations, the proliferation of hepatocytes was determined by BrdU incorporation. Clearly, hepatocyte proliferation decreased significantly in mice treated with the PARP inhibitor (DEN+DPQ mice group) compared with the non-DPQ-treated mice ($P < 0.05$, Fig. 7B). Furthermore, PARP inhibition decreased the NF-κB activation in whole livers as compared with the mice treated only with the hepatotoxic drug (Fig. 8A). Finally, treatment with DPQ also affected the expression of different genes in whole livers involved in inflammation and hepatocarcinogenesis (*Tnf, IL6, IL1b, Gstm3, Vegf, Spp1 [Opn]*, Fig. 8B).

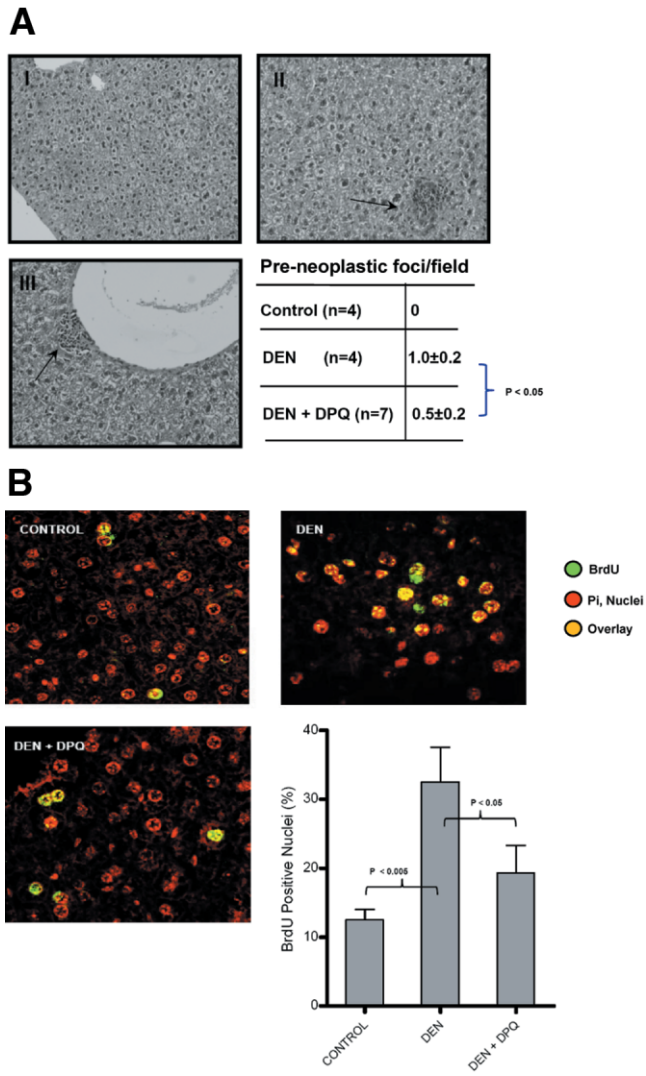

Fig. 7. Experimental liver carcinogenesis studies using DEN in C57BL/6 mice. (A) Histological (H&E) analysis of mice liver from (i) control mice with normal liver tissue, (ii) DEN-treated mice with preneoplastic foci, and (iii) DEN+DPQ mice with rare inflammatory focus. (B) Inhibitory effect of DPQ on the rate of proliferation (BrdU incorporation) in mouse liver. BrdU-positive cells were quantified by microscope observation, measuring 10 arbitrary fields.

Discussion

HCC is the third most common cause of cancer mortality worldwide and its prognosis is normally extremely poor. In view of the lack of an existing effective systemic therapy for this cancer, alternatives are required; for this reason, we suggest PARP inhibition as an approach that may be effective against HCC. PARP blockade has emerged as a promising treatment for a wide range of disorders, including stroke, ischemia/reperfusion injury, diabetes, shock, and various other forms of inflammation. In addition, it has been shown to alleviate different forms of hepatic injury.[6,7,15-18] The mechanisms involved in such protection are fairly heterogeneous, ranging from cellular energy preservation to transcription regulation. In the present study we provide the first experimental evidence suggesting that the pharmacologic inhibition of PARP decreases HCC tumoral xenograft growth and protects against DEN-induced hepatocarcinogenesis; it also produces a spectacular reduction in cell proliferation and new vessel formation, as well as an increase in the number of cells with apoptotic features, which suggests that PARP inhibition may be involved in diverse processes concerning tumor progression. The *in vitro* assays with tumoral cell lines confirm the previous data, indicating that both PARP inhibition and PARP-1 knockdown (siP912 vector, PARP-1-siRNA, PLC-PRF-5 C6 clone) reduce cell viability, cell motility, and vessel formation.

