HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Dissecting the Role of Epidermal Growth Factor Receptor Catalytic Activity During Liver Regeneration and Hepatocarcinogenesis

Judit López-Luque,[1]* Daniel Caballero-Díaz,[1]* Adoración Martinez-Palacián,[2]* César Roncero,[2]** Joaquim Moreno-Càceres,[1]** María García-Bravo,[3,4,5]** Esther Grueso,[3,4] Almudena Fernández,[4,6] Eva Crosas-Molist,[1] María García-Álvaro,[2] Annalisa Addante,[2] Esther Bertran,[1] Angela M. Valverde,[7,8] Águeda González-Rodríguez,[7,8] Blanca Herrera,[2] Lluis Montoliu,[4,6] Teresa Serrano,[9] Jose-Carlos Segovia,[3,4,5] Margarita Fernández,[2] Emilio Ramos,[10] Aránzazu Sánchez,[2]*** and Isabel Fabregat[1,11]***

Different data support a role for the epidermal growth factor receptor (EGFR) pathway during liver regeneration and hepatocarcinogenesis. However, important issues, such as the precise mechanisms mediating its actions and the unique versus redundant functions, have not been fully defined. Here, we present a novel transgenic mouse model expressing a hepatocyte-specific truncated form of human EGFR, which acts as negative dominant mutant (ΔEGFR) and allows definition of its tyrosine kinase–dependent functions. Results indicate a critical role for EGFR catalytic activity during the early stages of liver regeneration. Thus, after two-thirds partial hepatectomy, ΔEGFR livers displayed lower and delayed proliferation and lower activation of proliferative signals, which correlated with overactivation of the transforming growth factor-*β* pathway. Altered regenerative response was associated with amplification of cytostatic effects of transforming growth factor-*β* through induction of cell cycle negative regulators. Interestingly, lipid synthesis was severely inhibited in ΔEGFR livers after partial hepatectomy, revealing a new function for EGFR kinase activity as a lipid metabolism regulator in regenerating hepatocytes. In spite of these profound alterations, ΔEGFR livers were able to recover liver mass by overactivating compensatory signals, such as c-Met. Our results also indicate that EGFR catalytic activity is critical in the early preneoplastic stages of the liver because ΔEGFR mice showed a delay in the appearance of diethyl-nitrosamine-induced tumors, which correlated with decreased proliferation and delay in the diethyl-nitrosamine-induced inflammatory process. *Conclusion*: These studies demonstrate that EGFR catalytic activity is critical during the initial phases of both liver regeneration and carcinogenesis and provide key mechanistic insights into how this kinase acts to regulate liver pathophysiology. (HEPATOLOGY 2016;63:604-619)

See Editorial on Page 371

The liver is a unique organ in displaying reparative response and regenerative capacity. However, prolonged liver regeneration as a consequence of

Abbreviations: DEN, diethyl-nitrosamine; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; mRNA, messenger RNA; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PH, partial hepatectomy; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; TGF-α/-β, transforming growth factor (α/β); TNF-α, tumor necrosis factor-α; uPA, urokinase-type plasminogen activator; WT, wild type.

From the [1]Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, Barcelona, Spain; [2]Department of Biochemistry and Molecular Biology II, School of Pharmacy, Complutense University of Madrid, and Instituto de Investigación Sanitaria del Hospital Clínico San Carlos, IdISSC, Madrid, Spain; [3]Cell Differentiation and Cytometry Unit, Hematopoietic Innovative Therapies Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain; [4]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain; [5]Advanced Therapies Mixed Unit, CIEMAT/IIS Fundación Jiménez Díaz, Madrid, Spain; [6]Department of Molecular and Cellular Biology, National Centre for Biotechnology (CNB-CSIC), Madrid, Spain; [7]Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC/UAM), Madrid, Spain; [8]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, Spain; [9]Pathological Anatomy Service, University Hospital of Bellvitge, Barcelona, Spain; [10]Department of Surgery, Liver Transplant Unit, University Hospital of Bellvitge, Barcelona, Spain; [11]Department of Physiological Sciences II, School of Medicine, University of Barcelona, Spain.

chronic liver diseases produces the appearance of genetic/epigenetic alterations that finally concur in the development of hepatocarcinogenesis. The epidermal growth factor receptor (EGFR/ErbB1) pathway plays an essential role in virtually all steps of these processes.[1] However, many questions remain unanswered. It is not well understood which of its two main functions (cell proliferation and survival) plays more essential roles during liver regeneration or hepatocarcinogenesis. Additionally, EGF/EGFR and hepatocyte growth factor (HGF)/Met signaling play both redundant and specific roles in these phenomena.[2-4] Nevertheless, their specific mechanisms are not completely known. We previously reported that the EGFR pathway impairs the suppressor arm of the transforming growth factor-β (TGF-β) pathway in hepatocytes and liver tumor cells *in vitro*[5,6]; however, there is no evidence that this effect may be relevant *in vivo*. Furthermore, there is a lack of knowledge about how necessary the catalytic domain of the EGFR pathway is in its *in vivo* actions, which is very important for the design of therapeutic strategies targeting this pathway. It would be expected that the enzymatic phosphorylation capacity of the EGFR is essential. In this sense, targeting EGFR by small interfering RNA technology or inhibiting its enzymatic capacity with gefitinib produced similar effects in liver tumor cells.[7] However, it has been also proposed that hepatocyte priming during liver regeneration involves modulation of the EGFR-mediated growth responses without an increase in its receptor tyrosine kinase activity.[8]

Taking all this into consideration, we decided to generate a new experimental animal model to explore the role of hepatocyte EGFR tyrosine kinase activity in liver regeneration and hepatocarcinogenesis. A transgenic animal was generated which expresses specifically in hepatocytes a truncated form of the human EGFR that lacks the intracellular catalytic domain (previously generated by Schlessinger's group[9]). This truncated form is able to undergo EGF-induced dimerization with wild-type (WT) receptors, allowing the binding of EGFR ligands but acting as a negative dominant mutant.[10,11] This model has the advantage, compared with the knockout mouse model, that the EGFR ligands bind to the receptors in the hepatocyte membrane and no excess of EGFR ligand is available to the nonparenchymal cells. Furthermore, only the intracellular domain of the EGFR is missed, which means that the specific role of the phosphorylation-dependent EGFR activity will be analyzed. Partial hepatectomy (PH) and diethylnitrosamine (DEN) treatment were performed to analyze the specific roles of the EGFR catalytic activity during liver regeneration and hepatocarcinogenesis.

Materials and Methods

Generation of Transgenic EGFR Mice and Hepatocyte Cell Lines. A complementary DNA coding for a truncated form of the human EGFR, lacking the kinase domain in its intracytosolic region (amino acids 654-1186), was cloned in transference bacterial artificial chromosome clone RP23-279P6 (kindly provided by Prof. Dr. Günther Schütz, Molekularbiologie der Zelle I, Deutsches Krebsforschungszentrum (DKFZ) Heidelberg, Germany) carrying *Albumin* locus and *Chloramphenicol* resistance gene. The truncated EGFR was introduced just in the ATG starting codon of the albumin gene, surrounded by 160 kb of the albumin chromosomal genomic sequence, as described (Fig. 1A).[12]

E. Grueso's present address is: Division of Medical Biotechnology, Paul Ehrlich Institute, Langen, Germany

Received April 14, 2015; accepted August 21, 2015.

Additional Supporting Information may be found at http://onlinelibrary.wiley.com/doi/10.1002/hep.28134/suppinfo.

Cofunded by the Ministry of Economy and Competitiveness-MINECO and the European Regional Development Fund-FEDER, Spain (Contract grant numbers: BFU2012-35538 and ISCIII-RTICC RD12-0036-0029 to I.F.-IDIBELL; SAF2009-12477 to A.S.-UCM/IdISSC; RETICS-RD12/0019/0023 and SAF2011-30526-C02-01 to J.-C.S.-CIEMAT/CIBERER/IIS-FJD; SAF2012-33283 to A.M.V.-CSIC/CIBERDEM). People Programme (Marie Curie Actions) of the FP7-2012, under REA grant agreement #PITN-GA-2012-316549 (IT-LIVER) to I.F.-IDIBELL and to A.S.-UCM/IdISSC. AGAUR-Generalitat de Catalunya to I.F-IDIBELL (Contract grant number: 2009SGR-312). Dirección General de Investigación de la Comunidad de Madrid, Spain (Contract grant number S2010/BMD-2402 to A.S.-UCM/IdISSC). Fundación Eugenio Rodríguez Pascual to M.G.-B.-CIEMAT/CIBERER/IIS-FJD. J.L.-L. was recipient of a predoctoral fellowship from IDIBELL; D.C.-D. and A.M.-P. were recipients of predoctoral fellowships from MINECO, Spain (Contract grant number: FPI - BES-2013-064609 and BES-2007-16187, respectively). [Correction added October 21, 2015, after first online publication: funding information was added.]

**These authors contributed equally to this work as first authors.*

***These authors contributed equally to this work as second authors.*

****These authors contributed equally to this work as senior authors.*

Address reprint requests to: Isabel Fabregat, Bellvitge Biomedical Research Institute (IDIBELL), Gran via de l'Hospitalet, 199, L'Hospitalet, 08908 Barcelona, Spain. E-mail: ifabregat@idibell.cat; tel: +34 932607828; fax: +34 932607426.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28134

Potential conflict of interest: Nothing to report.

