Amphiregulin Contributes to the Transformed Phenotype of Human Hepatocellular Carcinoma Cells

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Abstract

Hepatocellular carcinoma is a major cause of cancer-related deaths. Current treatments are not effective, and the identification of relevant pathways and novel therapeutic targets are much needed. Increasing evidences point to the activation of the epidermal growth factor receptor (EGFR) as an important mechanism in the development of hepatocarcinoma. We previously described that amphiregulin (AR), a ligand of the EGFR, is not expressed in healthy liver but is up-regulated during chronic liver injury, the background on which most liver tumors develop. Now, we have studied the expression and role of AR in human hepatocarcinoma. AR expression and function was studied in human liver tumors and cell lines. AR is expressed in human hepatocellular carcinoma tissues and cell lines and behaves as a mitogenic and antiapoptotic growth factor for hepatocarcinoma cells. We provide several lines of evidence, including AR silencing by small interfering RNAs and inhibition of amphiregulin by neutralizing antibodies, showing the existence of an AR-mediated autocrine loop that contributes to the transformed phenotype. Indeed, interference with endogenous AR production resulted in reduced constitutive EGFR signaling, inhibition of cell proliferation, anchorage-independent growth, and enhanced apoptosis. Moreover, knockdown of AR potentiated transforming growth factor-β and doxorubicin-induced apoptosis. Conversely, overexpression of AR in SK-Hep1 cells enhanced their proliferation rate, anchorage-independent growth, drug resistance, and *in vivo* tumorigenic potential. These observations suggest that AR is involved in the acquisition of neoplastic traits in the liver and thus constitutes a novel therapeutic target in human hepatocarcinoma. (Cancer Res 2006; 66(12): 6129-38)

Introduction

Hepatocellular carcinoma is a malignancy of worldwide significance, being the fifth most common solid tumor and a leading cause of cancer-related death (1). Its incidence is high in developing countries and is continuously rising in western society, mainly due to the dissemination of hepatitis C virus infection, excessive alcohol intake, and the increase in nonalcoholic fatty liver disease-associated cirrhosis, major risk factors for hepatocellular carcinoma (1). Most hepatocellular carcinoma patients are diagnosed at advanced stages, when they are not suitable for curative therapies, such as resection and transplantation (1), and currently available chemotherapeutic options are not effective, with poor responses and low survival of patients with advanced hepatocellular carcinoma (1).

Hepatocellular carcinoma is strongly associated with chronic liver diseases, including chronic hepatitis and cirrhosis, which are regarded as preneoplastic conditions for this malignancy (2, 3). The genomic alterations leading to the development of the neoplastic phenotype of the hepatocyte seem to be heterogeneous, and diverse combinations of these aberrations may result in the malignization of the liver parenchymal cell (1–4). However, a common feature found in the preneoplastic liver is the increase in the proliferation of both regenerative and dysplastic hepatocytes compared with non-diseased liver (3). This enhanced cellular proliferation, accompanied by reduced rates of cell death, is thought to be essential for the development of the full neoplastic phenotype, facilitating the accumulation of genetic alterations in a large population of progeny cells (2, 3). One of the major molecular mechanisms that drive the hyperproliferative condition found in chronically injured liver is the elevated expression of mitogenic factors for the hepatocytes. Initially, this response can be part of the endogenous regenerative and defense mechanisms of the injured liver to the loss of parenchymal cells (5). However, its perpetuation is thought to participate in the multistep process of hepatocarcinogenesis through the establishment of autocrine mechanisms for self-sustaining cellular growth (2–4). The epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase, is highly expressed in normal and transformed hepatocytes and is believed to convey essential mitogenic and survival signals in transformed cells, including hepatocellular carcinoma cells (2, 3, 6, 7). In support of this notion are recent reports that describe the prevention of chemically induced hepatocellular carcinoma in rats and the growth of human hepatocellular carcinoma cell lines by gefitinib, an inhibitor of EGFR tyrosine kinase activity (8–10). The EGFR can be bound and activated by a broad family of ligands, that besides EGF include transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, and amphiregulin (AR; refs. 11–13). Overexpression of TGF-α and HB-EGF has been observed in liver cirrhosis and hepatocellular carcinoma, and these factors are believed to contribute to EGFR activation during hepatocarcinogenesis (14, 15). We have recently reported that AR, a heparin-binding EGFR ligand with undetectable expression in normal liver, is readily induced in the regenerating liver after partial hepatectomy and behaves as a potent mitogenic and antiapoptotic factor for normal hepatocytes (16, 17). The role played by AR in hepatocyte proliferation and cytoprotection seems to be unique because

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studies in AR knockout mice showed that the effects exerted by AR on liver parenchymal cells could not be compensated by other EGFR ligands, such as TGF-α and HB-EGF, also induced during liver injury (16–19). We have also observed that *AR* gene expression was up-regulated in the premalignant cirrhotic human liver and in experimental liver cirrhosis in rats (16). Together, these findings led us to speculate that AR might have a role in hepatocarcinogenesis, and thus that an AR-mediated autocrine or paracrine loop could contribute to the growth of hepatocellular carcinoma cells. Our present results support the notion that AR can be considered as one key activator of the EGFR signaling pathway in the unrestrained growth and apoptosis resistance of hepatocellular carcinoma cells.

Materials and Methods

Materials. Cell culture media, serum, glutamine, and antibiotics were from Life Technologies/Invitrogen (Barcelona, Spain). Human recombinant AR, forskolin, doxorubicin, goat serum IgG, and heparin were from Sigma (St. Louis, MO). TGF-β was from Roche (Penzberg, Germany). The metalloproteinase inhibitor GM6001 and the EGFR inhibitor PD153035 were from Calbiochem (San Diego, CA). Antibodies used were anti-human AR goat polyclonal antibody (AF262) from R&D Systems (Minneapolis, MN); phosphorylated Akt (Ser^{473}; 9271S), c-Jun NH_2-terminal kinase (JNK; 9252), phosphorylated JNK (Thr^{183}/Tyr^{185}; 9251), and active caspase-3 p17 subunit (9664S) from Cell Signaling (Beverly, MA); extracellular signal-regulated kinase (Erk1/2; 06-182) and phosphorylated EGFR (Tyr^{1148}; 4404) from Upstate Biotechnology (Charlottesville, VA); phosphorylated Erk1/2 (Tyr^{204}; sc7383), EGFR (sc03), Akt (sc5298), and β-actin from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-Bim antibody (AB17003) from Chemicon (Temecula, CA).

Patients. Liver tissue was obtained from three groups of subjects: (*a*) Control individuals ($n = 11$; all males; mean age, 58 years; range, 45-70 years) with normal or minimal changes in the liver. Tissue samples were collected at surgery of digestive tumors of from percutaneous liver biopsy done because of mild alteration of liver function tests. (*b*) Patients with liver cirrhosis of different etiology ($n = 14$; 9 males; mean age, 60 years; range, 42-77 years): 9 patients with hepatitis C viral cirrhosis, 3 patients with hepatitis B viral cirrhosis, and 2 patients with alcoholic cirrhosis. (*c*) Patients with primary hepatocellular carcinoma ($n = 19$; all males; mean age, 67 years; range, 39-79 years). Cancerous liver tissues were obtained during surgical resection. This study was approved by the University of Navarra Human Research Review Committee.

Cell culture and treatments. The human hepatocellular carcinoma cell lines HepG2, Hep3B, HuH7, PLC/PRF/5, and SK-Hep1 and the human non–small cell lung cancer cell lines H322 and H358 were grown in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Where indicated, serum was removed from the culture medium. Anchorage-independent growth of human hepatocellular carcinoma cells was assessed by colony formation ability in soft agar and was done as previously described (20). After 4 weeks, colonies were counted. Data are means of three independent experiments done in duplicates.

