EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation

Hanane Lanaya\textsuperscript{1,4}, Anuradha Natarajan\textsuperscript{1,4}, Karin Komposch\textsuperscript{1,4}, Liang Li\textsuperscript{2}, Nicole Amberg\textsuperscript{1}, Lei Chen\textsuperscript{3}, Stefanie K. Wculek\textsuperscript{1}, Martina Hammer\textsuperscript{1}, Rainer Zenz\textsuperscript{1}, Markus Peck-Radosavljevic\textsuperscript{3}, Wolfgang Sieghart\textsuperscript{3}, Michael Trauner\textsuperscript{3}, Hongyang Wang\textsuperscript{2} and Maria Sibilia\textsuperscript{1,5}

Hepatocellular carcinoma (HCC) is a frequent cancer with limited treatment options and poor prognosis. Tumorigenesis has been linked with macrophage-mediated chronic inflammation and diverse signalling pathways, including the epidermal growth factor receptor (EGFR) pathway. The precise role of EGFR in HCC is unknown, and EGFR inhibitors have shown disappointing clinical results. Here we discover that EGFR is expressed in liver macrophages in both human HCC and in a mouse HCC model. Mice lacking EGFR in macrophages show impaired hepatocarcinogenesis, whereas mice lacking EGFR in hepatocytes unexpectedly develop more HCC owing to increased hepatocyte damage and compensatory proliferation. Mechanistically, following interleukin-1 stimulation, EGFR is required in liver macrophages to transcriptionally induce interleukin-6, which triggers hepatocyte proliferation and HCC. Importantly, the presence of EGFR-positive liver macrophages in HCC patients is associated with poor survival. This study demonstrates a tumour-promoting mechanism for EGFR in non-tumour cells, which could lead to more effective precision medicine strategies.

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide\textsuperscript{1}. Main risk factors for HCC include hepatitis B or C infection, alcoholic liver injury, non-alcoholic steatohepatitis, environmental carcinogens and hereditary metabolic diseases\textsuperscript{2}, which can lead to chronic hepatitis or cirrhosis, conditions regarded as preneoplastic stages\textsuperscript{3}. Current treatment options are limited, which may be due to the lack of biomarkers for patient stratification, as there is limited use of biopsies for HCC diagnosis, which is largely based on radiological criteria\textsuperscript{4}. Therefore, a better understanding of the mechanisms driving HCC development is needed.

Persistent infections, activation of liver-resident macrophages (Kupffer cells) and recruitment of inflammatory cells can lead to chronic inflammation\textsuperscript{5,8} accompanied by many factors favouring HCC development\textsuperscript{9}. The molecular link between inflammation and HCC is not completely understood. Cytokines such as interleukin-1 and IL-6 (IL-1 and IL-6, respectively) play a central role in liver carcinogenesis. IL-6 is produced by Kupffer cells following stimulation with IL-1, which is released by dying hepatocytes\textsuperscript{10,11}. IL-6 is responsible for compensatory proliferation of damaged hepatocytes, leading eventually to HCC development\textsuperscript{12}. Many signalling pathways involved in HCC development, such as MYD88, JNK1/2, p38\alpha and IKK\beta, can regulate hepatic IL-6 production, although the precise mechanism is unclear. Moreover, JNK1, p38 and IKK\beta have been shown to be involved in human HCC (refs 13–15), and their deletion in parenchymal versus non-parenchymal cells can differentially affect hepatocarcinogenesis in mice\textsuperscript{7,10,12,13,16–19}.

Epidermal growth factor receptor (EGFR) overexpression, which occurs in 40–70\% of human HCCs, has been linked with tumorigenesis\textsuperscript{20}. Elevated expression of the EGFR ligand TGF\alpha has been reported in preneoplastic lesions, suggesting a role in early HCC (ref. 21). EGFR antagonists were effective in human HCC cells and in a rat HCC model\textsuperscript{22,23}. In clinical trials with unselected patients, erlotinib has shown moderate effects in phase II, whereas gefitinib and cetuximab have provided only disappointing results in advanced stage HCC patients\textsuperscript{2}. Moreover, the SEARCH trial, the only phase

\textsuperscript{1}Institute of Cancer Research, Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria.
\textsuperscript{2}National Center for Liver Cancer, International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute/Hospital, Shanghai, 225 Changhai Road, Shanghai 200438, China.\textsuperscript{3}Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Division of Gastroenterology and Hepatology, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria.\textsuperscript{4}These authors contributed equally to this work.
\textsuperscript{5}Correspondence should be addressed to M.S. (e-mail: maria.sibilia@meduniwien.ac.at)

Received 24 February 2014; accepted 18 July 2014; published online 31 August 2014; DOI: 10.1038/ncb3031
III study carried out, was unable to show survival improvement with erlotinib in advanced stage HCC (ref. 24). Therefore, a better understanding of the mechanisms whereby EGFR signalling influences HCC progression is needed. Genetically modified mouse models represent an invaluable tool to dissect the interplay between tumour and stromal cells during HCC development and to identify important signalling pathways in the respective cell types. Here, we employed mice lacking EGFR in different cell types of the liver to dissect the role of different cellular players and signalling pathways in HCC development.

RESULTS

HCC formation in mice lacking EGFR in all liver cells

To investigate the function of EGFR during HCC formation, we employed polyinosinic–polycytidylic acid (pIpC)-inducible Mx-Cre transgenic mice (EGFR\textsuperscript{\textsuperscript{f\textsubscript{f}}};Mx-Cre=EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}}; ref. 25; Supplementary Fig. 1a). Absence of EGFR from parenchymal cells was confirmed by immunohistochemistry and western blot analysis (Fig. 1a,b and Supplementary Fig. 1a,b). By 46 weeks, EGFR\textsuperscript{\textsuperscript{f\textsubscript{f}}} livers developed tumours, whereas EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}}; ref. 25; Supplementary Fig. 1a) mice showed a marked decrease in tumour mass, area and number (Fig. 1c,d). Analysis of EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} mice revealed a significant decrease in proliferation and an increase in apoptosis in HCC (Fig. 1e), but not in adjacent non-tumour tissue (Supplementary Fig. 1c). These results suggest that EGFR in liver cells promotes HCC formation by protecting them from DEN-induced apoptosis.