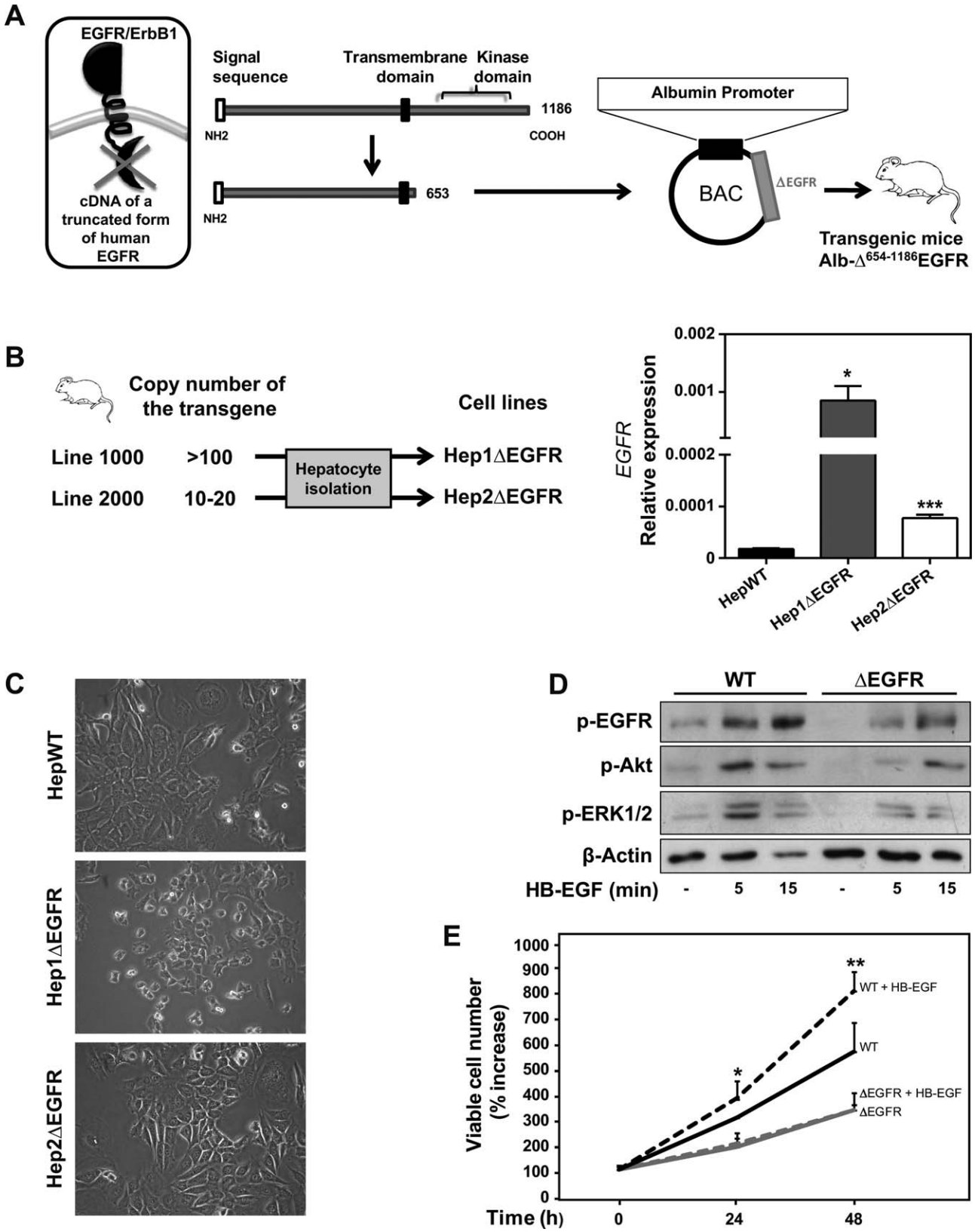

Fig. 1. Characterization of the transgenic model of Alb-$\Delta^{654\text{-}1186}$EGFR (abbreviated as ΔEGFR) mice. (A) A complementary DNA coding for a truncated form of the EGFR that had a deletion in its intracytosolic region (amino acids 654-1186) was cloned in a transference plasmid under the control of a specific hepatocyte promoter formed by the albumin locus just before ATG of the albumin mouse gene. Three different lines of transgenic mice were established and named as 1000, 2000, and 4000, which presented different copy number of the transgene. (B) Left: Primary hepatocytes were isolated from WT and ΔEGFR of the two mouse lines that presented a higher transgene copy number and immortalized as described in Materials and Methods. Right: Levels of human EGFR transcripts in mice analyzed by qRT-PCR. Mean ± standard error of the mean of three independent experiments. (C) Morphology of the isolated hepatocytes. (D) Response to heparin binding EGF-like growth factor in terms of EGFR phosphorylation (fetal bovine serum depleted medium) was attenuated in the Hep1ΔEGFR hepatocytes. Western blot analysis, a representative experiment of five is shown. (E) Differences in the response to heparin binding EGF-like growth factor (fetal bovine serum depleted medium) in terms of cell proliferation between WT and Hep1ΔEGFR hepatocytes. Cell number was analyzed by trypan blue staining. Mean ± standard error of the mean of two independent experiments performed in triplicate. (B,E) Student *t* test was used: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Abbreviations: BAC, bacterial artificial chromosome; cDNA, complementary DNA; HB-EGF, heparin binding EGF-like growth factor.

Linearized bacterial artificial chromosome DNA was microinjected into the pronuclei of mouse B6CBAF2 zygotes according to reported protocols,[13] which were later transferred to pseudopregnant recipient females, finally obtaining transgenic mice Alb-$\Delta^{654\text{-}1186}$EGFR (from now abbreviated as ΔEGFR). Six different founder mice were bred with C57BL/6 WT mice. Three of them gave littermates and showed germline transmission of the transgene. The ΔEGFR F1 mice obtained were interbred to generate stable transgenic mouse lines, which were kept heterozygous. Transgenic lines are archived in the European Mouse Mutant Archive as B6CBA-Tg(Alb-deltaEGFR). Genotyping was assessed by polymerase chain reaction as specified in the European Mouse Mutant Archive. Using specific primers that exclusively detect expression of the human EGFR gene, we demonstrated the transgene expression in ΔEGFR mice by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis, which was confirmed at the protein level by western blot (Supporting Fig. S1).

For generation of immortalized hepatocytes, pools of four to six livers from WT and ΔEGFR neonates (5-7 days old) were used. Isolation of the cells (by collagenase dispersion) and immortalization were achieved as described.[14] For further details, culture conditions, cell viability, and apoptosis analyses, see the Supporting Information.

PH in Mice. PH was performed in animals at 8-12 weeks of age by removal of two-thirds of the liver, as described by Higgins and Anderson.[15] Mice were killed at 6, 12, 24, 48, and 72 hours and 7 days after surgery; and tissue samples were immediately frozen in liquid nitrogen for RNA and protein extraction, cryopreserved in optimal cutting temperature compound for oil red O staining, or fixed in 4% paraformaldehyde for immunohistochemical analysis. Sham-operated mice were used as control.

DEN-Induced Hepatocarcinogenesis in Mice. Male mice at day 15 of age received intraperitoneal injections of DEN (10 mg/kg) diluted in saline buffer. At 9 and 12 months of age, mice were sacrificed and their livers removed. For histological studies, liver lobes were fixed in 4% paraformaldehyde overnight and paraffin-embedded for immunohistochemical staining. Total RNA and protein were isolated from frozen tissues for qRT-PCR and western blotting analyses. Three to four animals/condition and at least two different tissue pieces/animal were processed for RNA extraction.

Western Blot Analysis. Total protein extracts and western blotting procedures were carried out as described.[16,17]

For more details and other methods, ethics statement, and statistical analysis, see the Supporting Information.

Results

Generation of Transgenic Mice Expressing a Truncated Form of the EGFR, Which Lacks the Catalytic Intracellular Domain in Hepatocytes. Three different lines of transgenic mice were established from founder mice expressing different copy numbers of the transgene (Fig. 1A). Primary hepatocytes were isolated and immortalized, as described above. EGFR messenger RNA (mRNA) expression levels were proportional to the copy number of the transgene (Fig. 1B). Analysis of cell morphology revealed that hepatocytes from line 1000 (highest expression of the transgene) presented lower viability (Fig. 1C) and decreased basal proliferative capacity compared with the WT ones. Response to heparin binding EGF-like growth factor was significantly attenuated in terms of EGFR phosphorylation/signaling, at either short (Fig. 1D) or long (results not shown) time points, as well as in terms of cell proliferation (Fig. 1E). TGF-β transactivates the EGFR in hepatocytes, which inhibits its proapoptotic activity.[7,18] Hepatocytes isolated from ΔEGFR mice did not phosphorylate the EGFR under TGF-β treatment, which correlated with impairment in the activation of survival signals, such as extracellular signal–regulated kinase (ERK) mitogen-activated protein kinases (Supporting Fig. S2A), higher growth inhibition, and apoptosis (Supporting Fig. S2B-D). Interestingly, ΔEGFR hepatocytes showed higher basal apoptosis compared with WT ones. These results indicate the suitability of the animal and cell model to study the specific implication of the hepatocyte EGFR catalytic domain in liver physiology and pathology.

ΔEGFR Mice Show Delayed Hepatocyte Proliferation After Two-Thirds PH Coincident With a Higher Activation of the TGF-β Pathway. To explore the specific role of hepatocyte EGFR activity during liver regeneration, we performed two-thirds PH in WT and ΔEGFR mice, selecting the two mouse lines with a higher expression of the transgene. WT animals activated the EGFR pathway in the remaining liver after PH, analyzed as EGFR phosphorylation in hepatocytes by immunohistochemical analysis of paraffined tissues (Fig. 2A). ΔEGFR animals, in contrast, did not show phosphorylation of the EGFR. This was not due to differences in expression of the endogenous EGFR or its ligand TGF-α, the EGFR ligand highly expressed in hepatocytes during liver regeneration[19] (Supporting Fig. S3A), which proves that the human ΔEGFR transgene indeed acts as a negative dominant, inhibiting the hepatocyte response to EGFR ligands. No compensatory increases in the expression of other members of the EGFR family