RNA isolation and analysis of gene expression. Total RNA was extracted using the TRI Reagent (Sigma). Reverse transcription and real-time PCR was done using an iCycler (Bio-Rad, Hercules, CA) and the iQ SYBR Green Supermix (Bio-Rad) as previously described (17). The amount of each transcript was expressed as the *n*-fold difference relative to the control gene *β-actin* ($2^{\Delta Ct}$, where ΔC_t represents the difference in threshold cycle between the control and target genes), as previously described (21). Multiplex reverse transcription-PCR (RT-PCR) was carried out as reported (22). The primers used for AR, HB-EGF, and TGF-α determinations were the same used in previous publications (16, 17). Primers used to assess *TACE/ADAM17* gene expression were as reported (23).

Western blot analysis and AR ELISA. Hepatocellular carcinoma cells were lysed, and homogenates were subjected to Western blot analyses as reported (16, 17). Soluble AR was measured in hepatocellular carcinoma cells' conditioned media (CM) by ELISA. For this purpose, cells were seeded into six-well plates (1.5×10^5 per well) and incubated for 48 hours in culture medium with or without serum. CM were collected and, after addition of phenylmethylsulfonyl fluoride (1 mmol/L), was precleared by centrifugation (15 minutes; 14,000 rpm) and lyophilized. The concentration of AR in CM was determined by sandwich ELISA from R&D Systems, using a monoclonal anti-AR capture antibody (MAB262) and a biotinylated polyclonal detection antibody (BAF262). A standard curve using recombinant human AR was used to calculate AR concentrations in CM.

Transfections. SK-Hep1 cells grown in 60-mm dishes until 70% confluence were transfected with a pcDNA3 plasmid (Invitrogen, Carlsbad, CA) harboring the complete murine AR cDNA previously described (24), or the empty pcDNA3 vector. Transfections were carried out using calcium phosphate, and transfectants were selected in complete medium containing 0.6 mg/mL of G418 sulfate (Geneticin; Invitrogen) as described (20). After 2 weeks, individual colonies were harvested, and clones transfected with the empty vector pcDNA3 (SK-EV) or the AR expressing construct (SK-AR) were expanded.

RNA interference. The sequences of the small interfering RNAs (siRNA) targeting different regions of the human *AR* gene (siAR1 and siAR2) and the control siRNA (siGL2) along with their specificity have been described (23). These siRNAs were obtained from Dharmacon Research (Lafayette, CO). Transfections of the 21-nucleotide siRNA duplexes (100 nmol/L) were carried out using the Dharmafect reagent (Dharmacon Research) following the manufacturer's instructions. Silencing of AR was confirmed by RT-PCR and by quantitating the amount of AR in CM.

Cell growth and apoptosis assays. Cell proliferation and viability was estimated using the cell proliferation reagent WST-1 from Roche. Apoptosis was estimated by the determination of soluble histone-DNA complexes using the Cell Death Detection Assay (Roche). Specific enrichment of mono-nucleosomes and oligonucleosomes released into the cytoplasm (enrichment factor) was calculated as the ratio between the absorbance values of the samples obtained from treated and control cells as previously described (17).

Tumor growth in nude mice. Five million cells were inoculated s.c. in the dorsal flanks of 4-week-old male BALB/c athymic nude mice (Harlan, Barcelona, Spain).

Statistical analysis. Normally distributed data were compared among groups using the Student's *t* test. Non-normally distributed data were compared using the Mann-Whitney test. Data are means ± SE. $P < 0.05$ was considered significant.

Results

Expression of AR is up-regulated in human hepatocellular carcinoma tissue and hepatocellular carcinoma cell lines. We have previously reported that *AR* gene expression is up-regulated in human and experimental liver cirrhosis (16). It has been proposed that persistent activation of growth and survival pathways during liver cirrhosis may contribute to the pathogenesis of hepatocellular carcinoma (2, 3, 6). These notions led us now to evaluate whether the expression of AR was also elevated in the neoplastic liver. As shown in Fig. 1*A*, whereas real-time PCR analysis of control samples showed barely detectable levels of AR mRNA, AR expression was significantly increased in about 70% of hepatocellular carcinoma tissue samples (13 of 19). All EGFR ligands, including AR, are synthesized as membrane-anchored precursors that can be proteolytically released from the cell surface, allowing their autocrine and paracrine interactions with the EGFR (25). This shedding is carried out by a family of membrane-anchored metalloproteases known as "a disintegrin and metalloprotease" (ADAM) proteins (26). The shedding of AR from the cell membrane is carried out by ADAM17, also known as tumor necrosis factor-α converting enzyme (TACE; ref. 24). We have measured the expression of ADAM17 in samples from human hepatocellular carcinoma tissue by real-time PCR (Fig. 1*B*) and found it to be