HCC formation in mice lacking EGFR in parenchymal cells

Given the complexity of HCC and the involvement of different cell types, we induced HCC in mice lacking EGFR specifically in hepatocytes and bile duct cells (EGFR\textsuperscript{\textsuperscript{f\textsubscript{f}}};Alfp-Cre=EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}}; ref. 25; Supplementary Fig. 1a). Absence of EGFR from parenchymal cells was confirmed by immunohistochemistry and western blot analysis in tumour and non-tumour tissue (Fig. 1f,g). In EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} tumours, EGFR was detectable in non-parenchymal cells (Fig. 1f), whereas EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} livers showed a marked decrease in tumour mass, area and number (Fig. 1c,d). Analysis of EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} mice revealed a significant decrease in proliferation and an increase in apoptosis in HCC (Fig. 1e), but not in adjacent non-tumour tissue (Supplementary Fig. 1c). These results suggest that EGFR in liver cells promotes HCC formation by protecting them from DEN-induced apoptosis.

EGFR expression in Kupffer cells/liver macrophages promotes HCC development

We hypothesized that the difference in HCC formation between EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} and EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{P}}} mice was caused by EGFR function in non-parenchymal cells. We therefore carried out immunohistochemistry for non-parenchymal cell markers and observed a fourfold increase of F4/80-positive cells, which could be Kupffer cells or infiltrating macrophages in EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{P}}} tumours (Fig. 2a,b). There was significant upregulation of serum CCL2, a chemokine known to attract F4/80-positive cells, in EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{P}}} tumours, but not in EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{M}}} tumours (Fig. 2a,b). To test if EGFR-expressing liver macrophages could contribute to increased HCC formation in EGFR\textsuperscript{\textsuperscript{\Delta\textsubscript{P}}} mice, we
Figure 1  HCC formation in mice lacking EGFR in hepatocytes or all liver cells. (a) EGFR staining on tumour sections of EGFRf/f and EGFRΔMx mice. Scale bar, 100μm. (b) Western blot of tumour (T) and non-tumour tissue (NT). (c) Representative livers (top; scale bar, 1 cm) and haematoxylin and eosin (H&E) stainings of liver sections (bottom; scale bar, 1 mm). (d) Tumour mass (left), area (middle) and number (right) of EGFRf/f (n=8) and EGFRΔMx (n=10) mice. Two pooled independent experiments. (e) Proliferation marker Ki67-positive (left, EGFRf/f, n=103; EGFRΔMx, n=27) and TUNEL-positive cells (right, EGFRf/f, n=153; EGFRΔMx, n=49) in tumours. n=high power field (HPF). Six mice per genotype were analysed. (f) EGFR staining on liver tumour sections of EGFRf/f and EGFRΔMx mice. Arrows point to EGFR-expressing non-parenchymal cells. Scale bar, 100μm. (g) Western blot of tumour (T) and non-tumour tissue (NT). (h) Representative livers (top; scale bar, 1 cm) and H&E stainings of liver sections (bottom; scale bar, 1 mm). Dotted lines in c and h mark tumour nodules. (i) Tumour mass (left) and area (right) of EGFRf/f (n=12) and EGFRΔMx (n=14) mice. Two pooled independent experiments. (j) Ki67-positive (left, EGFRf/f, n=26; EGFRΔMx, n=68) and TUNEL-positive cells (right, EGFRf/f, n=19; EGFRΔMx, n=48) in tumours. n=HPF, three EGFRf/f and four EGFRΔMx mice were analysed. (k) Damaged liver areas after DEN intoxication: 36 h (EGFRf/f, n=5; EGFRΔMx, n=4; EGFRΔhep, n=4); 48 h (EGFRf/f, n=4; EGFRΔMx, n=3; EGFRΔhep, n=4); and 72 h (EGFRf/f, n=4; EGFRΔMx, n=3; EGFRΔhep, n=2). n=number of mice. (l) Caspase-3-positive cells after DEN intoxication: 0 h, EGFRf/f, n=23 (six mice), EGFRΔMx, n=23 (six mice), EGFRΔhep, n=23 (five mice); 24 h, EGFRf/f, n=19 (four mice), EGFRΔMx, n=10 (two mice), EGFRΔhep, n=20 (four mice); 48 h, EGFRf/f, n=5 (one mouse), EGFRΔMx, n=12 (two mice), EGFRΔhep, n=15 (three mice); 96 h, EGFRf/f, n=32 (six mice), EGFRΔMx, n=21 (three mice), EGFRΔhep, n=6 (one mouse). n=HPF. (m) qRT-PCR showing IL-1β and IL-1α expression in isolated hepatocytes after DEN intoxication in vivo (n=3 mice per genotype and time). (n) qRT-PCR for IL-1β and IL-1α of total liver (n=6 mice per genotype) 24 h after DEN intoxication. Two pooled independent experiments. (o) qRT-PCR for IL-1β in liver tumours of EGFRf/f (n=5), EGFRΔMx (n=3) and EGFRΔhep (n=5) mice. Data (d,e,i,l,n,o) represent mean ± s.e.m. Data in k-o represent mean ± s.d. Student’s t-test for independent samples and unequal variances was used to assess statistical significance (‘∗’P<0.05, ‘∗∗’P<0.01, ‘∗∗∗’P<0.001). Original data are provided in Supplementary Table 1 and uncropped blots in Supplementary Fig. 6.
EGFR expression is induced in activated Kupffer cells/liver macrophages when compared with control and EGFRΔmac mice (Fig. 2c). Similarly to EGFRΔhepΔmac and EGFRΔhep mice, EGFRΔmac mice showed significantly smaller tumours when compared to the respective controls (Fig. 2d,e). Thus, our results reveal an unexpected tumour-promoting role for EGFR in Kupffer cells/liver macrophages during HCC formation.