In view of these encouraging results, we studied the gene expression profile in tumoral xenografts after DPQ treatment, taking into account that it has been reported that loss of PARP-1 affects the gene expression profile in liver cells.[19] The mechanisms involved in PARP-1 transcriptional regu-

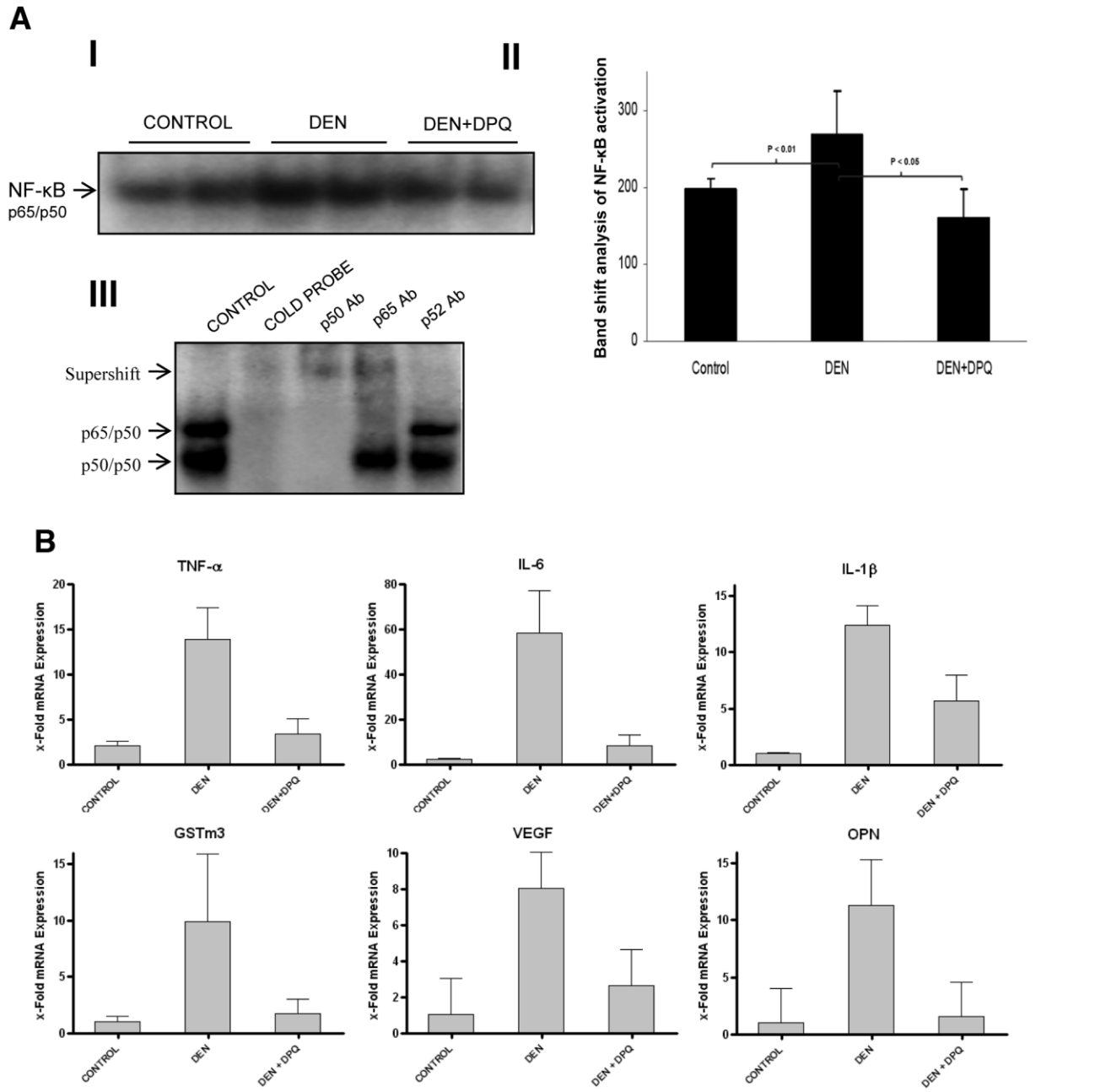

Fig. 8. Analysis of NF-κB activation and inflammatory/related-tumoral genes in DEN-treated mice. (A) (I) Band shift analysis of NF-κB activation using NF-κB consensus oligonucleotides (Promega, Madison, WI). (II) Band shift quantification. DEN-treated mice (n = 6) showed an increased band shift analysis of NF-κB activation with respect to untreated (control group, n = 6) or DPQ-treated mice (DEN+DPQ group, n = 6). (III) A preliminary competitive study was carried out for the identification of NF-κB complexes. The NF-κB complexes (p65/p50 and p50/p50) were identified using cold probe, as well as anti-p65, p50, and p52 antibodies in control sample. The images are representative of three independent experiments. (B) Expression levels of some genes involved in liver inflammation and tumor progression determined by quantitative RT-PCR (18 samples, control n = 6, DEN n = 6, and DEN + DPQ n = 6). Values are normalized to the level of 18s ribosomal RNA (rRNA).

lation are fairly heterogeneous, ranging from epigenetic regulation, chromatin structure modulation, enhancer-binding, insulation to its role as coactivator of several transcription factors.[3,20] The global analysis of gene expression during DPQ treatment revealed striking differences between the two groups analyzed (Fig. 3; Supporting Tables S2 and S3). Genes up-regulated in the nontreated control xenograft group include several human tumor-associated genes (angiogenesis, cell proliferation, cell death, metastasis, DNA repair, signal transduction, etc.). PARP inhibition was found to produce a considerable reduction in mRNA levels of *EGFR, MDM2, ANGPT2 (ANG-2), HGF,* and *MYC*. These genes have been reported to contribute to hepatocarcinogenesis by stimulating mitogenesis, survival, the inva-

siveness of HCC, etc., and have been proposed as a potential target for biological therapies aimed at HCC cells.[21-23] Of particular interest is the reduction in the induction of *EPAS1 (HIF2A), FLT1 (VEGFR1), SPP1 (OPN)*, and *ENG (END)* as well as of genes that are regulated by HIF-1α; for example, *EGLN1/PHD2, ANXA3,* and *ADORA3* after treatment with DPQ. These genes play a large part in HCC tumor progression by promoting a global response to hypoxia, including new vessel formation, and are expressed in most cases of HCC with capsular infiltration and portal vein invasion, which indicates a possible role of these genes in HCC metastasis.