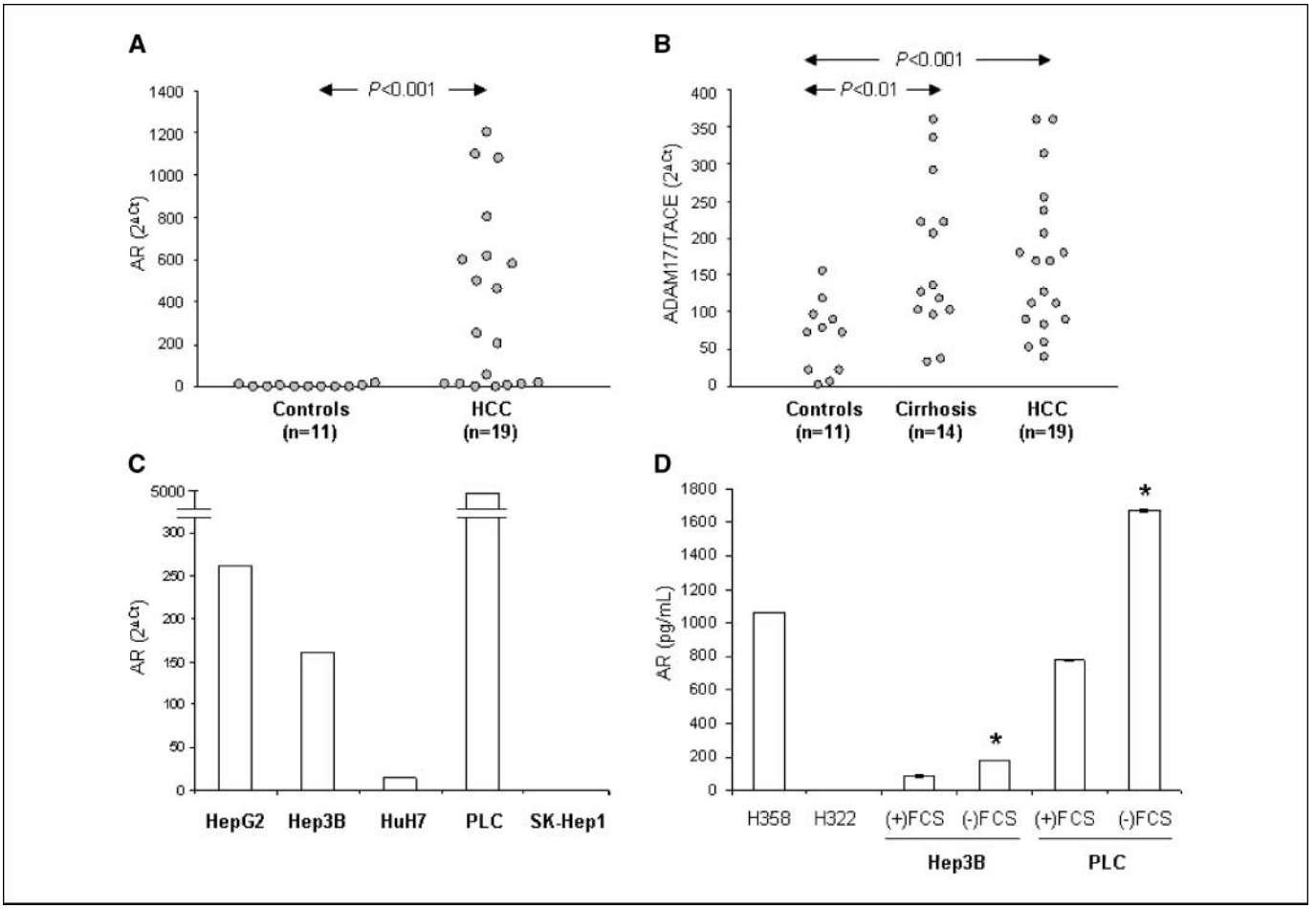

Figure 1. *A, AR* gene expression in human hepatocellular carcinoma tissues. *B, ADAM17/TACE* gene expression in human liver cirrhosis and hepatocellular carcinoma tissues. *C, AR* gene expression in human hepatocellular carcinoma cell lines. Gene expression was measured by real-time PCR. *D,* AR protein contents in CM obtained from Hep3B and PLC human hepatocellular carcinoma cell lines grown in the presence (+*FCS*) or absence (−*FCS*) of 10% FCS for 48 hours. CM from the human non–small cell lung carcinoma cell lines H358 and H322 were used as positive and negative controls for AR production, respectively. AR in CM was measured by ELISA. *Points*/*columns,* means of three independent experiments done in duplicates; *bars,* SE. *, $P < 0.05$.

significantly higher than in controls. Interestingly, we observed that the mRNA levels of ADAM17 were already significantly elevated in cirrhotic liver tissue compared with healthy controls, and that there were no differences between cirrhotic liver and hepatocellular carcinoma samples in terms of *ADAM17* gene expression. There was no association between *ADAM17* gene expression levels and the etiology of cirrhosis.

Next, we tested the expression of AR in five human hepatocellular carcinoma cell lines by real-time PCR. AR was found to be expressed, albeit to different extent, in all but one of the human hepatocellular carcinoma cell lines examined (Fig. 1*C*). Shedding of AR is essential for autocrine and paracrine signaling through the EGFR (26). To establish whether such autocrine system could occur in hepatocellular carcinoma, it was important to ascertain if hepatocellular carcinoma cells not only expressed but also released AR to the culture medium. We did an ELISA assay for AR using the human lung cancer cell lines H322 and H358 as controls for AR producing and nonproducing cells, respectively (27), and we were able to detect AR protein accumulation in the CM of the two hepatocellular carcinoma cell lines tested (PLC and Hep3B; Fig. 1*D*). Interestingly, we observed that when cells were cultured in the absence of serum, AR production was enhanced (Fig. 1*D*).

AR treatment of hepatocellular carcinoma cells stimulates cell growth and confers apoptosis resistance. We have previously shown that AR is a mitogenic factor for normal rodent hepatocytes in primary culture (16). However, AR was originally described as a bifunctional protein not only capable of stimulating the growth of normal epithelial cells but also able to inhibit that of certain carcinoma cell lines (28); therefore, it was critical to establish the effect of AR on hepatocellular carcinoma cells. We observed that treatment of Hep3B and PLC cells with AR in the absence of serum increased the growth of these cells in a dose-dependent manner, and that this effect was blocked by the EGFR-specific inhibitor PD153035 (Fig. 2*A*). Similar results were obtained using HuH7 (data not shown). Interestingly, in the presence of PD153035, cell growth was reduced below levels found in serum-starved cells, in agreement with the crucial role played by EGFR-conveyed growth signals for hepatocellular carcinoma cells (8–10). Besides providing growth-promoting signals, activation of the EGFR enhances survival of hepatocytes and hepatocellular carcinoma cells (9, 17). In agreement with this, we observed that AR efficiently prevented TGF-β-induced apoptosis in Hep3B cells (Fig. 2*B*). Consistent with the observed biological effects of AR, treatment of Hep3B cells with AR resulted in the rapid

phosphorylation of the EGFR and the downstream effector kinases Erk1/2 and Akt in an EGFR-dependent manner, as indicated by the inhibitory effect of PD153035 (Fig. 2*C*). In agreement with previous observations, the potent activation of Erk2 phosphorylation elicited by AR treatment was accompanied by a shift in mobility of the phosphorylated protein. Similar results were obtained with PLC and HuH7 cells (data not shown).

In human colon carcinoma cells, *AR* gene expression is promoted by cyclooxygenase-2-generated prostaglandin E_2, through the stimulation of protein kinase A (PKA), in conjunction with the activation of the EGFR by TGF-α (29). In agreement with this, and our previous observations in primary hepatocytes (16), we observed that the PKA activator forskolin induced *AR* gene expression in Hep3B cells (data not shown), and that AR itself could promote its own expression through the activation of the EGFR (Fig. 1*D*).

Characterization and functional relevance of an AR-mediated autocrine loop in hepatocellular carcinoma cells. We also examined the effects of self-produced AR on hepatocellular carcinoma cells. To this end, we interfered with AR autocrine signaling at different levels in serum-free cultures of hepatocellular carcinoma cells. First, we showed that Hep3B and PLC cell growth was attenuated by an AR-neutralizing antibody (Fig. 3*A*). As previously mentioned, AR is synthesized as a membrane-anchored precursor that is released by the action of the metalloprotease ADAM17 (25, 26). We observed that addition of the metalloprotease inhibitor GM6001 reduced the serum-independent growth of Hep3B and PLC cells (Fig. 3*A*). Similar to HB-EGF, AR contains a heparin-binding domain, and it has been reported that addition of heparin inhibits AR-mediated autocrine growth of human keratinocytes (30). When Hep3B and PLC cells were cultured in serum-free medium, we observed that the presence of heparin significantly inhibited the proliferation of these hepatocellular carcinoma cell lines (Fig. 3*A*). The effect of these two pharmacologic interventions on the autocrine growth of hepatocellular carcinoma cells was mimicked by PD153035 and was accompanied by a significant attenuation of the enhanced basal levels of Erk1/2 phosphorylation found in hepatocellular carcinoma cells (ref. 31; Fig. 3*B*). We did an additional experiment in which CM obtained from PLC cells was added to serum-depleted cultures of HuH7 cells, and cellular proliferation was subsequently measured. We observed that CM obtained from PLC cells contained a growth-promoting activity for HuH7 cells, and that this proliferative response was completely blunted by the