The presence of EGFR-expressing Kupffer cells/liver macrophages in human HCC correlates with poor prognosis

We next investigated if EGFR expression in Kupffer cells/liver macrophages is relevant for human HCC, and analysed EGFR expression in 129 surgically resected HCC samples, predominantly alcohol-, non-alcoholic steatohepatitis- and hepatitis C (HCV)-induced liver cirrhosis (Table 1b). EGFR staining for cancerous tissue and in 15 ‘normal’ livers (Table 1a,b). As chronic HBV is a less common cause of HCC in Europe, we furthermore investigated EGFR expression in 108 European HCC patients, who underwent liver transplantation for HCC (Table 1a).

Healthy and untreated EGFRΔlivers contained very few EGFR-positive Kupffer cells (Fig. 3d,f). However, 5 days after DEN injection, prominent EGFR expression was detected in F4/80-positive cells (Fig. 3e,f). Interestingly, increased levels of EGFR were also present in hepatocytes following DEN treatment (Fig. 3g). We next investigated whether EGFR was induced in Kupffer cells/liver macrophages of HCCs. Immunohistochemistry on serial sections of EGFRΔlivers and EGFRΔhepΔmac tumours revealed co-expression of EGFR and F4/80 in tumour and adjacent tissue of EGFRΔlivers (Fig. 3h,i), but not of EGFRΔhepΔmac mice (Fig. 3j,k). These findings suggest that EGFR expression is induced in activated Kupffer cells/liver macrophages under pathological conditions.
Liver, untreated

EGFR

EGFR

EGFR

EGFR

h

α

F4/80

DAPI

-EGFR

976 NATURE CELL BIOLOGY

normal tissue (Table 1a and Supplementary Fig. 3a), which confirms (hepatocytes of the adjacent non-cancerous tissue and normal livers C(0) for EGFR expression in hepatocytes, and there was no difference

pathologists using a previously published scale

both cohorts was examined and blindly scored by two independent pathologists using a previously published scale.

In the Chinese cohort, about 73% of normal livers were negative (0) for EGFR expression in hepatocytes, and there was no difference in EGFR expression levels (+, ++, ++++) among tumour cells and hepatocytes of the adjacent non-cancerous tissue and normal livers (Table 1a and Supplementary Fig. 3a). High expression of EGFR (+++, +++++) was more prevalent in HCC when compared with normal tissue (Table 1a and Supplementary Fig. 3a), which confirms previous reports. Similar results were observed for EGFR expression in hepatocytes and tumour cells of the European cohort (Table 1a).

For both cohorts, EGFR expression in tumour cells did not show significant prognostic value for patients' overall (OS) or disease-free survival (DFS) after surgery (Supplementary Fig. 3be and Table 1a), and no relationship between EGFR expression in hepatocytes and clinicopathological characteristics was found.

To analyse EGFR expression in liver macrophages we stained for CD68 and F4/80 in Kupffer cells/liver macrophages of EGFR(+/+) mice. White arrows indicate EGFR-positive Kupffer cells. Scale bar, 50 μm. Representative western blot showing EGFR expression in isolated hepatocytes and Kupffer cells confirmed by F4/80 staining. Scale bar, 50 μm. Representative anti-EGFR (α-EGFR) hepatocytes (EGFR(+/+) untreated, EGFR negative, n = 9, EGFR positive, n = 10; EGFR(+/+) 5 days after DEN, EGFR negative, n = 4, EGFR positive, n = 26) and (g) hepatocytes (EGFR(+/+) untreated, n = 12; EGFR(+/+) 5 days after DEN, n = 13). Two pooled independent experiments. Representative anti-EGFR (α-EGFR) and anti-F4/80 (β-F4/80) staining carried out on serial sections of control and EGFR(+/+) HCC and no EGFR expression in EGFR(+/+) tumours. Scale bar, 50 μm. Nuclei (4',6-diamidino-2-phenylindole, DAPI), blue; EGFR (Alexa 488), green, and F4/80 (Alexa 594), red; merge, bottom right. Data in f and g represent mean ± s.d. Student’s t-test for independent samples and unequal variances was used to assess statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001). Original data are provided in Supplementary Table 1 and uncorrected plots in Supplementary Fig. 6.

To analyse EGFR expression in liver macrophages we stained adjacent tissue sections for EGFR and the macrophage marker CD68. In 'normal' liver tissue, all CD68-positive cells did not express EGFR (Table 1a). In contrast, 45% of Chinese and 34% of European samples