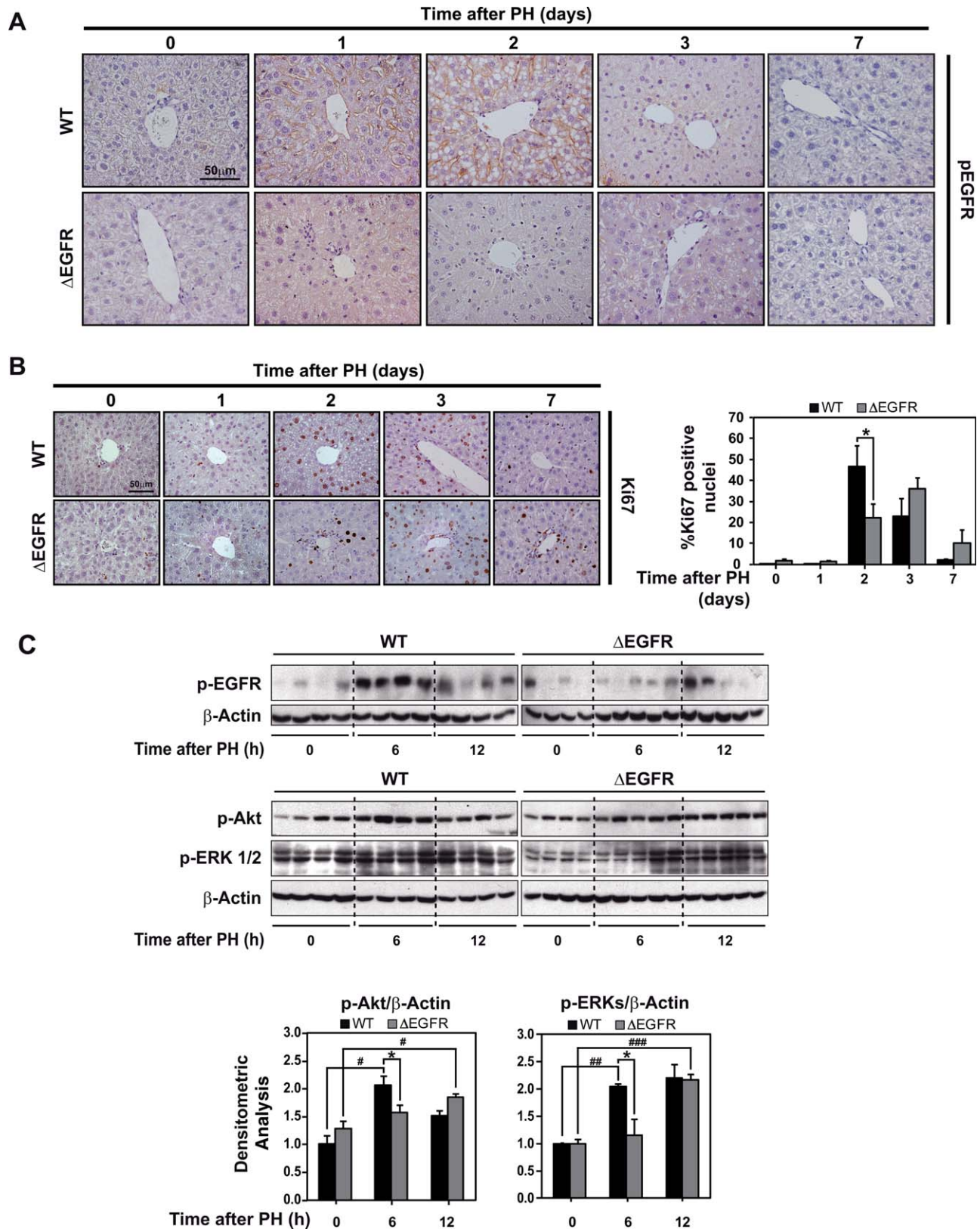

Fig. 2. Hepatocyte proliferation induced after two-thirds partial PH is delayed in ΔEGFR transgenic mice. Immunohistochemical analysis of phospho-EGFR (A) and Ki67 (B, left) in paraffined liver tissues from WT and ΔEGFR mice at the indicated times after PH. (B) Right: Quantification of the percentage of Ki67-labeled nuclei. (C) Upper: Western blot analysis of the protein levels of phospho-EGFR, phospho-Akt, and phospho-ERKs as early proliferative signals. Lower: Quantification of the densitometric analysis. (B, right; C, lower) Data are mean ± standard error of the mean of at least four animals per group. Student *t* test was used: $*P < 0.05$ compared to WT; $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ compared to time 0.

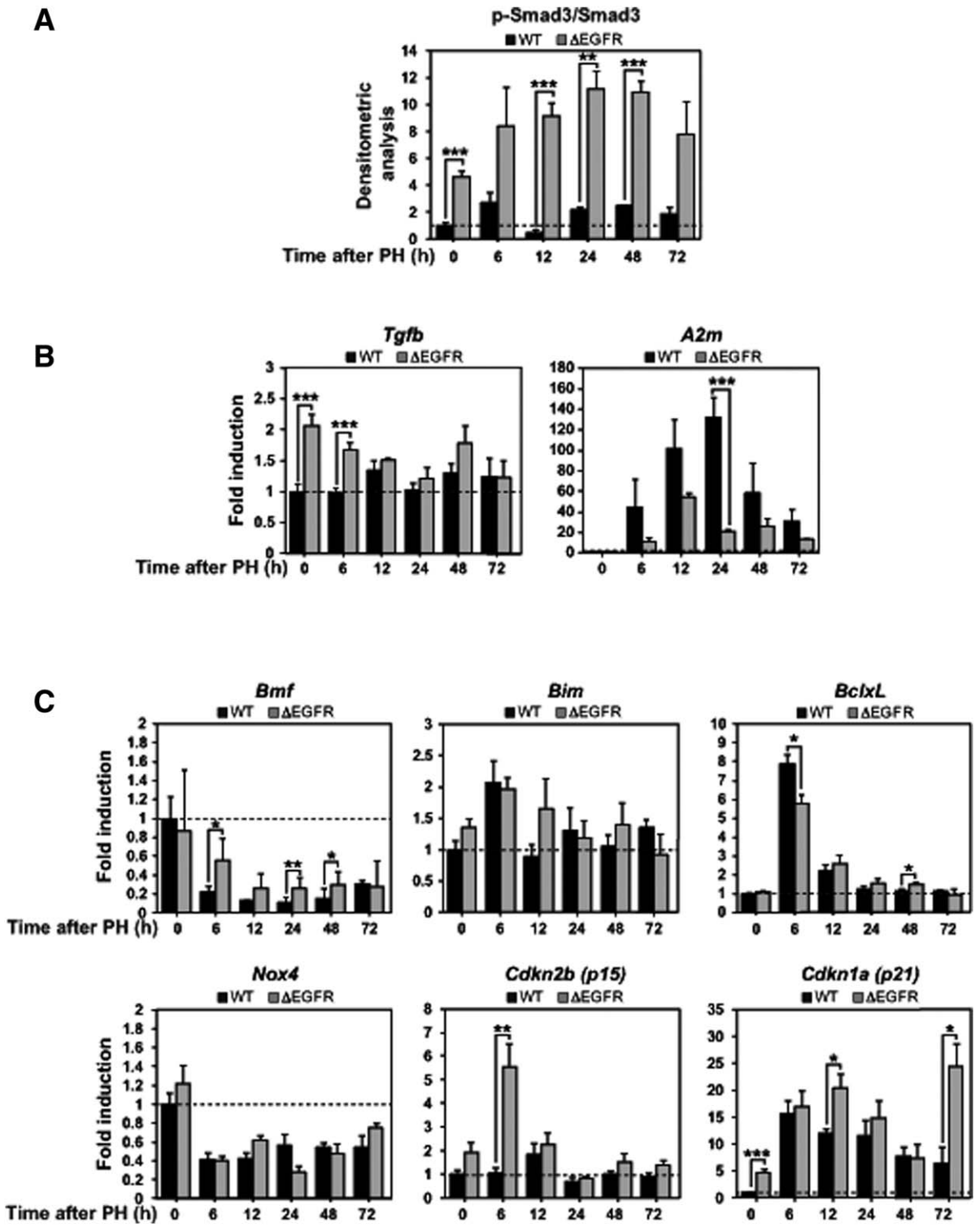

Fig. 3. Overactivation of the TGF-β pathway in ΔEGFR mice under basal conditions and during liver regeneration after PH. (A) Ratio of phospho-Smad3 versus Smad3 protein levels (densitometric analyses of western blots shown in Supporting Fig. S4) at basal conditions and at the indicated times after PH. (B) qRT-PCR analysis of *TGF-β1* and *A2m* mRNA levels. (C) Analysis of the mRNA levels of the indicated genes by qRT-PCR at different times after PH. (A-C) Data are mean ± standard error of the mean of at least four animals per group. Student *t* test was used: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Abbreviation: A2m, α_2-macroglobulin.

(*Erbb2*, *Erbb3*, and *Erbb4*), due to inactivation of the EGFR function, were found (Supporting Fig. S3B). Immunohistochemical analysis of Ki67 revealed lower and delayed hepatocyte proliferation in ΔEGFR mice (Fig. 2B). WT animals showed an early increase in the levels of intracellular signals that mediate hepatocyte

proliferation, such as p-AKT and p-ERK mitogen-activated protein kinases (Fig. 2C). However, ΔEGFR livers presented a significantly lower response. Cytokines such as interleukin-6 and tumor necrosis factor-α (TNF-α) are considered relevant for priming hepatocytes to proliferate[2-4]; however, both WT and ΔEGFR livers showed similar changes in the expression of these cytokines at 12-24 hours after PH, which correlated with a similar inflammatory response (results not shown).

Considering the relevant role played by EGF in counteracting the TGF-β suppressor effects,[6] we wondered whether ΔEGFR animals may show higher Smads phosphorylation and/or higher activation of TGF-β downstream pathways. Results indicated that Smad3 phosphorylation, a hallmark of TGF-β activation, is higher in ΔEGFR animals at basal levels and at all the times analyzed after PH (Fig. 3A; Supporting Fig. S4). Interestingly, the decrease in Smad3 phosphorylation observed in WT animals at 12 hours after PH was not observed in ΔEGFR mice, which maintained a high activation of the pathway. Levels of TGF-β1 transcripts were significantly higher in ΔEGFR livers before PH as well as at short times after PH compared with WT ones (Fig. 3B, left). Furthermore, α_2-macroglobulin, a plasma protein previously proposed as a binding protein for TGF-β that attenuates its effects,[20] was up-regulated in WT, but not in ΔEGFR, animals after PH (Fig. 3B right). Indeed, the EGFR pathway may up-regulate α_2-macroglobulin, which could in turn counteract TGF-β activity. All these data suggest a stronger activation of the TGF-β pathway in ΔEGFR livers compared with WT ones.