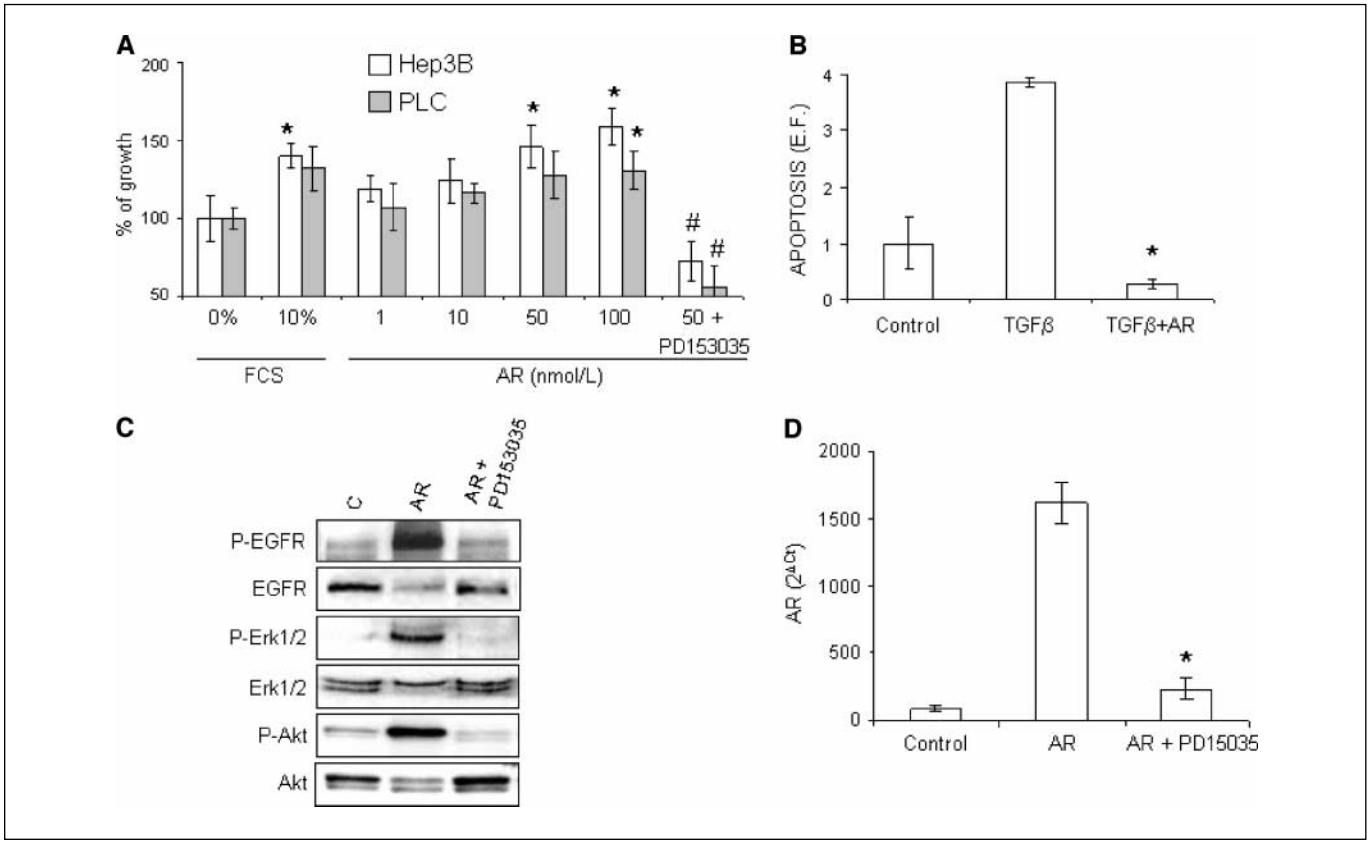

Figure 2. *A,* AR treatment induces cell growth in Hep3B and PLC cells. Hep3B and PLC cells were treated with increasing concentrations of AR for 24 hours, and growth was measured as described in Materials and Methods. Where indicated, cells were treated with the EGFR inhibitor PD153035 (1 μmol/L). Growth of cells in the absence of serum was given the arbitrary value of 100%. Cell growth in the presence of 10% FCS is shown as control. *, $P < 0.05$ versus cells maintained in 0% FCS. #, $P < 0.05$ versus cells treated with 50 nmol/L AR. *B,* AR protects Hep3B cells from TGF-β-induced apoptosis. Apoptosis was induced by TGF-β (1 nmol/L) treatment for 24 hours. Where indicated, cells were pretreated with AR (50 nmol/L) for 2 hours. Apoptosis was estimated by specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm as described in Materials and Methods. *, $P < 0.05$ versus TGF-β alone. *C,* AR treatment activates the EGFR and EGFR-dependent downstream signaling (Erk1/2 and Akt) in Hep3B cells. Cells were treated with AR (20 nmol/L) for 10 minutes. Where indicated, cells were pretreated with the EGFR inhibitor PD153035 (1 μmol/L) for 30 minutes. Representative Western blots. *D,* AR (20 nmol/L) stimulates its own gene expression in Hep3B cells through the activation of the EGFR. PD153035 was added 30 minutes before AR, and treatment was continued for 6 hours. AR mRNA levels were measured by real-time PCR. *, $P < 0.05$ versus AR alone. *Points*/*columns,* means of three independent experiments done in duplicates; *bars,* SE.