Figure 3 EGFR expression is induced in activated Kupffer cells/liver macrophages under pathological conditions. (a,b) Representative immunofluorescent confocal microscopy image showing co-staining for F4/80 and EGFR in cultured Kupffer cells/liver macrophages isolated from (a) EGFR(+/+) and (b) EGFR(+/+) mice and stimulated with IL-1β for 24 h. Cultures contained 98% or more Kupffer cells/liver macrophages as confirmed by F4/80 staining. Scale bar, 50 μm. (c) Representative western blot showing EGFR expression in isolated hepatocytes and Kupffer cells of EGFR(+/+), EGFR(+/+) and EGFR(+/+) mice. (d,e) Representative immunofluorescent confocal microscopy images showing F4/80 and EGFR expression in liver sections of (d) untreated and (e) DEN treated (5 days) EGFR(+/+) mice. (f,g) Mean fluorescence intensity (FI) showing EGFR expression levels (Alexa 488, green) in (f) liver macrophages (EGFR(+/+) untreated, EGFR negative, n = 9, EGFR positive, n = 10; EGFR(+/+) 5 days after DEN, EGFR negative, n = 4, EGFR positive, n = 26) and (g) hepatocytes (EGFR(+/+) untreated, n = 12; EGFR(+/+) 5 days after DEN, n = 13). Two pooled independent experiments. Representative anti-EGFR (α-EGFR) hepatocytes (EGFR(+/+) untreated, EGFR negative, n = 9, EGFR positive, n = 10; EGFR(+/+) 5 days after DEN, EGFR negative, n = 4, EGFR positive, n = 26) and (g) hepatocytes (EGFR(+/+) untreated, n = 12; EGFR(+/+) 5 days after DEN, n = 13). Two pooled independent experiments. Representative anti-EGFR (α-EGFR) and anti-F4/80 (β-F4/80) staining carried out on serial sections of control and EGFR(+/+) HCC and no EGFR expression in EGFR(+/+) tumours. Scale bar, 50 μm. Nuclei (4',6-diamidino-2-phenylindole, DAPI), blue; EGFR (Alexa 488), green, and F4/80 (Alexa 594), red; merge, bottom right. Data in f and g represent mean ± s.d. Student’s t-test for independent samples and unequal variances was used to assess statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001). Original data are provided in Supplementary Table 1 and uncorrected plots in Supplementary Fig. 6.
EGFR expression in tumour cells and Kupffer cells of human HCCs.

(a) EGFR expression in hepatocytes and Kupffer cells of the Chinese (n = 129 patients) and European (n = 108 patients; in brackets) cohorts.

| EGFR expression | Hepatocytes | Kupffer cells |
|-----------------|-------------|---------------|
|                 | HCC         | Tissue adjacent to carcinoma | Normal liver | HCC | Tissue adjacent to carcinoma | Normal liver |
| 0               | 70/129 (69/108) | 83/129 (57/108) | 11/15 | 71/129 (71/108) | 114/129 (76/104*) | 15/15 |
| +               | 35/129 (31/108) | 30/129 (40/108) | 3/15 | 33/129 (36/108) | 8/129 (27/104*) | 0/15 |
| ++              | 18/129 (6/108) | 13/129 (7/108) | 1/15 | 20/129 (1/108) | 5/129 (1/104*) | 0/15 |
| +++             | 6/129 (2/108) | 3/129 (0/108) | 0/15 | 5/129 (0/108) | 2/129 (0/104*) | 0/15 |

(b) Relationship between EGFR-positivity in Kupffer cells and the clinicopathological characteristics.

| Variable | 0 | +, ++, +++ | P value |
|----------|---|------------|--------|
| Age      | 49 (30–78) | 49 (21–68) | 0.8646 |
| Sex      | 57 (33–68) | 55 (28–67) | 0.349 |
| AFP      | 65.6 | 51.7 | 0.4971 |
| Etiology | 40.9 | 50.9 | 0.666 |
| Diameter | 42.29 | 16.42 | 0.0004 |
| Microvascular invasion | n.d. | n.d. | n.d. |
| Recurrence of HCC, | 55.16 | 53.5 | 0.0332 |
| TNM staging | 15:25:9 | 18:28:13 | 0.884 |
| Recurrence of HCC, | 31:14:26 | 13:21:24 | 0.0222 |
| Afte | 2.5 (1–13) | 4.5 (1–24) | <0.001 |
| Etiology: | 11:60 | 18:40 | 0.0354 |
| sex | 7:42 | 19:40 | 0.030 |
| TNM staging | 60:8:3 | 38:12:8 | 0.0339 |
| Recurrence of HCC, | 11:12:26:0 | 2:12:44:1 | 0.008 |
| EU: n.d. | n.d. | n.d. | n.d. |
| China: HBsAg, positive:negative | 55.16 | 53.5 | 0.0332 |
| EU: alc:viral:other | 15:25:9 | 18:28:13 | 0.884 |
| Diameter | 31:14:26 | 13:21:24 | 0.0222 |
| Microvascular invasion | 2.5 (1–13) | 4.5 (1–24) | <0.001 |
| China: yes:no | 11:60 | 18:40 | 0.0354 |
| EU: yes:no | 7:42 | 19:40 | 0.030 |
| TNM staging | 60:8:3 | 38:12:8 | 0.0339 |
| Recurrence of HCC, | 11:12:26:0 | 2:12:44:1 | 0.008 |
| EU: n.d. | n.d. | n.d. | n.d. |

AFP, alpha-fetoprotein; TNM, classification of malignant tumours (tumour, node, metastasis); *In four patients, no adjacent tissue was available. † includes samples where tumour and tissue adjacent to carcinoma were negative for EGFR in Kupffer cells; ‡ includes samples where either tumour or tissue adjacent to carcinoma (or both) were positive for EGFR in Kupffer cells; § Mann–Whitney test.