[24-28] The antiangiogenic effect of the PARP inhibitor has been demonstrated by our group and by others,[5,29] by reference to HIF1A modulation by PARP-1 during carcinogenesis of the skin. Thus, the blocking of PARP-1 may be an important and useful strategy for targeting these molecules for tumor prevention and therapy by way of its antiangiogenesis property.

The role of PARP-1 in carcinogenesis is controversial and further studies are required to determine whether its involvement in DNA repair (protective effect against carcinogens),[30] or whether its role in transcription modulation and as an inflammatory process regulator (carcinogen enhancer effect),[5] is the role determinant or, conversely, whether the determinant is the tumoral model used.[31] Our data using PARP inhibitor show that this protein is involved in the DEN-induced carcinogenesis by means of its function in the expression control of genes involved in inflammation and carcinogenesis (Fig. 8B) as well as by means of the modulation of transcription factors correlated with carcinogenesis (NF-κB, Fig. 8A). Therefore, targeting PARP-1 is not only a way to prevent efficient DNA repair during treatment with classical chemotherapy and radiotherapy, as has been traditionally envisaged, but is also significant because of its effects on the transcription of key genes involved in tumor progression and carcinogenesis.

A significant aspect of this research is the question of why PARP-1 is overexpressed in HCC in contrast to normal liver cells and whether this affects HCC formation and progression. The data reported in the present study are in accordance with those reported previously by Shimizu et al.[32] Moreover, it is known that various pathologies with inflammatory characteristics (diabetes and ischemia-reperfusion injury) are capable of producing a pathological increase in PARP-1 expression.[33,34] We consider these aspects to be of great interest and they are currently under investigation in our laboratory.

In summary, this study shows that PARP inhibition, in itself, as monotherapy, limits HCC xenograft growth and prevents tumor vasculogenesis through its ability to contribute to the regulation of the expression of different genes involved in tumor progression. In consequence, the use of PARP inhibitors may be a novel and effective therapy against HCC.

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