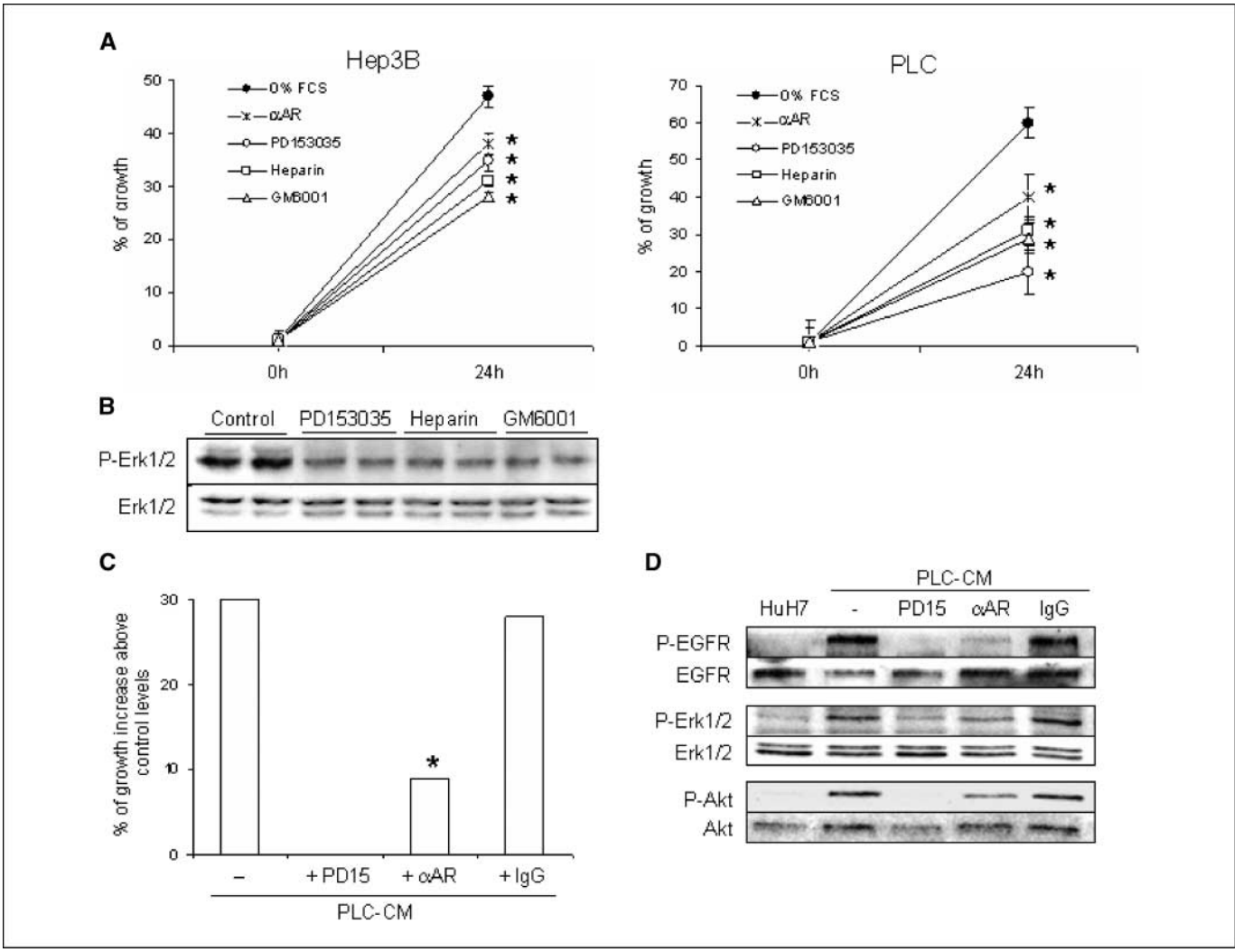

Figure 3. *A,* serum-free (0% FCS) growth of Hep3B and PLC cells in the presence of an AR-neutralizing antibody (αAR, 20 μg/mL), the EGFR inhibitor PD153035 (1 μmol/L), heparin (40 μg/L), or the ADAM17/TACE inhibitor GM6001 (40 μmol/L). *, $P < 0.05$ versus cells grown in 0% FCS. *B,* PD153035, heparin, and GM6001 down-regulate the basal levels of Erk1/2 phosphorylation. Hep3B cells were treated with the above-indicated concentrations of these compounds for 40 minutes, and Erk1/2 phosphorylation levels were assessed by Western blotting. Representative blot. *C,* growth induced by PLC CM (PLC-CM) in HuH7 cells is AR dependent. CM was obtained from PLC cells kept in serum-free conditions for 48 hours, and then it was added to subconfluent HuH7 cells. HuH7 cell growth was estimated 24 hours later as described in Materials and Methods. Where indicated, before treatment of HuH7, PLC-CM was incubated for 1 hour at 37°C with PD153035 (1 μmol/L), AR-neutralizing antibody (αAR, 20 μg/mL), or purified goat IgG (IgG, 20 μg/mL). *, $P < 0.05$ versus HuH7 cells treated with PLC-CM alone. *D,* EGFR-dependent signaling elicited by PLC-CM in HuH7 cells is inhibited by an AR-neutralizing antibody (αAR). HuH7 cells were treated for 10 minutes with PLC-CM; subsequently, EGFR, Erk1/2, and Akt phosphorylation was estimated by Western blotting. Where indicated, before HuH7 treatment, PLC-CM was incubated with PD153035, AR-neutralizing antibody, or purified goat IgG as described above. Representative blots.

EGFR inhibitor PD153035, or significantly attenuated by an AR-neutralizing antibody (Fig. 3*C*). Consistent with these observations, treatment of HuH7 cells with PLC cells CM activated the EGFR and EGFR-dependent downstream signaling (Erk1/2 and Akt phosphorylation), responses that were specifically blunted by an AR neutralizing antibody (Fig. 3*D*).

More compelling evidence on the importance of AR in maintaining the hepatocellular carcinoma malignant phenotype was obtained through two complementary strategies. First, the endogenous expression of AR was silenced by siRNAs oligos in the AR-overexpressing PLC and Hep3B cells. Cell lysates and CM were harvested at different time points after transfection and analyzed by RT-PCR and ELISA, respectively. We confirmed the knockdown of *AR* gene expression at the mRNA level and the reduced accumulation of AR protein in CM from PLC cells (Fig. 4*A*) and Hep3B cells (data not shown). The specific silencing of the *AR* gene expression was further confirmed by the lack of effect of AR siRNAs on the mRNA levels of the other EGFR ligands HB-EGF and TGF-α, which are constitutively expressed in both PLC (Fig. 4*A*) and Hep3B cells (data not shown). We next evaluated the functional consequences of AR depletion in hepatocellular carcinoma cells. Blunted AR production by AR siRNA transfection resulted in significantly reduced basal EGFR activation, as determined by EGFR and Erk1/2 phosphorylation levels, in PLC and Hep3B cells (Fig. 4*B*). We also examined the proliferation and anchorage-independent growth of hepatocellular carcinoma cells in which AR expression was down-regulated. As shown in Fig. 4*C*, PLC and Hep3B siAR transfectants presented significantly inhibited cell growth compared with control cultures transfected with siGL2 (control siRNA). Interfering with AR production also dramatically reduced the aggressiveness of

hepatocellular carcinoma cancer cells, as shown by the impaired anchorage-independent growth in soft agar (Fig. 4*D*). Similar results were also obtained in Hep3B cells (data not shown).

To further explore the influence of AR gene expression on the behavior of hepatocellular carcinoma cells, we undertook a complementary approach. It consisted in the transfection of SK-Hep1 cells, the only human hepatocellular carcinoma cell line tested that lacked *AR* gene expression, with a plasmid vector harboring murine AR cDNA, and the generation of stable transfected clones. As shown in Fig. 5*A*, transfected SK-Hep1 cells (SK-AR1 and SK-AR2 clones) were confirmed to express AR mRNA and to release AR to the CM. Next, we examined the growth properties of SK-AR1 and SK-AR2 cells compared with the control SK-EV cells. We observed that AR expressing cells exhibited enhanced growth rate than SK-EV cells (Fig. 5*B*). Furthermore, the expression of AR significantly enhanced the tumorigenic behavior of SK-Hep1 cells, as assessed both *in vitro* and *in vivo*. The ability to form colonies in soft agar and the tumorigenic potential of AR-expressing SK-Hep1 cells in athymic nude mice were significantly higher than those of control cells that did not express AR (Fig. 5*C* and *D*).

AR expression contributes to the resistance of hepatocellular carcinoma cells to TGF-β and cytostatic drugs. Normal hepatocytes do not express TGF-β and, when exposed to this cytokine, undergo cell cycle arrest and apoptosis (5). In contrast, hepatocellular carcinoma cells produce TGF-β and have lost the sensitivity to TGF-β-induced apoptosis (2–4). This altered response seems to be crucial for the establishment of a highly malignant and invasive phenotype (32). To evaluate if the expression of AR in hepatocellular carcinoma cells could contribute to TGF-β resistance, we measured apoptosis in PLC cells after AR knockdown by AR siRNA transfection. Consistent with our previous observations using recombinant AR (Fig. 2*B*), we found that AR down-regulation significantly increased the sensitivity of PLC cells towards TGF-β-induced apoptosis (Fig. 6*A*). Interestingly, silencing of AR resulted in enhanced basal apoptosis (Fig. 6*A*), which is in agreement with the inability of PLC cells to survive in semisolid medium when AR is down-regulated (Fig. 4*D*). The levels of the active caspase-3 p17 subunit, shown in Fig. 6*B*, correlated well with the extent of apoptosis induced by AR silencing and TGF-β treatment (Fig. 6*A*). A common cellular response associated with