We next analysed the mechanism whereby EGFR signalling in macrophages promotes tumorigenesis. IL-6 is produced at high levels by Kupffer cells in response to IL-1 derived from damaged hepatocytes, harboured EGFR-expressing CD68-positive cells in the tumours ranging from + to +++ (Fig. 4a,b and Supplementary Fig. 3f and Table 1a). EGFR-expressing macrophages were also present within the non-cancerous tissue adjacent to the carcinoma: 12% for the Chinese and 27% for the European cohort. The higher number of EGFR-positive macrophages in adjacent tissue of the European samples might reflect the more advanced cirrhosis in these livers. The specificity of EGFR and CD68 co-expression was confirmed by co-staining in fresh frozen human HCC samples (Supplementary Fig. 3g). EGFR-expressing macrophages in HCCs were associated with poor clinical outcome of Chinese patients after surgical tumour resection and of European patients after liver transplantation, mirrored by significantly reduced OS and DFS (Fig. 4c–f). EGFR-deficient Kupffer cells fail to produce IL-6

© 2014 Macmillan Publishers Limited. All rights reserved.
Figure 4  EGFR expression in Kupffer cells of HCC patients correlates with poor prognosis. (a,b) Representative EGFR and CD68 staining (brown) on serial sections revealing Kupffer cells in human HCC samples. Scale bar, 50 μm. (c–f) OS (c,e) and DFS (d,f) of HCC patients with (+, ++, ++++) or without (0) EGFR expression in Kupffer cells/liver macrophages of the Chinese (c,d; 129 patients; n=71 negative for EGFR; n=58 positive for EGFR) and European cohorts (e,f; 108 patients; n=49 negative for EGFR; n=59 positive for EGFR). (g–j) OS (g,i) and DFS (h,j) of HCC patients with low or high numbers of Kupffer cells/liver macrophages in tumours in the Chinese (g,h; 129 patients; n=52 with low and n=77 with high counts) and European cohorts (i,j; 108 patients; n=50 with low and n=58 with high counts). For the respective patient cohorts, low was classified as values below or at the 50th percentile and high was classified as values above the 50th percentile. Scoring system: 0, negative staining (0–10% positive); 1, weak signal (10–20% positive); 2, intermediate signal (20–50% positive); 3, strong signal (>50% positive) as previously described. A log-rank test was used to assess statistical significance.

and stimulates compensatory hepatocyte proliferation through IL-6R activation\cite{10,11}. We found IL-6 serum levels to be strongly induced after DEN injection in $EGFR^\Delta Mx$ and $EGFR^{\Delta Mx}$ mice, but not in $EGFR^{\Delta Mx}$ or $EGFR^{\Delta Mx}/\Delta Muc$ mice, which are deficient for EGFR in macrophages (Fig. 5a). Importantly, we also found significantly higher IL-6 levels in the plasma of Chinese HCC patients showing EGFR-positive Kupffer cells in their tumours (Fig. 5b). This occurred in association with HBV infection (Fig. 5c), suggesting that infections and inflammatory conditions lead to upregulation of EGFR in Kupffer cells with consequent increased IL-6 production. For the European cohort, patient plasma was not available. Thus, on DEN-induced liver damage, EGFR in Kupffer cells/liver macrophages is required to induce expression of IL-6.

To further investigate whether IL-1 can induce IL-6 production in liver macrophages in an EGFR-dependent manner, we quantified IL-6 levels after incubation of isolated Kupffer cells/liver macrophages with IL-1β in vitro. IL-1β was able to induce IL-6 secretion in EGFR-expressing, but not in EGFR-deficient liver macrophages (Fig. 5d). IL-6 production in Kupffer cells could be prevented by treatment with EGFR inhibitors in a dose-dependent manner (Supplementary Fig. 4a,b). Inflammatory cytokines such as IL-17A, IL-22 and IL-23 were not detectable in the supernatants of $EGFR^{\Delta Mx}$, $EGFR^{\Delta Mx}$ or $MYD88^{\Delta Mx}$ Kupffer cells following IL-1β or EGF stimulation (Supplementary Fig. 4d). IL-6 production was comparable between EGFR-expressing and deficient Kupffer cells after stimulation with toll-like receptor (TLR) agonists such as PolyIC, imiquimod and LPS (Supplementary Fig. 4c) showing that EGFR-deficient Kupffer cells are not intrinsically impaired in IL-6 production. Thus, EGFR-deficient Kupffer cells cannot produce IL-6 in response to IL-1, indicating that EGFR-dependent IL-6 production is downstream of IL-1R signalling.

As IL-6 stimulates compensatory proliferation, we next analysed hepatocyte proliferation after DEN treatment in mice (Fig. 5e). The strongest 5-bromodeoxyuridine (BrdU) incorporation was observed in $EGFR^{\Delta Mx}$ livers, probably because DEN-induced damage is high (because hepatocytes lack EGFR) and EGFR-expressing Kupffer cells/liver macrophages produce IL-6 to stimulate proliferation. In contrast, proliferation was lower in $EGFR^{\Delta Mx}$ and control livers, probably because of impaired IL-6 production by EGFR-negative Kupffer cells/liver macrophages, and less severe DEN-induced damage in control (EGFR-expressing) hepatocytes (Fig. 5e). Together, these results show that increased compensatory proliferation correlates with increased IL-6 levels and increased HCC formation.