As *in vitro* experiments had indicated that EGF counteracts TGF-β-induced apoptosis by inhibiting its effects on *Bim* and *Bmf* up-regulation,[7] we tested for potential differences in the expression of these genes between WT and ΔEGFR mice. *Bmf*, but not *Bim*, expression was down-regulated after PH in both animals; but, interestingly, levels remained higher in ΔEGFR compared with WT mice (Fig. 3C). Expression of *Bcl-x$_L$*, an antiapoptotic Bcl-2 member known to be a TGF-β target gene in hepatocytes,[6] increased early after PH in both animal models, although the increase in ΔEGFR mice was slightly lower. However, in spite of these differences, analysis of the active form of caspase-3 by immunohistochemistry or caspase-3 activity in tissues revealed no differences between WT and ΔEGFR livers at any of the different times analyzed (Supporting Fig. S5). Because *Bim* and *Bcl-x$_L$* expression undergoes also posttranscriptional regulation, we analyzed their protein levels; but we could not find significant differences between WT and ΔEGFR mice (results not shown). Additionally, we searched for changes in the expression of *Nox4*, a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase whose expression is up-regulated by TGF-β and mediates its apoptotic actions,[21] an effect that is impaired by EGF.[5] *Nox4* expression significantly decreased after PH, as we have recently described.[22] However, no significant differences were observed between WT and ΔEGFR animals (Fig. 3C).

As apoptosis failed to explain the observed delay in the hepatocyte proliferative response, we next focused on the expression of cell cycle regulators modulated by TGF-β. We found a significant increase in the expression of *Cdkn2b* (the gene encoding p15INK4b) at 6 hours after PH in ΔEGFR mice that was not observed in WT animals (Fig. 3C). Levels of *Cdkn1a* transcripts (the gene encoding p21/WAF1/CIP1) increased at short times after PH in both animals, but whereas in WT animals levels slowly returned to basal ones, in ΔEGFR mice levels remained high, the differences being particularly relevant at 72 hours after PH. These results suggest that high levels of p15 could be responsible for the delay in the early proliferative response of ΔEGFR hepatocytes to PH and that high levels of p21 at later times could slow the proliferation during the entire regenerative process. Interestingly, both p15 and p21 levels were increased in nonhepatectomized ΔEGFR livers compared with WT ones. Indeed, a functional EGFR pathway may be necessary to counteract the cytostatic effects of TGF-β in hepatocytes, a process that is particularly crucial after two-thirds PH of the liver.

Regulation of Lipid Metabolism After Two-Thirds PH Is Altered in ΔEGFR Mice. When histological analysis by hematoxylin and eosin was performed, we observed that WT livers 48 hours after PH showed vacuolated cells, with the appearance of multifocal drops (Fig. 4A), whereas ΔEGFR livers did not show this appearance. Suspecting that they could be deposits of lipids, we performed oil red O staining, confirming the accumulation of lipid drops, which correlated with an increase in triglyceride levels in WT, but not in ΔEGFR, livers (Fig. 4B,C). Among different regulatory genes analyzed, the increase in lipid accumulation in WT livers was coincident with up-regulation of the mRNA levels of fatty acid synthase, a key enzyme involved in *de novo* fatty acid synthesis, and glucose-6-phosphate dehydrogenase, the regulatory enzyme of the pentose-phosphate cycle, responsible for NADPH production. However, up-regulation of these enzymes was altered in ΔEGFR animals (Fig. 4D). These results suggest that the EGFR pathway regulates lipid metabolism in hepatocytes after PH.

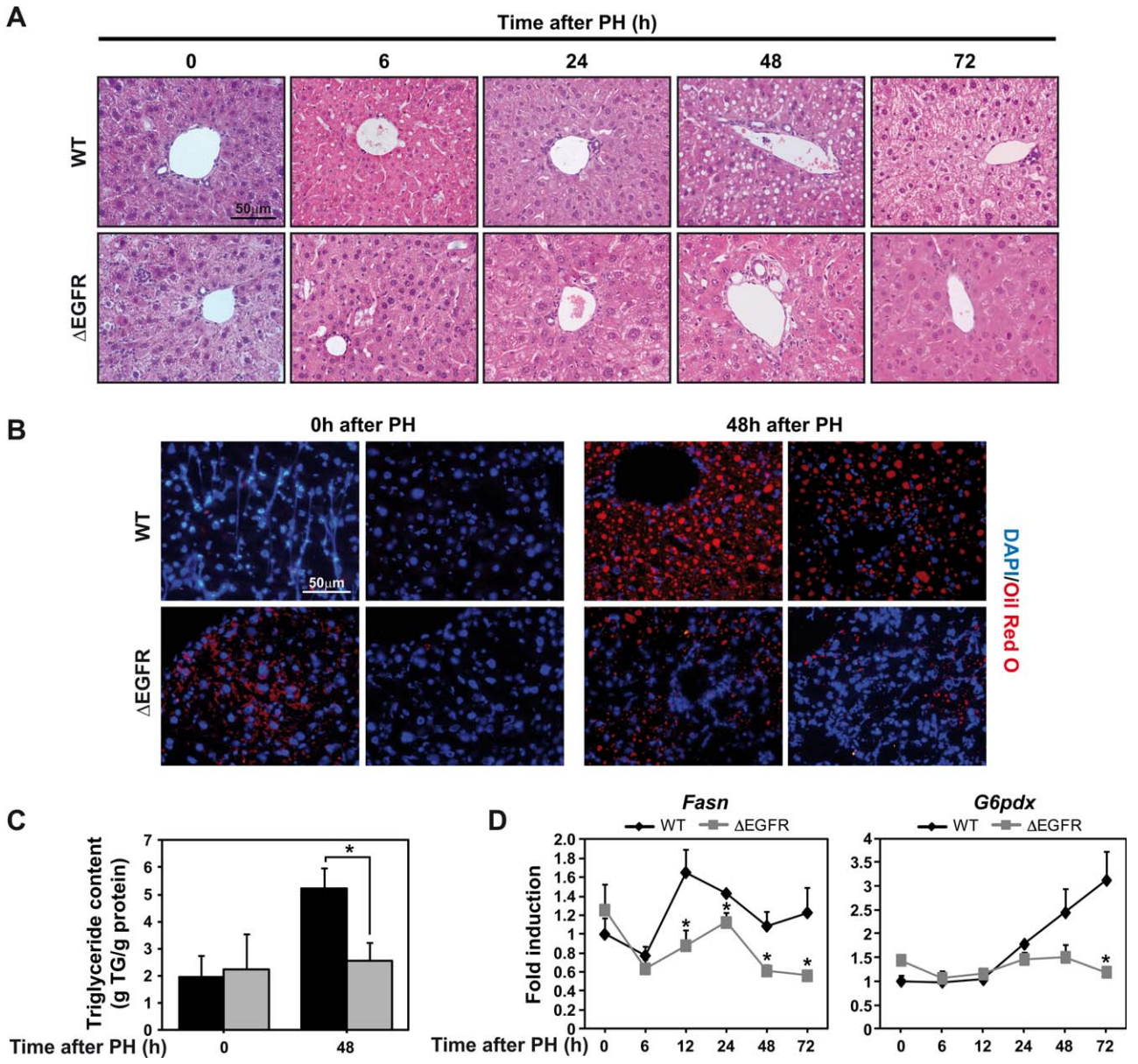

Fig. 4. ΔEGFR mice show differences in lipid metabolic changes during liver regeneration. (A) Histological analysis of the livers from WT and ΔEGFR mice by hematoxylin and eosin staining. (B) Immunohistochemical analysis of the lipid content by oil red O staining performed in frozen liver sections. (C) Analysis of triglyceride content in WT and ΔEGFR livers at basal conditions and 48 hours after PH. (D) qRT-PCR analysis of the levels of expression of *Fasn* and *G6pdx* at the indicated times after PH. (C) Data are mean ± standard error of the mean of three animals per group. Student *t* test was used: $*P < 0.05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; TG, triglyceride.

ΔEGFR Mice Are Able to Regenerate the Liver: Potential Role of HGF/Met in This Process. Regardless of the described alterations, ΔEGFR mice showed only a slight, but not significant, increase in the mortality after two-thirds PH (Fig. 5A). Remaining animals overcame liver injury (analyzed by serum parameters; Supporting Table S2) and were able to fully regenerate the liver with a similar kinetic to WT animals (Fig. 5B). These results led us to state that in spite of the delay in hepatocyte proliferation, the capacity of the liver to recover its mass was not affected by the inhibition of EGFR signaling. Hence, we focused on identifying other potential mitogenic signals that could be overactivated in ΔEGFR livers. We observed that ΔEGFR mice expressed higher levels of *Hgf* mRNA than WT mice, both under basal conditions and shortly after PH (Fig. 5C, left). Interestingly, we also found differences in the expression of urokinase-type plasminogen activator (uPA), which mediates the release of HGF from extracellular matrix during liver regeneration.[23] uPA mRNA levels were elevated in ΔEGFR livers, compared with WT mice, this difference being particularly significant at