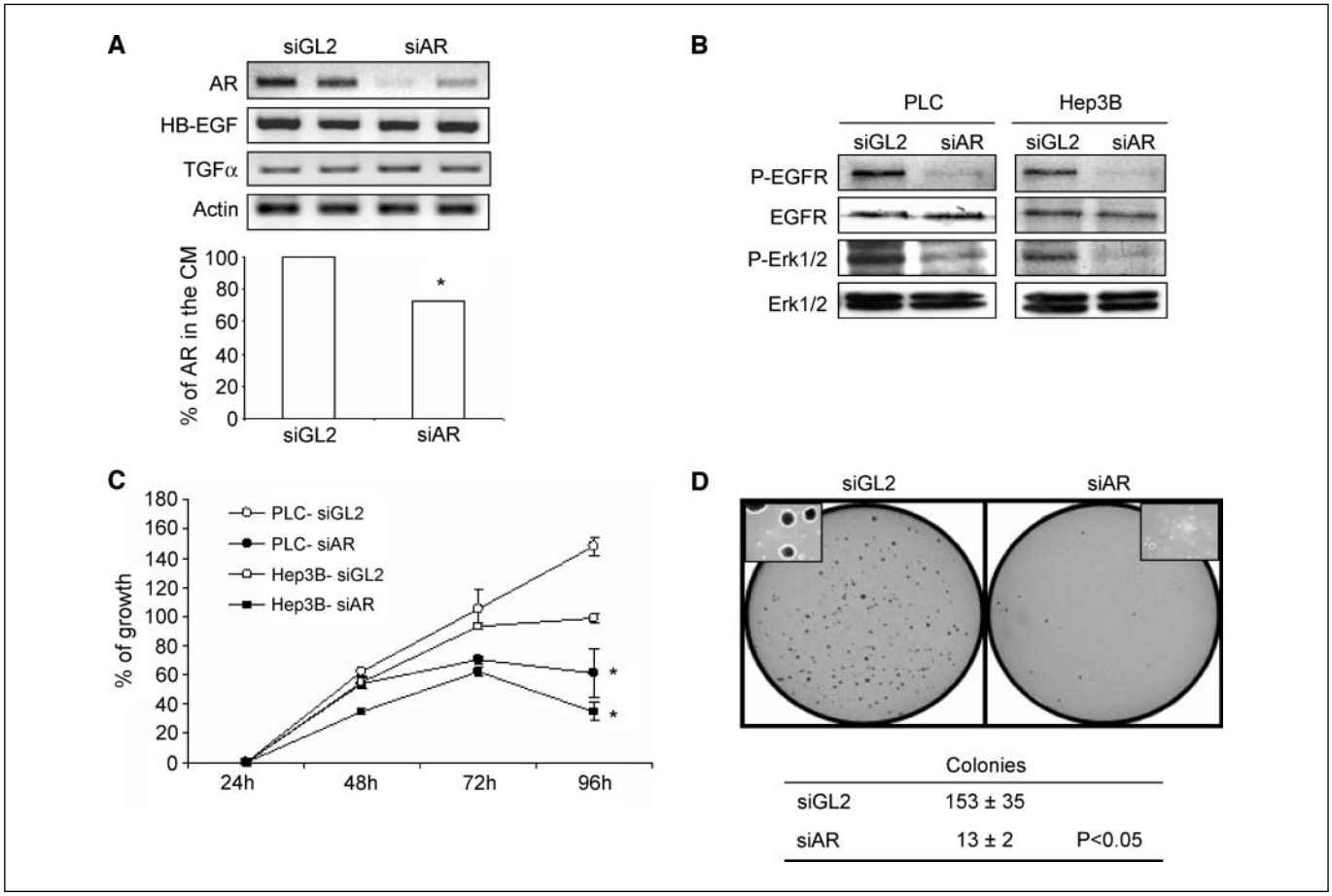

Figure 4. *A,* specific AR silencing by siRNAs in PLC cells. PLC cells were transfected with a mixture of two AR-specific siRNAs (50 nmol/L each; siAR), or a control siRNA (100 nmol/L; siGL2). AR, HB-EGF, TGF-α, and actin gene expression was measured by RT-PCR 72 hours after transfections. Representative gels. *Bottom,* relative AR protein levels in PLC CM 72 hours after transfection with siGL2 (given the arbitrary value of 100%) or siAR, as determined by ELISA. *, $P < 0.05$ versus siGL2. *B,* basal levels of EGFR and Erk1/2 phosphorylation in PLC and Hep3B cells 96 hours after transfection with either siGL2 or siAR as determined by Western blotting. Representative blots. *C,* effect of AR silencing on the growth of PLC and Hep3B cells. Cell growth was measured at different time points after transfections as described in Materials and Methods. *, $P < 0.05$ versus cells transfected with siGL2. *D,* effect of AR silencing on anchorage-independent cell growth in soft agar. PLC cells were transfected with either siGL2 or siAR siRNAs. After 24 hours, cells were harvested, counted, resuspended in 0.2% soft agar, and seeded onto 0.4% soft agar in DMEM supplemented with 10% FCS (10^4 per plate). After 4 weeks, colonies were stained with crystal violet and counted. *Points/columns,* means of three experiments done in duplicates; *bars,* SE. Representative images.

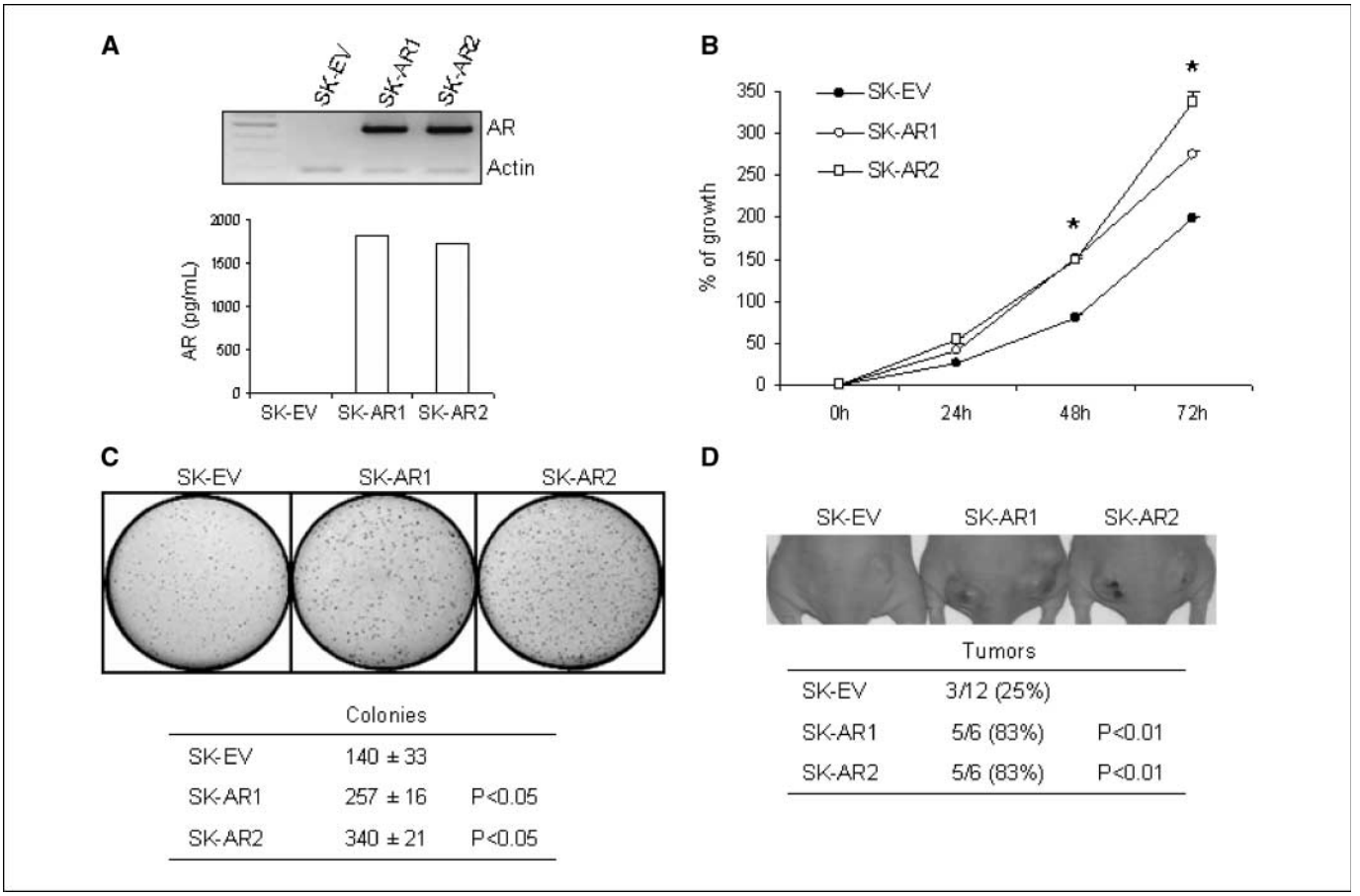

Figure 5. *A,* generation of AR stable transfectants in SK-Hep1 cells. SK-Hep1 cells were stably transfected with a vector harboring murine AR cDNA and colonies were selected as described in Materials and Methods. AR mRNA and protein were measured by multiplex RT-PCR (*top*) and ELISA analysis (*bottom*) of CM from two AR-expressing clones (SK-AR1 and SK-AR2) and a control clone (cells transfected with the empty vector, SK-EV). *B,* effect of AR expression on the growth of SK-Hep1 cells. The growth of SK-EV, SK-AR1, and SK-AR2 clones in complete medium was measured at different time points as described in Materials and Methods. Growth is referred to values obtained after attachment to the plates (time, 0 hour). *Points*/*columns,* means of three experiments done in triplicates; *bars,* SE. *, $P < 0.05$ versus control cells (SK-EV). *C,* anchorage-independent growth in soft agar of SK-EV, SK-AR1, and SK-AR2 cells. For each cell line, 10^4 cells were resuspended in 0.2% soft agar and seeded onto 0.4% soft agar in DMEM supplemented with 10% FCS. Colonies were counted 4 weeks later after staining with crystal violet. *Points*/*columns,* means of three experiments done in triplicates; *bars,* SE. *D,* tumorigenic potential of SK-EV, SK-AR1, and SK-AR2 cells. For each cell line, 5×10^6 cells were s.c. injected in the dorsal flanks of nude mice. Tumor incidence was estimated 8 weeks after injections. Tumor incidence is indicated as the number of tumors developed with respect to the total number of injections.