**Mechanism of IL-1β-induced IL-6 production by EGFR**

To investigate the molecular mechanism by which EGFR signalling leads to IL-1-induced IL-6 production in Kupffer cells, we measured EGFR ligand expression following IL-1β stimulation and found that, except for betacellulin (BTC), all other EGFR ligands were significantly induced (Fig. 5f). TACE (also known as ADAM17), a metalloprotease proteolytically releasing EGFR ligands\cite{31}, was also induced by IL-1β (Fig. 5f). TACE and EGFR ligands were not expressed in $MYD88^{\Delta Mx}$ Kupffer cells, indicating that their induction is under direct control of IL-1R signalling (Fig. 5f). EGFR ligands and TACE were also induced by IL-1β in Kupffer cells lacking EGFR ($EGFR^{\Delta Mx}$), suggesting EGFR-independent...
Figure 5 EGFR-dependent IL-6 production and release. (a) Enzyme-linked immunosorbent assay (ELISA) showing IL-6 serum levels in EGFR$^{+/+}$, EGFR$^{+/hep}$, EGFR$^{+/-}$ and EGFR$^{-/-}$/mac mice (n=3 for each genotype and time) 6, 24 and 48 h after DEN injection in vivo. (b,c) IL-6 plasma levels in HCC patients (n=104) of the Chinese cohort grouped according to (b) the presence of EGFR-positive (n=31) or EGFR-negative (n=73) Kupffer cells in tumours or additionally considering (c) positivity for hepatitis B surface antigen (HBsAg). HBsAg negative/EGFR negative, n=37; HBsAg negative/EGFR positive, n=11; HBsAg positive/EGFR negative, n=36; HBsAg positive/EGFR positive, n=20. (d) IL-6 release into the supernatant by cultured Kupffer cells (KC) 24 h after incubation with IL-1β in vitro (n=3 Kupffer cell isolates per genotype). (e) Quantification of BrdU-positive cells in liver sections of mice of the indicated genotypes 5 and 8 days after DEN injection (sum of counted HPF of n=3 mice for each genotype and time). (f) qRT-PCR showing expression of heparin-binding EGF-like growth factor (HB), TGfα, AR, epiregulin (ER), BTC, ADAM17 (also known as TACE) and ADAM12 (A12) in Kupffer cells after IL-1β stimulation in vitro (n=4 Kupffer cell isolates per genotype and condition). Two pooled independent experiments. KO, knockout. (g) AR release into the supernatant of cultured Kupffer cells 24 h after IL-1β stimulation in the presence of the indicated inhibitors (n=3 Kupffer cell isolates per genotype; each n is the average of three technical replicates). Three pooled independent experiments. (h,i) IL-6 release by cultured Kupffer cells 24 h after incubation with (h) IL-1β (n=3 Kupffer cell isolates per genotype; each n is the average of three technical replicates, three pooled independent experiments) or (i) EGF (n=3 Kupffer cell isolates per genotype; each n is the average of three technical replicates, three pooled independent experiments) in the presence of the indicated inhibitors. (g-i) Inhibitors: JNK, SP600125; p38, SB203580; IKK2, Sc-514; TACE, TAPI-1; EGF, BIBW2992. Data in a-i represent mean ± s.d. Student’s t-test for independent samples and unequal variances was used to assess statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001). Original data are provided in Supplementary Table 1.
transcriptional regulation (Fig. 5f). Consistent with this, the release of amphiregulin (AR) into the culture medium of IL-1β stimulated Kupffer cells was significantly increased in both EGFR-expressing and deficient Kupffer cells, but did not occur in MYD88/-/- Kupffer cells or when inhibiting TACE (TAPI-1) (Fig. 5g). AR release could be blocked by inhibiting IKK (TAPI-1) (Fig. 5g), but not by inhibiting JNK (SP600125) or p38 (SB203580) signalling (Fig. 5g), demonstrating that release of AR occurs through IKK-dependent activation of TACE.

These results demonstrate that IL-1R signalling in Kupffer cells controls expression of EGFR ligands and TACE, which probably leads to EGFR activation and downstream IL-6 production. To test this, we analysed IL-6 production in isolated Kupffer cells/liver macrophages in the presence of various inhibitors. TACE-1 and EGFR inhibitors blocked IL-1β-induced release of IL-6 in EGFR-expressing Kupffer cells to a similar extent as observed in MYD88/-/- and EGFR-deficient Kupffer cells (Fig. 5h). This demonstrates a linear pathway from IL-1R signalling through MYD88, TACE and EGFR ligands to EGFR signalling and IL-6 production.

On the basis of these results, we hypothesized that direct stimulation of EGFR would induce IL-6 in Kupffer cells. Indeed EGF was equally potent to IL-1β in inducing IL-6 production in EGFR-expressing Kupffer cells (Fig. 5i). Importantly, EGF, but not IL-1β, was able to fully restore IL-6 production in MYD88/-/- Kupffer cells (Fig. 5i). These results indicate that EGFR activation and IL-6 production are downstream of IL-1R/MYD88 signalling. Consistent with this, IL-6 production by Kupffer cells/liver macrophages induced by either EGF or IL-1β was prevented in the absence of EGFR. Precubination of EGFR-positive liver macrophages with JNK, p38 or IKK inhibitors also inhibited IL-6 production induced by either EGF or IL-1β, indicating the importance of JNK, p38 and NFκB signalling downstream of EGFR in mediating IL-6 production (Fig. 5i).

To demonstrate activation of EGFR, we analysed EGFR phosphorylation in isolated Kupffer cells. IL-1β stimulation was able to induce EGFR phosphorylation in EGFR-expressing Kupffer cells to a similar extent to EGF. This did not occur in EGFR-deficient (EGFR/-/-) Kupffer cells (Supplementary Fig. 4e,f). IL-1β-induced EGFR transactivation was blocked by p38 inhibition but not by JNK or IKK inhibition, suggesting that activation of p38 is necessary for EGFR activation (Supplementary Fig. 4e). A similar requirement for p38 for EGFR transactivation by LPS has been recently described.35 JNK, p38, IKKα/β and NFκB phosphorylation following IL-1β stimulation of EGFR-expressing and deficient Kupffer cells could be efficiently blocked by respective inhibitors (Supplementary Fig. 4e). In addition to IKK and NFκB, JNK and p38 were also activated following EGFR stimulation of EGFR wildtype Kupffer cells (Supplementary Fig. 4f).

Together, our data show that IL-1β stimulation of Kupffer cells leads to induction of EGFR ligands and ADAM17 with subsequent p38-dependent EGFR transactivation required for IL-6 production through JNK, p38 and IKK (Supplementary Fig. 5).