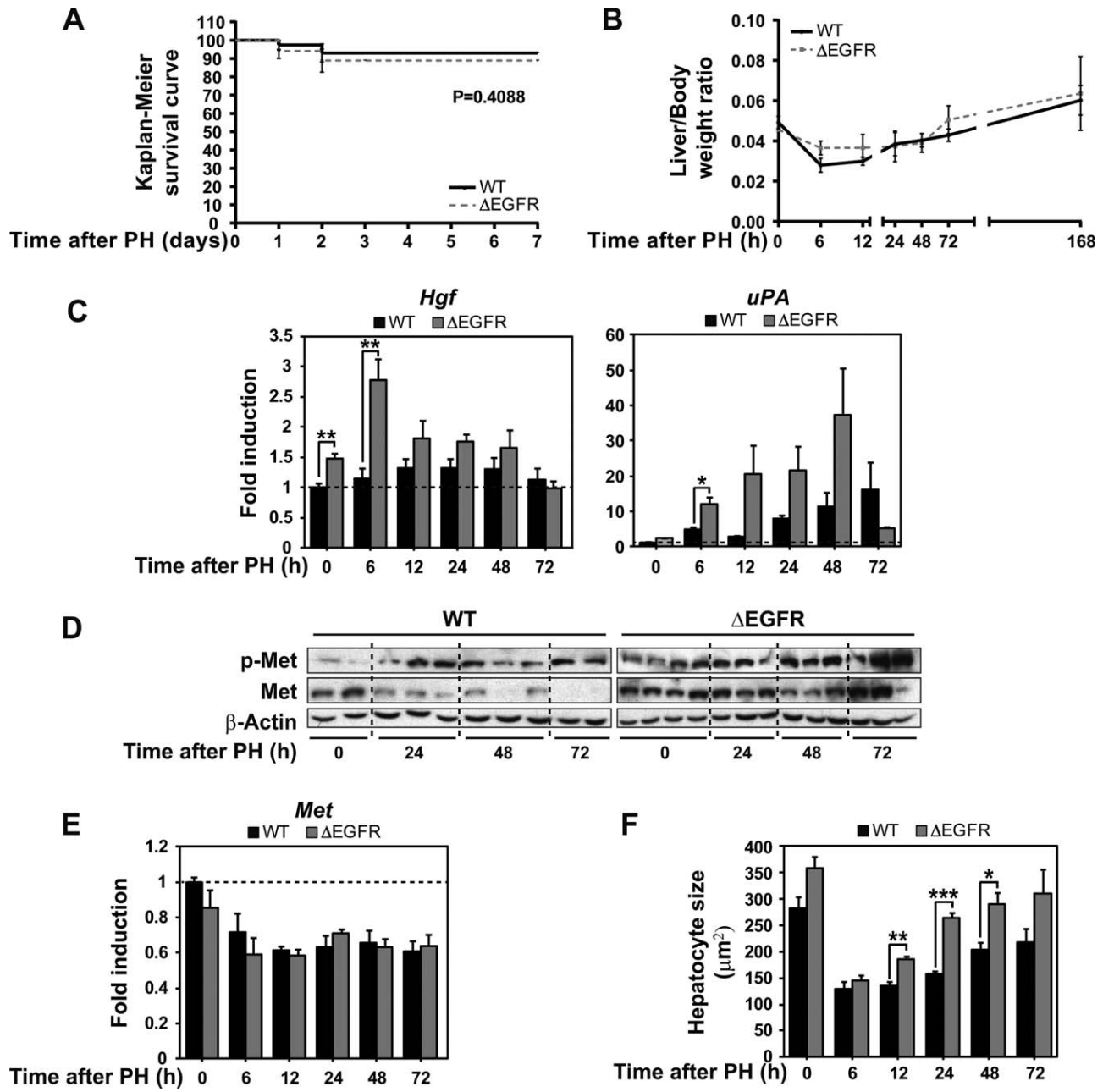

Fig. 5. ΔEGFR livers that are able to regenerate after PH show overactivation of the HGF/Met pathway and a significantly greater cell size compared with WT mice. (A) Kaplan-Meier survival curve. ΔEGFR mice showed a slight increase in mortality after two-thirds PH, although differences were not statistically significant. (B) Remaining animals fully regenerate the liver. Liver/body weight ratio. (C) qRT-PCR analysis of the mRNA levels of *Hgf* (left) and *uPA* (right). (D) Analysis of phospho-Met and total Met by western blot at the indicated times after PH in WT and ΔEGFR livers. (E) qRT-PCR analysis of the mRNA levels of *Met*. (F) Quantification of hepatocyte size at the indicated times (after immunostaining of E-cadherin to label the cell membrane; Supporting Fig. S6) using ImageJ software. (B,C,E,F) Data are mean ± standard error of the mean of at least three animals per group. Student *t* test was used: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

6 hours after PH (Fig. 5C, right). The increase in *Hgf* expression correlated with higher levels of c-Met phosphorylation in ΔEGFR livers. Notably, levels of phosphorylated c-Met remained high at 3 days after PH in ΔEGFR livers (Fig. 5D). It is worth noting that, although c-Met mRNA levels decreased after PH in both WT and ΔEGFR livers (Fig. 5E), the decrease in protein levels was only observed in WT animals, whereas ΔEGFR mice maintained, or even increased, c-Met levels at all time points analyzed (Fig. 5D). This suggests that a posttranscriptional regulation of c-Met may allow the maintenance of high protein levels after PH in ΔEGFR mice, resulting in a sustained activation of this pathway. All these results indicate that the HGF

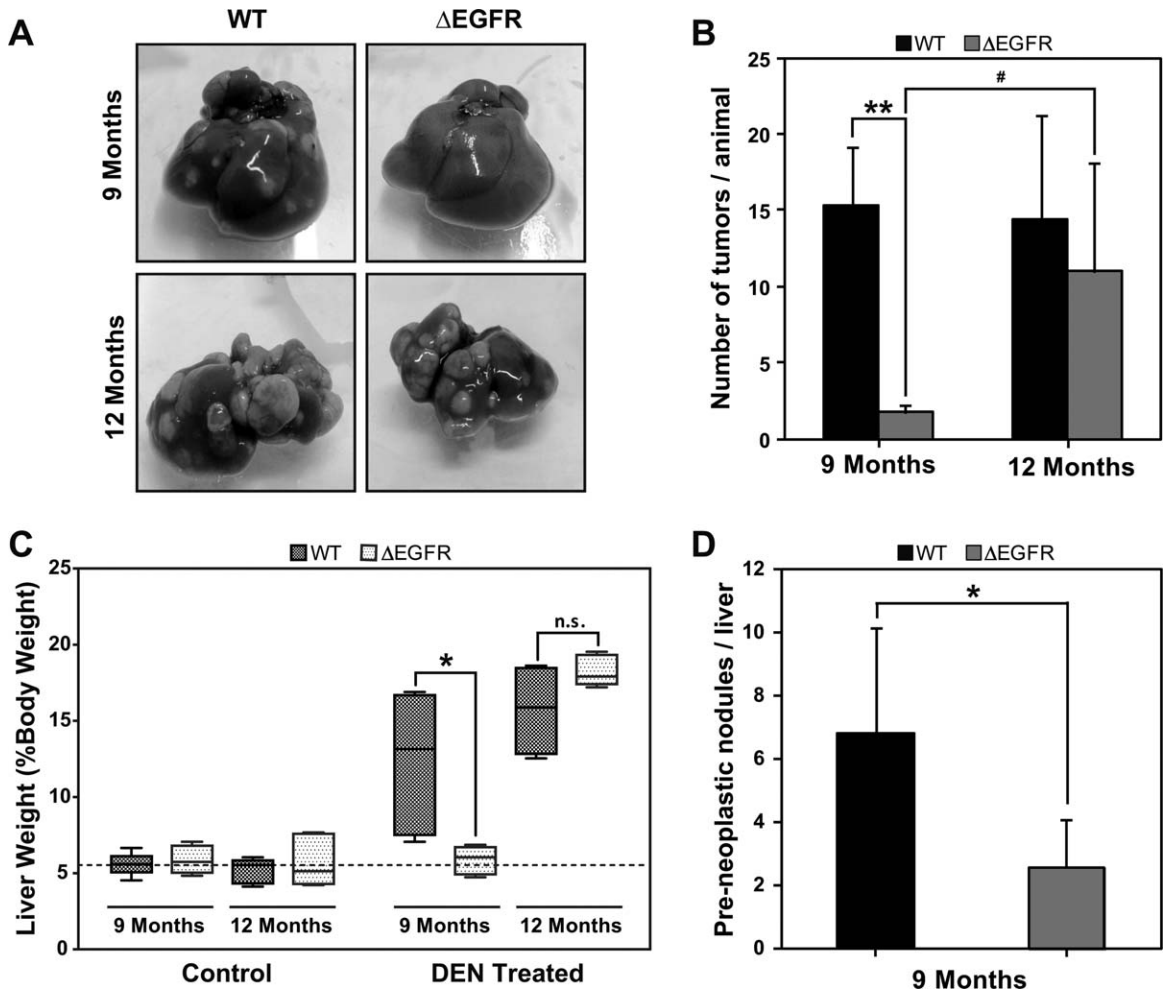

Fig. 6. ΔEGFR mice show a delay in the appearance of DEN-induced tumors. WT and ΔEGFR male mice were treated at day 15 of age with phosphate-buffered saline or DEN, and livers were collected at 9 and 12 months of age. (A) Representative images of livers from DEN-treated mice at the time of sacrifice. (B) Quantification of macroscopic tumors per animal. (C) Analysis of the liver/body weight ratio of WT and ΔEGFR mice. (D) Appearance of microscopic preneoplastic lesions at 9 months of age. (B-D) Data are mean ± standard error of the mean of at least four animals per group. Student *t* test was used: $*P < 0.05$ and $**P < 0.01$ compared to WT mice; $\#P < 0.05$ compared to 9 months. Abbreviation: n.s., no significance.

pathway is overactivated in ΔEGFR livers after PH and could justify that, although delayed, an efficient regeneration is observed in these animals.

It is worth mentioning that hepatocyte size significantly decreased at short times after PH in both WT and ΔEGFR animals; however, ΔEGFR mice showed a quick recovery of size such that after 24 hours hepatocyte size in ΔEGFR livers was significantly higher than that observed in WT livers (see Supporting Fig. S6 for images of the cells and Fig. 5F for quantification of the size). This hypertrophy could also contribute to the maintenance of liver functions and the low percentage of failure observed in ΔEGFR mice.

ΔEGFR Mice Show Delayed Appearance of Liver Tumors Induced by DEN Treatment. When we submitted 15-day-old WT and ΔEGFR mice to an acute treatment with DEN, liver injury (analyzed by serum parameters; Supporting Table S2) was higher in ΔEGFR animals, indicating a role for EGFR as a survival pathway in hepatocytes when submitted to a toxic insult. However, at 9 months of age, WT animals developed macroscopically visible tumors, which were barely observed in ΔEGFR mice (Fig. 6A,B). Consistently, the liver to body weight ratio significantly increased in WT, but not in ΔEGFR, mice (Fig. 6C). However, 12 months after DEN injection, ΔEGFR animals presented macroscopic tumors (Fig. 6A,B), and the liver to body weight ratio increase was quite similar in mice of both phenotypes (Fig. 6C). Expression of the truncated form of the *hEGFR* was maintained at this age in the ΔEGFR animals, which did not show changes in the mRNA levels of any of the EGFR family members (Supporting Fig. S7).