apoptosis induced by growth factor withdrawal and TGF-β treatment is the activation of the expression of the BH3-only protein Bim (33–35). Bim is a proapoptotic member of the Bcl-2 family that binds and neutralizes its prosurvival relatives, such as Bcl-X_L, priming the cell for apoptosis (33). We observed that upon AR silencing and TGF-β treatment, *Bim* gene expression was up-regulated at the mRNA (data not shown) and the protein level (Fig. 6*B*). Bim expression is controlled at various levels through the coordinate action of different signaling pathways (33). It is known that sustained activation of EGFR and Erk signaling prevents Bim up-regulation, whereas JNK activation triggers Bim expression and activation (33, 36–38). Consistent with the reported mode of regulation of Bim expression, we observed that concomitant with the attenuation of Erk1/2 phosphorylation, JNK activity was induced by AR down-regulation and TGF-β treatment (Fig. 6*B*). The same results were obtained in Hep3B cells (data not shown).

Accumulating evidences underscore the relevance of EGFR expression and activity for tumor cell drug resistance, including human hepatocellular carcinoma cells (39). This fact, together with our current observations on the promitogenic and antiapoptotic properties of AR for hepatocellular carcinoma cells, prompted us to evaluate whether AR expression could contribute to the resistance of these cells to doxorubicin, one of the most commonly used anticancer agents for solid tumors like hepatocellular carcinoma. To this end, we examined the effect of doxorubicin on PLC cells transfected with either control siRNA (siGL2) or with AR siRNAs (siAR). As shown in Fig. 6*C*, doxorubicin treatment induced apoptosis in PLC cells, and this effect was significantly potentiated by AR down-regulation. Similar to our previous findings with TGF-β, AR silencing potentiated the activation of caspase-3 and the up-regulation of Bim protein levels induced by doxorubicin treatment (Fig. 6*C*).

Discussion

Recent observations point to the EGFR signaling system as a prominent player in hepatocarcinogenesis. These observations include the overexpression and hyperactivation of the EGFR in cirrhotic and hepatocellular carcinoma tissues and in experimental models of hepatocellular carcinoma (8, 40–42). Targeting

the EGFR is emerging as a novel therapeutic approach for hepatocellular carcinoma, and encouraging experimental data and early clinical studies have been recently published (8, 9, 39, 41, 43). Nevertheless, more advanced experiences with EGFR inhibitors in other types of solid tumors have shown only modest clinical responses, suggesting the need to implement combination therapies with conventional cytostatics or with novel targeted molecules to improve efficacy (7, 41, 44). To this end, a profound knowledge of the biology of hepatocellular carcinoma is essential.

In the present work, we have identified the EGFR ligand AR as a novel growth and survival factor for hepatocellular carcinoma cells. As we previously reported, AR gene expression is undetectable in the healthy human liver (16); however, now, we observe that AR is up-regulated in a significant proportion of human hepatocellular carcinoma samples. In agreement with a recent publication (45), we also found that ADAM17, the transmembrane metalloprotease that cleaves the membrane-anchored AR precursor (26), was overexpressed in hepatocellular carcinoma tissue samples. Furthermore, we observed that ADAM17 mRNA levels were also up-regulated in the cirrhotic liver when compared with healthy controls. This finding, together with our previous observation on the activation of AR gene expression in human liver cirrhosis (16), suggests an enhanced availability of soluble AR from the early stages of hepatocarcinogenesis.

We provide several lines of evidence supporting the existence of a functional AR-mediated autocrine/paracrine loop in human hepatocellular carcinoma cells. First, we show that treatment with recombinant AR induced EGFR-dependent intracellular signaling that stimulated hepatocellular carcinoma cell proliferation and resistance to TGF-β-induced apoptosis. Interestingly, we also observed that AR was able to stimulate its own gene expression in hepatocellular carcinoma cells through the activation of the EGFR, suggesting the existence of a positive feedback loop for AR production. A similar response has been reported in other tumor cell types, such as colon cancer cells, where AR expression is induced by the EGFR ligand TGF-α in conjunction with the cyclooxygenase-2-derived prostaglandin E_2 (29). AR was constitutively expressed and released to the CM of hepatocellular carcinoma cells, on which it exerted promitogenic and antiapoptotic effects through the activation of the EGFR. Indeed, pharmacologic inhibition of AR shedding or interference with AR binding, including the use of neutralizing antibodies, resulted in reduced EGFR signaling and cellular proliferation. Moreover, when hepatocellular carcinoma cells were cultured in the absence of serum, AR protein further accumulated in the CM, suggesting that this growth factor could be involved in the self-sufficiency in growth signals characteristic of tumor cells, including hepatocellular carcinoma cells (6).

To directly examine the role of AR expression in hepatocellular carcinoma, we used two complementary approaches. First, we used AR-specific siRNAs to knockdown the expression of endogenous AR in two different human hepatocellular carcinoma cell lines (PLC and Hep3B). Down-regulation of AR expression resulted in substantial attenuation of basal EGFR and Erk1/2 phosphorylation, which have been found to be activated in a significant proportion of human hepatocellular carcinomas (3, 31). Consistent with the role of Ekr1/2 in cell proliferation, hepatocellular carcinoma growth in serum-free media was markedly attenuated by AR siRNAs. Nonetheless, probably the most compelling phenotypic alteration induced by AR silencing in hepatocellular carcinoma cells was the abrogation of anchorage-independent growth. The ability to survive and grow under nonadhesive conditions by inhibiting anoikis-related apoptotic pathways is a characteristic of transformed cells that is absent in normal epithelial cells (46). Although the mechanisms that allow tumor cell survival under these conditions are not completely understood, activation of the EGFR/Erk1/2 signaling pathway has been shown to play an essential function (36). It should be considered that the expression of other EGFR ligands, such as TGF-α and HB-EGF, remained unaltered upon AR silencing in hepatocellular carcinoma cells. In light of these considerations, we could propose that AR exerts a nonredundant role in the development of the full neoplastic phenotype that cannot be compensated for by other EGF family members. The reasons for this predominant role of AR are not clear at present and deserve further studies. However, this situation seems not to be limited only to human hepatocellular carcinoma cells because a similar unique role for AR in the transactivation of the EGFR in head and neck carcinoma cells, which also express other EGFR ligands, has been reported (23).

The ability of AR to influence hepatocellular carcinoma cell behavior was further shown in SK-Hep1 cells, the only hepatocellular carcinoma cell line tested that did not express AR but did express TGF-α and HB-EGF (data not shown). We established two SK-Hep1 cell lines that constitutively expressed and secreted AR and tested their growth and tumorigenic potential. By this approach, we could confirm that AR expression enhances key phenotypic characteristics of hepatocellular carcinoma cancer cells. The growth rate and colony-forming ability in semisolid agar medium were significantly increased in AR-producing cells, as well as the incidence of tumor development when s.c. injected in nude mice. These observations further support a prominent role of AR in the determination of hepatocellular carcinoma aggressive behavior.