**DISCUSSION**

EGFR is frequently overexpressed in human HCC, but its relevance for malignant progression is poorly understood. Our finding on the tumour-promoting role of EGFR in Kupffer cells might provide a possible explanation for the poor response of unstratified advanced stage HCC patients to EGFR targeted therapies. On the basis of our results, we would predict that only HCC patients with EGFR expression in liver macrophages will show a therapeutic effect with EGFR inhibitors (provided that liver macrophages are targeted by EGFR inhibitors). If EGFR is expressed only in tumour cells of HCC, we anticipate that EGFR inhibitors may even promote tumorigenesis, because our genetic results revealed that loss of EGFR in hepatocytes promotes HCC. Clinical follow-up studies are needed to re-evaluate the use of EGFR inhibitors in HCC, and to consider the possibility of targeting specific cell populations. At this stage, it is also possible that EGFR-expressing Kupffer cells play a tumour-promoting role only in the early stages of HCC development. Should this be true, patients with advanced stage HCC would probably not benefit from EGFR-targeted therapies. However, patients with HBV or HCV infections might benefit from EGFR inhibitor treatment in early disease stages to prevent HCC development. It will therefore be interesting to explore the predictive power of EGFR expression in Kupffer cells also in patients in more advanced disease stages. Treatment of patients with EGFR-positive Kupffer cells with EGFR inhibitors selectively targeting Kupffer cells could allow for improved HCC treatment in preselected patient populations.

Our results also highlight the complexity and provide mechanistic insights of EGFR signalling in hepatocarcinogenesis (Supplementary Fig. 5). We show that EGFR plays a hepatoprotective role during DEN-induced liver damage, as absence of EGFR renders hepatocytes more susceptible to DEN-induced damage, leading to increased IL-1β secretion and subsequent enhanced stimulation of Kupffer cells. This occurs in both mouse models lacking EGFR in parenchymal cells (EGFR<sup>Δhep</sup> and EGFR<sup>ΔMx</sup> mice). However, IL-6 production in Kupffer cells is strictly dependent on EGFR expression and occurs in a bimodal way involving first IL-1R/MYD88 signalling followed by TACE/EGFR-L production and p38-dependent EGFR transactivation. In mice lacking EGFR only in hepatocytes (EGFR<sup>Δhep</sup>), IL-6 secretion and compensatory proliferation are elevated, leading ultimately to increased HCC formation. Further deletion of EGFR from Kupffer cells/macrophages (EGFR<sup>ΔMx</sup> and EGFR<sup>Δhep/Δmam</sup> mice) impairs HCC development, despite increased liver damage, as EGFR-deficient Kupffer cells cannot produce IL-6 to stimulate compensatory proliferation (Supplementary Fig. 5). In conclusion, we have discovered a crucial role for EGFR signalling in Kupffer cells/macrophages during inflammation-driven HCC formation, demonstrating that EGFR signalling plays a tumour-promoting function in non-tumour cells. Thus, EGFR-positive Kupffer cells might constitute a future prognostic marker and could potentially represent a target for HCC therapy.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We are grateful to L. Bakiri, D. P. Barlow, R. Eferl, M. Oft and E. F. Wagner for critical reading of the manuscript. We thank T. Baykuscheva-Gentscheva and S. Bardaki for genotyping and E. Hücke and G. Heinze for statistical support. This work was supported by the EC programme LSHC-CT-2006-017731 (Growthstop), the Austrian Science Fund (FWF) grants SFB F3318-B20 and 037731 (Growthstop), the Austrian Science Fund (FWF) grants SFB F3518-B20.
AUTHOR CONTRIBUTIONS

A.N. designed, carried out and analysed in vivo tumour experiments with the EGFR<sup>−/−</sup>, EGFR<sup>del/−</sup> and EGFR<sup>−/−</sup> mice. H.L. designed, carried out and analysed in vitro experiments and some western blot analysis and carried out in vivo tumour experiments with EGFR<sup>del/−</sup> mice. K.K. carried out in vivo analyses with EGFR<sup>del/−</sup> mice and EGFR<sup>−/−</sup> mice and in vitro analyses with Kupffer cells including western blot analysis. N.A. helped with histology, immunohistochemistry and immunofluorescence. S.K.W. helped with qRT-PCRs. M.H. helped with histology, mouse colony and animal experiments. L.L. and L.C. carried out stainings on all human samples (Chinese and European cohorts) and analysed the Chinese cohort with the supervision of H.W. W.S. analysed the European cohort together with M.T. and M.P-R. R.Z. and M.S. wrote the manuscript and analysed the Chinese cohort with the supervision of H.W. W.S. analysed the whole project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
METHODS

Mice and genotyping. EGFR, EGFR<sup>Shp1</sup> and EGFR<sup>AlfpCre</sup> mice have been previously described<sup>13</sup>. EGFR<sup>AlfpCre</sup> and EGFR<sup>AlfpCre/lm</sup> mice were generated by crossing EGFR<sup>C57BL/6</sup> or EGFR<sup>129</sup> with LysM-Cre (ref 33) transgenic male mice. Male mice used in this study were housed in the facilities of the Medical University of Vienna in accordance with institutional policies and federal guidelines. Inducible EGFR deletion in EGFR<sup>AlfpCre/lm</sup> mice was achieved by three consecutive intraperitoneal injections with pLpc (400 µg) every third day in adult mice or by three consecutive intraperitoneal injections with pLpc (150 µg) at days 9, 11 and 13 after birth. To genotype Cre transgenic a mouse forward primer (Cre-F (5'-CAT ACC CGG AAA ATG CTC CGG TCC-3')) and a reverse primer situated in the Cre transgene (Cre-R (5'-CCGACAAATGCCAGGATTGC-3')) were combined. To genotype Alfp-Cre, LysM-Cre double transgenic mice, promoter-specific primers for the particular transgenes (Alb (5'-GCAAAACTGAGAGAAGGATT-3')) or LysM (5'-GAGGAGGTGAATATTCTGCAAA-3')) were used in combination with the Cre-R primer. The primers -EGFR-F (5'-GGTCGTTATGCTGTTGCTTG-3') and -EGFR-R (5'-CAACCAGGTCAAGCTGGCAGGTCG-3') were used to detect deletion of EGFR. The mice were of mixed 129/5 x C57BL/6 x CBAJ genetic background, and in all experiments EGFR-expressing littersmates (EGFR<sup>+/+</sup> or Cre<sup>-/+</sup>) served as controls to the respective EGFR deleted mice. For tumour experiments, age of mice is indicated in the relevant results section and the figure legends. For all short-term experiments, mice were between 8 and 12 weeks of age.