Immunohistological and proliferative (Ki67 staining) analysis of tissues at 9 months after DEN treatment

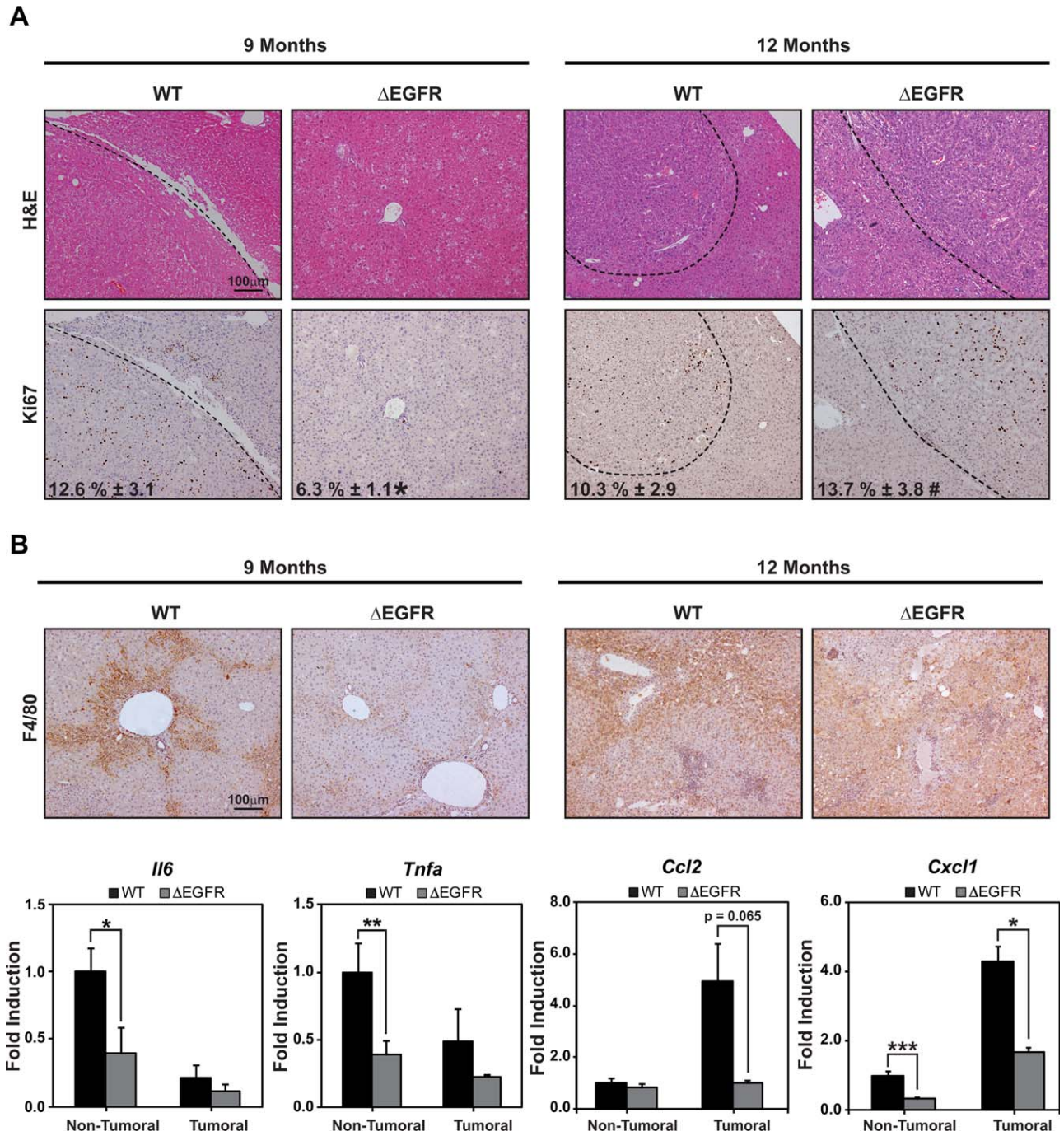

Fig. 7. Immunohistochemical analysis of proliferative and inflammatory areas in livers from DEN-treated WT and ΔEGFR mice. (A) Representative hematoxylin and eosin and immunohistochemical analyses of proliferation (Ki67 staining) in preneoplastic areas in livers from WT and ΔEGFR mice. Quantification of Ki67-positive cells (percentage) is in the bottom of each image. (B) Upper: Inflammatory lesions analyzed by F4/80 staining (macrophage marker). Lower: qRT-PCR analysis of mRNA levels of the proinflammatory cytokines interleukin-6 (*Il6*) and TNF-α (*Tnfa*) and chemokines CCL2 (*Ccl2*) and CXCL1 (*Cxcl1*) in macroscopically selected tumoral areas as well as in the surrounding tissue at 9 months of age in WT and ΔEGFR animals. (A,B) Data are mean ± standard error of the mean of at least four animals per group (three sections/animal). Student *t* test was used: $*P < 0.05$ and $**P < 0.01$ compared to WT mice; $\#P < 0.05$ compared to 9 months. Abbreviation: H&E, hematoxylin and eosin.

revealed the appearance of early preneoplastic lesions detectable only under the microscope. The number of lesions at 9 months of age in WT mice was much higher than that in ΔEGFR ones (Fig. 6D). Analysis of cell proliferation rate by Ki67 immunohistochemistry in the neoplastic areas revealed a lower proliferative rate in ΔEGFR tumors at 9 months compared with WT tumors but a similar proliferative rate at 12 months of

age (Fig. 7A). These results suggest that lack of the catalytic activity of the EGFR delays the appearance of tumors but cannot fully prevent the hepatocarcinogenic process.

DEN-Induced Hepatic Inflammatory Process Is Delayed in ΔEGFR Mice. Considering that hepatocellular carcinoma (HCC) is a clear example of inflammation-related cancer, we evaluated inflammatory markers in histological sections of nontumoral areas of WT and ΔEGFR livers. As shown in Fig. 7B, WT livers showed strong positive areas for F4/80 staining, a macrophage marker, 9 months after DEN treatment, while ΔEGFR mice did not. At 12 months of age the situation in WT and ΔEGFR livers was comparable as both animals showed similar extent of inflammation. Furthermore, some fibrotic areas could be observed at 9 months of age in WT mice, whereas in ΔEGFR mice collagen deposits were only seen around the portal triads and perivascular regions (Supporting Fig. S8), as occurs in healthy liver. Larger collagen deposits were found in ΔEGFR livers at 12 months of age, similar to the situation in WT animals. These results indicate that lack of EGFR catalytic activity in the hepatocytes provokes a delay in the DEN-induced inflammatory process in the liver. Analysis of the expression of classical cytokines involved in liver inflammation revealed lower *Il6* and *TNFa* mRNA levels (Fig. 7B) but no changes in *Il1b* or *Il17a* (results not shown) in the tumor-surrounding tissues of ΔEGFR livers compared with WT ones. Furthermore, among different inflammatory chemokines for which some evidence exists that may be controlled by the EGFR pathway in HCC,[24] we found decreased expression of *Ccl2* in the tumoral tissues and of *Cxcl1* in both tumoral and nontumoral areas in ΔEGFR livers (Fig. 7B). No differences were found in other analyzed chemokines (*Ccl20*, *Cxcl2*, *Cxcl3*, or *Cxcl5*; results not shown).

Following the previous hypothesis about crosstalk between TGF-β and the EGFR, we next analyzed the expression of *TGF-β1* mRNA and the levels of phosphorylation of Smads in livers from WT and ΔEGFR mice at 9 months of DEN treatment. We could not find significant differences in either *TGF-β1* levels (Fig. 8A) or phospho-Smad3 levels (results not shown). Of relevance, none of the apoptosis or cell cycle regulatory genes analyzed showed differences in ΔEGFR livers (in either tumor or surrounding areas) that could correlate with their lower tumor progression (Fig. 8A). Nonetheless, it was really interesting to find a significant difference in the levels of *Nox4*, the NADPH oxidase that mediates some of the suppressor actions of TGF-β and for which we have recently found a relevant liver tumor suppressor role.[22] Finally, analysis of *Hgf* mRNA levels revealed a significant increase in the livers of ΔEGFR mice (nontumoral tissue). No evidence was observed for increases in *uPA* or *Met* at the mRNA level (Fig. 8B).

These results together indicate that delay in the inflammation process is the most evident alteration observed in ΔEGFR mice that justifies the retarding of the appearance of DEN-induced tumors. The increased levels of Nox4, a liver growth suppressor protein, could also contribute. The increase in HGF levels might replace the EGF-induced growth once the tumor is formed.

Discussion

The purpose of this study was to elucidate the specific role of the EGFR catalytic activity in a physiological (liver regeneration) and a pathological (hepatocarcinogenesis) situation of the liver where proliferation of adult hepatocytes takes place. Our results demonstrate that although EGFR signaling plays crucial roles in these processes, its absence only results in an overall delay in both regeneration and carcinogenesis, suggesting the existence of at least partial functional compensation by alternative routes.

First of all, we propose an essential role for EGFR in the early phases of the regenerative response after PH in mice, mediating a fast and efficient process. In a previous study, Natarajan et al.[25] generated a mouse model carrying a floxed EGFR allele to inactivate the EGFR in the adult liver. They proposed that EGFR is a critical regulator of hepatocyte proliferation in the initial phases of liver regeneration. Here, we confirm these results using a novel and unique animal model, but we also associate these effects with the catalytic activity of the EGFR and provide new insights about the molecular mechanisms by which this occurs, particularly overactivation of the TGF-β pathway and alterations in lipid metabolism.