Our findings on the modulation exerted by AR on the response of hepatocellular carcinoma cells to TGF-β can be of particular relevance regarding the biology of liver tumor cells. TGF-β is not expressed in normal hepatocytes and, when exposed to this cytokine normal liver cells, undergo growth arrest and apoptosis (5). Conversely, TGF-β is expressed in hepatocellular carcinoma cells; however, transformed cells have developed resistance towards TGF-β-induced cell death, and this trait is regarded as one important determinant in the malignant transformation of the liver (32). Moreover, resistance to TGF-β-induced apoptosis is intimately linked to the acquisition of self-sustained migratory and matrix-invasive phenotypes. This is a complex process known as epithelial-to-mesenchymal transition and is also promoted by TGF-β during liver tumor progression (32). In other cancers, such as colon and pancreatic cancers, resistance to TGF-β has been attributed to defects in the components of the TGF-β signaling pathway. However, such mutations seem to be rare in hepatocellular carcinoma, indicating that other pathways may be responsible for TGF-β resistance (47). Our present observations suggest that AR expression may play a role in the protection of hepatocellular carcinoma cells against TGF-β-mediated apoptosis. From a mechanistic point of view, AR down-regulation resulted in enhanced activation of the stress-activated protein kinase JNK, which has been involved in triggering hepatocellular carcinoma cell apoptosis (48). Concomitantly, AR silencing resulted in reduced Erk1/2 phosphorylation. As mentioned before, the

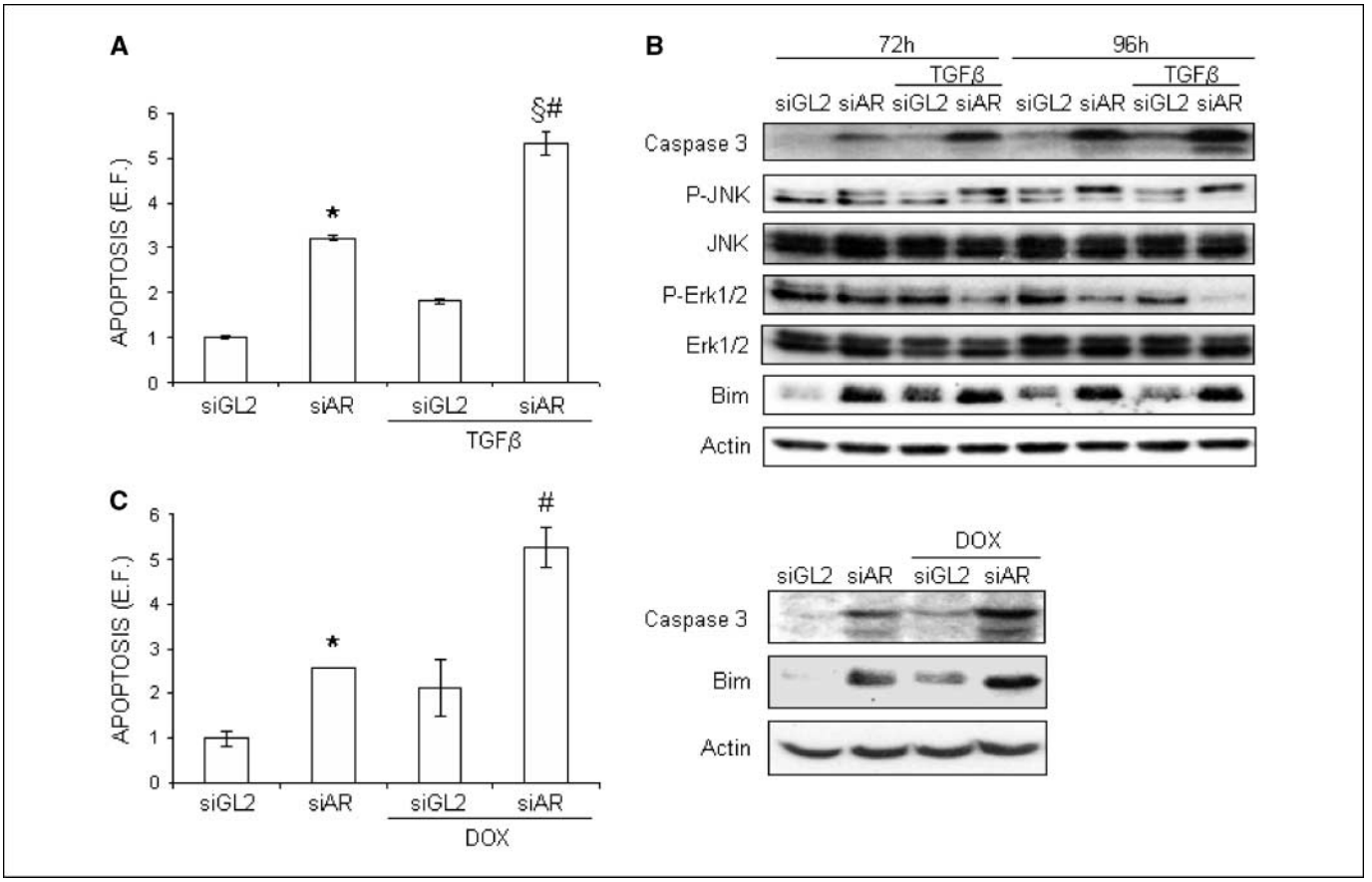

Figure 6. *A,* effect of AR silencing on TGF-β-induced apoptosis in PLC cells. PLC cells were transfected with either siAR or the control siGL2. After 48 hours, cells were treated or not with TGF-β (0.5 nmol/L) for 24 hours more, and apoptosis was measured as described in Materials and Methods. *, $P < 0.05$ versus siGL2; #, $P < 0.05$ versus siGL2 plus TGF-β; §, $P < 0.05$, versus siAR. *B,* Western blot analyses of the active caspase-3 p17 subunit, the phosphorylation of JNK and Erk1/2, and the expression of Bim in PLC cells upon AR silencing and TGF-β (0.5 nmol/L) treatment. PLC cells were transfected with either siGL2 or siAR; after 48 hours, cells were treated or not with TGF-β (0.5 nmol/L) for 24 or 48 hours more. Representative blots. *C,* effect of AR silencing on doxorubicin (*DOX*)–induced apoptosis in PLC cells. Treatment with DOX (0.25 μg/mL) was started 48 hours after transfection with either siGL2 or siAR, and apoptosis was measured 24 hours later. At this, time the active caspase-3 p17 subunit and Bim protein levels were assessed by Western blotting (*right*). Representative blots.

EGFR/Erk1/2 pathway is crucial in preventing anoikis (36) and has been recently identified as a key pathway in the protection from TGF-β-induced apoptosis in late-stage liver tumorigenesis (49). Interestingly, both protein kinases (JNK and Erk1/2) play opposing roles in the regulation of the expression and activity of the proapoptotic BH3-only protein Bim (33, 35–38). Consistent with this, we observed a potent induction in Bim expression upon AR silencing in hepatocellular carcinoma cells. BH3-only proteins, such as Bim, target all prosurvival Bcl-2 family members and prime the cell for apoptosis elicited by cytokines, including TGF-β (33, 34), or cytotoxic drugs like doxorubicin (33). Interestingly, AR silencing in the absence of any pro-apoptotic stimuli also resulted in a significant degree of apoptosis. Together, these findings fit well with the hypothesis of intrinsic tumor suppression recently formulated by Lowe et al. (50). According to this concept, cancer arises when the innate molecular networks that connect proliferation and tumor suppression (apoptosis) become uncoupled by the acquisition of oncogenic lesions, such as those that constitutively activate survival pathways. Consequently, targeting those survival programs in cancer cells can expose and reengage the innate apoptotic mechanisms triggered by aberrant proliferation (50). In our case, this could be exemplified by the strong up-regulation of *Bim* expression, which is regarded as a tumor suppressor gene (33), upon AR silencing.

In summary, we have identified AR as a novel player in human hepatocellular carcinoma cell biology. Targeting AR expression and/or activity may thus be useful to increase the efficacy of chemotherapy in hepatocellular carcinoma. Moreover, inhibiting AR activity may be necessary to overcome the resistance to EGFR-targeted therapies in hepatocellular carcinoma (9), as recently suggested for patients with non–small cell lung cancers in which AR overexpression correlated with a poor response to gefitinib (44).

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