Inactivation of TGF-β signaling in hepatocytes results in an increased proliferative response after PH.[26,27] One of the main effects of TGF-β in liver cells is the induction of apoptosis through up-regulation of *Bim* and *Bmf*, proapoptotic members of the Bcl-2 family. The EGF pathway counteracts TGF-β proapoptotic effects by impairing up-regulation of both genes and up-regulating $Bclx_L$ and *Mcl-1*, two antiapoptotic members of the Bcl-2 family,[6,7] an effect mediated by survival signals such as the phosphoinositide 3-kinase/Akt and ERK pathways. However, our study demonstrates that the major role of EGF during liver regeneration after PH is not cell survival, probably because apoptosis is not relevant in a model where there is no hepatocyte damage. In contrast, the decrease and delay in activation

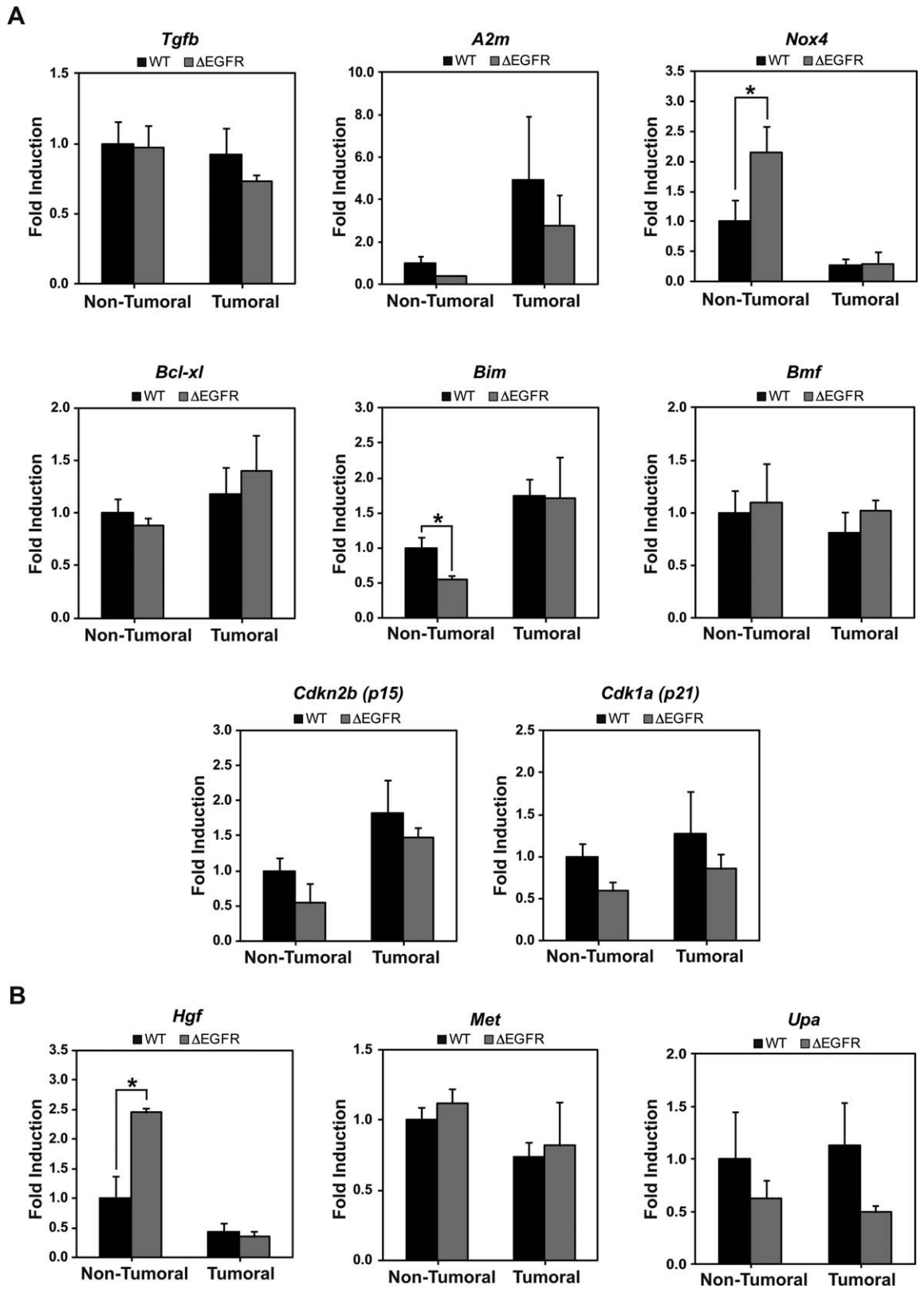

Fig. 8. Transcriptomic (qRT-PCR) analysis in tumoral and nontumoral areas in liver tissues from 9-month DEN-treated WT and ΔEGFR mice. (A) *Tgfb*, apoptosis, and cell cycle regulatory genes. (B) HGF signaling pathway. (A,B) Data are mean ± standard error of the mean of at least four animals per group (three sections/animal). Student *t* test was used: $*P < 0.05$.

of the phosphoinositide 3-kinase/Akt and ERK pathways at early times after PH in ΔEGFR animals, concomitant with high expression of the cyclin-dependent kinase inhibitor p15INK4, suggest a relevant role for the EGFR pathway in priming hepatocytes to enter into the cell cycle. We suggest that this delay could be related, at least partially, to the overexpression of TGF-β1 and overactivation of the TGF-β pathway observed in animals with attenuated EGFR signaling. Although initially it was supposed that TGF-β could play a relevant role in mediating termination of liver regeneration, the latest results indicate that it is not necessary during this stage.[26] In contrast, a higher and accelerated DNA synthesis peak after PH was found in a *Tgfbr2* knockout (R2LivKO) animal model.[26] Indeed, in contrast to previous predictions, TGF-β could play an essential role in the first stages of liver regeneration; and here we show that the EGFR pathway may be essential in regulating its expression and signaling.

It is well known that after PH hepatocellular fat accumulation occurs, concomitant with up-regulation of genes related to the adipogenic program,[28] which has been suggested to be a mammalian target of rapamycin–dependent process.[29] Disruption of hepatic adipogenesis is associated with impaired hepatocellular proliferation following PH[28] (for review, see Rudnick and Davidson[30]). Here we show for the first time that the EGFR pathway is required for fat accumulation and up-regulation of two key enzymes related to the *de novo* lipid synthesis. Further studies will be required to determine the precise molecular mechanisms mediating these effects.

In spite of these alterations, most of the ΔEGFR mice survived to the PH and no significant differences could be observed in the serum parameters related to liver injury or in the liver to body mass ratio. It is true that a higher mortality was observed in the first 48 hours after PH, as also suggested by Natarajan et al. in the mice carrying a floxed EGFR[25]; but differences were not statistically significant. Our results are in agreement with the work of Michalopoulos's group where the consequences of *in vivo* silencing of the EGFR on rat liver regeneration using EGFR-specific short hairpin RNAs were analyzed.[31] Despite suppression of hepatocyte proliferation lasting into day 3 after PH, liver restoration occurred. Interestingly, hepatocytes in short hairpin EGFR–treated rats were considerably larger compared with short hairpin RNA–treated controls, an effect that we also observed in the ΔEGFR animals. Furthermore, we show that ΔEGFR mice presented higher phosphorylation levels of c-Met that could compensate for the lack of EGFR-mediated proliferative signals, a possibility that was also suggested in Michalopoulos et al.'s work.[31] Interestingly, we demonstrate up-regulation not only of the expression of *Hgf* in ΔEGFR livers after PH but also of its activator uPA, whose deficiency was shown to retard liver regeneration by impairing the HGF pathway.[23] However, even though the HGF/Met pathway is clearly overactivated in ΔEGFR animals, the contributions made by EGFR are unique in some aspects and not compensated by c-Met. In particular, early activation of phosphoinositide 3-kinase/Akt and ERK signaling would require EGFR signaling. Interestingly, Factor et al. elegantly demonstrated that c-Met signaling in hepatocytes is essential for sustaining long-term ERK1/2 activation throughout liver regeneration, and alternative pathways may account for the early ERK1/2 activation.[32] Furthermore, here we show that compensation of the cytostatic effects of the TGF-β pathway and regulation of lipid metabolism after PH appear to be fully dependent on EGFR and may be necessary for more efficient liver regeneration but dispensable for restoring liver mass in those animals that survive the first hours after PH. In agreement with these results, lack of Nogo-B (reticulon 4B) produces overactivation of the TGF-β pathway after PH in mice, coincident with a delay in hepatocyte proliferation, but did not affect the liver to body mass ratio in the regenerative process.[33]

We also show here that attenuation of EGFR catalytic activity induces a significant delay in the appearance of tumorigenic lesions in DEN-treated animals. However, once tumors appear, the proliferative rate is similar in ΔEGFR and WT animals. Surprisingly, no significant differences were observed in the TGF-β pathway or in the expression of apoptosis or cell cycle regulatory genes, which could justify the delay in tumorigenesis. The only significant change was the mRNA levels of *Nox4*, a member of the NADPH oxidase family that is down-regulated in HCC patients and negatively modulates hepatocyte proliferation.[22] Interestingly, EGF inhibits *Nox4* expression, acting at the transcriptional level on the *Nox4* promoter.[21] Indeed, the more significant difference lies in the appearance of preneoplastic lesions. In agreement with this result, a previous study using gefitinib (an EGFR inhibitor) in DEN-induced hepatocarcinogenesis in mice revealed that the gefitinib-treated animals showed significantly lower numbers of HCC nodules but that the mean tumor size was not different between untreated and gefitinib-treated mice.[34] Similar to what was found during liver regeneration, levels of HGF were much higher in ΔEGFR animals, which suggests that this cytokine might replace the mitogenic function of the EGFR ligands.

Our study indicates that the delay in the appearance of tumoral lesions might be associated with attenuation of the inflammatory process. In support of these results, pharmacological inhibition of EGFR signaling effectively prevented the progression of cirrhosis and regressed fibrosis in animal models.[35] EGFR signaling has been proposed as a critical junction between inflammation-related signals and potent cell regulating machineries.[36] Recent evidence indicates that the EGFR pathway regulates the expression of inflammatory factors and chemokine ligands produced by liver tumor cells.[24] Our results indicate differences in the expression of interleukin-6 and TNF-α in the tumor surrounding tissue, which suggests that the transgene expression in hepatocytes affects the production of inflammatory cytokines by themselves or other surrounding cells. Interestingly, the EGFR pathway plays an essential role in liver macrophages to mediate the inflammatory process in DEN-induced HCC.[37] Indeed, we cannot exclude that the overexpression of a truncated form of the EGFR that binds its ligands, but does not transduce the signal, may decrease the number of free EGFR ligands able to act on the nonparenchymal cells. Furthermore, it is worth pointing out that CCL2, a chemokine considered to be a central coordinator of hepatocyte-mediated inflammation,[38] showed decreased expression in ΔEGFR tumors. Finally, CXCL1, considered a critical player in both inflammation and tumor growth in HCC,[39] presented significantly decreased mRNA levels in ΔEGFR tumoral and nontumoral areas. Regardless of the mechanism and considering together our results and others in the literature, there is no doubt that one of the most essential functions of the EGFR pathway during hepatocarcinogesis should be regulation of inflammation addressed by parenchymal and/or nonparenchymal cells.

In conclusion, here we provide a novel research tool, a ΔEGFR transgenic mouse model, which has proved to be very useful in deciphering the molecular mechanisms underlying the functions of EGFR signaling in the adult liver, in particular uncovering its tyrosine kinase–dependent functions in liver regeneration and carcinogenesis. We believe this model will open new possibilities for exploring the EGFR pathway as a targeted therapeutic strategy in chronic liver diseases and carcinogenesis.

Acknowledgment: We thank Aurora de la Cal and Sergio Losada for their collaboration in the administrative work at the CIEMAT/CIBERER/ISS-FJD and Filip Radom and Alba Marin (graduate students at the IDIBELL) for their technical support and help.

References

1. **Berasain C**, **Avila MA**. The EGFR signalling system in the liver: from hepatoprotection to hepatocarcinogenesis. J Gastroenterol 2014;49:9-23.
2. Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 2010;176:2-13.
3. Riehle KJ, Dan YY, Campbell JS, Fausto N. New concepts in liver regeneration. J Gastroenterol Hepatol 2011;26(Suppl. 1):203-212.
4. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. J Hepatol 2012; 57:692-694.
5. Carmona-Cuenca I, Herrera B, Ventura J-J, Roncero C, Fernández M, Fabregat I. EGF blocks NADPH oxidase activation by TGF-beta in fetal rat hepatocytes, impairing oxidative stress, and cell death. J Cell Physiol 2006;207:322-330.
6. Fabregat I, Herrera B, Fernández M, Alvarez AM, Sánchez A, Roncero C, et al. Epidermal growth factor impairs the cytochrome C/caspase-3 apoptotic pathway induced by transforming growth factor beta in rat fetal hepatocytes via a phosphoinositide 3-kinase-dependent pathway. Hepatology 2000;32:528-535.
7. Caja L, Sancho P, Bertran E, Fabregat I. Dissecting the effect of targeting the epidermal growth factor receptor on TGF-*β*-induced-apoptosis in human hepatocellular carcinoma cells. J Hepatol 2011;55:351-358.
8. Skarpen E, Oksvold MP, Grøsvik H, Widnes C, Huitfeldt HS. Altered regulation of EGF receptor signaling following a partial hepatectomy. J Cell Physiol 2005;202:707-716.
9. Livneh E, Prywes R, Kashles O, Reiss N, Sasson I, Mory Y, et al. Reconstitution of human epidermal growth factor receptors and its deletion mutants in cultured hamster cells. J Biol Chem 1986;261: 12490-12497.
10. Kashles O, Yarden Y, Fischer R, Ullrich A, Schlessinger J. A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. Mol Cell Biol 1991;11:1454-1463.
11. Murillas R, Larcher F, Conti CJ, Santos M, Ullrich A, Jorcano JL. Expression of a dominant negative mutant of epidermal growth factor receptor in the epidermis of transgenic mice elicits striking alterations in hair follicle development and skin structure. EMBO J 1995;14:5216-5223.
12. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 2005;33:e36.
13. Giraldo P, Montoliu L. Size matters: use of YACs, BACs and PACs in transgenic animals. Transgenic Res 2001;10:83-103.
14. Valverde AM, Burks DJ, Fabregat I, Fisher TL, Carretero J, White MF, et al. Molecular mechanisms of insulin resistance in IRS-2-deficient hepatocytes. Diabetes 2003;52:2239-2248.
15. Higgins GM, Anderson R. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931;12:186-202.
16. Sancho P, Bertran E, Caja L, Carmona-Cuenca I, Murillo MM, Fabregat I. The inhibition of the epidermal growth factor (EGF) pathway enhances TGF-beta-induced apoptosis in rat hepatoma cells through inducing oxidative stress coincident with a change in the expression pattern of the NADPH oxidases (NOX) isoforms. Biochim Biophys Acta 2009;1793:253-263.
17. Caja L, Ortiz C, Bertran E, Murillo MM, Miró-Obradors MJ, Palacios E, et al. Differential intracellular signalling induced by TGF-beta in rat adult hepatocytes and hepatoma cells: implications in liver carcinogenesis. Cell Signal 2007;19:683-694.
18. Murillo MM, del Castillo G, Sánchez A, Fernández M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF-beta1 in hepatocytes. Oncogene 2005;24:4580-4587.
19. Mitchell C, Nivison M, Jackson LF, Fox R, Lee DC, Campbell JS, et al. Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. J Biol Chem 2005;280:2562-2568.

20. Arandjelovic S, Freed TA, Gonias SL. Growth factor-binding sequence in human alpha2-macroglobulin targets the receptor-binding site in transforming growth factor-beta. Biochemistry (Mosc) 2003;42:6121-6127.
21. Carmona-Cuenca I, Roncero C, Sancho P, Caja L, Fausto N, Fernández M, et al. Upregulation of the NADPH oxidase NOX4 by TGF-beta in hepatocytes is required for its pro-apoptotic activity. J Hepatol 2008;49:965-976.
22. Crosas-Molist E, Bertran E, Sancho P, López-Luque J, Fernando J, Sánchez A, et al. The NADPH oxidase NOX4 inhibits hepatocyte proliferation and liver cancer progression. Free Radic Biol Med 2014;69:338-347.
23. Shimizu M, Hara A, Okuno M, Matsuno H, Okada K, Ueshima S, et al. Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: impaired activation of hepatocyte growth factor after Fas-mediated massive hepatic apoptosis. Hepatology 2001;33:569-576.
24. **Huang P**, **Xu X**, Wang L, Zhu B, Wang X, Xia J. The role of EGF-EGFR signalling pathway in hepatocellular carcinoma inflammatory microenvironment. J Cell Mol Med 2014;18:218-230.
25. Natarajan A, Wagner B, Sibilia M. The EGF receptor is required for efficient liver regeneration. Proc Natl Acad Sci USA 2007;104:17081-17086.
26. **Oe S**, **Lemmer ER**, Conner EA, Factor VM, Levéen P, Larsson J, et al. Intact signaling by transforming growth factor beta is not required for termination of liver regeneration in mice. Hepatology 2004;40:1098-1105.
27. **Romero-Gallo J**, **Sozmen EG**, Chytil A, Russell WE, Whitehead R, Parks WT, et al. Inactivation of TGF-beta signaling in hepatocytes results in an increased proliferative response after partial hepatectomy. Oncogene 2005;24:3028-3041.
28. **Shteyer E**, **Liao Y**, Muglia LJ, Hruz PW, Rudnick DA. Disruption of hepatic adipogenesis is associated with impaired liver regeneration in mice. Hepatology 2004;40:1322-1332.
29. Obayashi Y, Campbell JS, Fausto N, Yeung RS. Impaired lipid accumulation in the liver of Tsc2-heterozygous mice during liver regeneration. Biochem Biophys Res Commun 2013;437:146-150.
30. **Rudnick DA**, **Davidson NO.** Functional relationships between lipid metabolism and liver regeneration. Int J Hepatol 2012;2012:549241.
31. Paranjpe S, Bowen WC, Tseng GC, Luo J-H, Orr A, Michalopoulos GK. RNA interference against hepatic epidermal growth factor receptor has suppressive effects on liver regeneration in rats. Am J Pathol 2010;176:2669-2681.
32. Factor VM, Seo D, Ishikawa T, Kaposi-Novak P, Marquardt JU, Andersen JB, et al. Loss of c-Met disrupts gene expression program required for G_2/M progression during liver regeneration in mice. PloS One 2010;5(9).
33. **Gao L**, **Utsumi T**, **Tashiro K**, Liu B, Zhang D, Swenson ES, et al. Reticulon 4B (Nogo-B) facilitates hepatocyte proliferation and liver regeneration in mice. Hepatology 2013;57:1992-2003.
34. Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, et al. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. Hepatology 2005;41:307-314.
35. Fuchs BC, Hoshida Y, Fujii T, Wei L, Yamada S, Lauwers GY, et al. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. Hepatology 2014;59:1577-1590.
36. Berasain C, Perugorria MJ, Latasa MU, Castillo J, Goñi S, Santamaría M, et al. The epidermal growth factor receptor: a link between inflammation and liver cancer. Exp Biol Med (Maywood) 2009;234:713-725.
37. **Lanaya H**, **Natarajan A**, **Komposch K**, Li L, Amberg N, Chen L, et al. EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. Nat Cell Biol 2014;16:972-981.
38. **Ziraldo C**, **Vodovotz Y**, Namas RA, Almahmoud K, Tapias V, Mi Q, et al. Central role for MCP-1/CCL2 in injury-induced inflammation revealed by in vitro, in silico, and clinical studies. PloS One 2013;8:e79804.
39. Han K-Q, He X-Q, Ma M-Y, Guo X-D, Zhang X-M, Chen J, et al. Inflammatory microenvironment and expression of chemokines in hepatocellular carcinoma. World J Gastroenterol 2015;21:4864-4874.

Author names in bold denote shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at http://onlinelibrary.wiley.com/doi/10.1002/hep.28134/suppinfo.