Liver tumour induction in mice by DEN/PB. Liver tumours were induced by chemical carcinogenesis in male mice according to the scheme shown in Supplemental Fig. 1a. Mice were sacrificed when liver tumours were visible in EGFR<sup>+/+</sup>, EGFR<sup>+/AlfpCre</sup> or EGFR<sup>+/Cre</sup> littermate control mice, which occurred around 36 weeks in the Alfp-Cre, around 46 weeks in the Mx-Cre and around 63 weeks in the Alfp-Cre; LysM-Cre double transgenic background. The genetic background of the mice was mixed (C57BL/6 x 129/Sv x CBA/J), but varied between the different Cre lines, thus explaining the difference in the timing of tumour development. For each experiment, we performed EGFR<sup>littermates as controls. To exclude Cre-mediated effects, we confirmed that EGFR<sup>+/+</sup> or EGFR<sup>+/AlfpCre</sup> mice, LysM-Cre mice developed the same defects as EGFR<sup>+/+</sup> controls. Liver injury after DEN injection (100 mg kg<sup>-1</sup> body weight) was determined by measuring circulating aspartate transaminase/alanine transaminase (Reflotron, Roche) and by quantifying necrotic areas seen by H&E stained sections at the times indicated.

Histology. Liver tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin and sectioned (5 µm). Sections were stained with H&E (Sigma) for quantification of necrosis after DEN-induced damage and for quantification of liver tumours. Images were obtained with a Nikon eclipse 80i microscope and quantification was done by Adobe Photoshop CS4 (Adobe). Quantification of liver tumours was carried out on two H&E sections per liver, which were at least 200 µm apart, as previously described<sup>13</sup>.

Immunohistochemistry and immunofluorescence. Protocols for Ki67 staining, BrdU in situ detection, TUNEL and immunoblotting have been previously described<sup>13,27</sup>. In brief, for antigen retrieval, paraffin-embedded tissue was treated with Target Retrieval Solution (Dako) unless otherwise stated in the manufacturer's instructions, and further processed for immunohistochemistry according to the manufacturer's recommendation. To analyse cell proliferation, mice were injected intraperitoneally with 100 µg g<sup>-1</sup> body weight of BrdU. Staining for BrdU (Callag) and Ki67 were carried out using an ABC staining kit (Vector Laboratories). An in situ cell-death detection kit (Roche) was used for TUNEL staining. The number of positive cells was determined by manual counting of the indicated number of high-power fields. For immunofluorescence staining, livers were embedded in optimal cutting temperature compound (Sakura) for frozen section preparation of high-power fields. For immunofluorescent staining, liver sections were embedded in paraffin and sectioned (5 µm). Sections were stained with H&E (Sigma), BCT-R (5'-GTTCGTTATGCTGTTGCTTG-3'), TAFE-R (5'-ACCACCCCTTTGCTTGT-3'), TACE-R (5'-GTCCGAACGTGATGATCCCT-3'), ADAM12-F (5'-GCCGACCTGTGTTGCTTG-3'), EGFR-F (5'-CCAGGCGCAAATCTACGAAGCT-3'), tubulin-F (5'-AGAAAGCTGGAGGGACTACA-3') and tubulin-R (5'-GTGTTGTTCTACAGTTGGGACG-3'). PCRs were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: an initial incubation at 50 °C for 20 s and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 54 °C for 1 min. Relative quantification of RNA was calculated by the ΔΔ Ct method. Omission of cDNA was used as a negative control.

Hepatocyte and Kupffer cell culture. Hepatocytes and Kupffer cells were isolated after liver perfusion according to previously published protocols<sup>9,10</sup>. In brief, livers were perfused at 7 ml min<sup>-1</sup> through the portal vein with perfusion buffer containing collagenase (Gibco). The resulting cell suspension was passed through a 70 µm filter and cultured as follows. Hepatocytes were plated onto collagen pre-coated cell culture dishes and cultured in HepatoZYME-SFM (Gibco) supplemented with 10% FCS, 2 mM glutamine and 1% penicillin-streptomycin for 24 h followed by overnight starvation (in 0.5% FCS containing medium for cytokine induction, serum-free medium for signalling experiments). Kupffer cells were stimulated with IL-1b (10 ng ml<sup>-1</sup>, eBioscience), EGF (10 ng ml<sup>-1</sup>, Lonza), polyIC (20 µg ml<sup>-1</sup>, GE Healthcare), imiquimod (12 µg ml<sup>-1</sup>, InvivoGen) and LPS (10 ng ml<sup>-1</sup>, InvivoGen) for the time period indicated. Whenever indicated, Kupffer cells were pre-incubated for 5 h (for cytokine secretion) or 1 h (for signalling experiments) before stimulation with the following inhibitors: IBM2292 (0.005-20 µM, Selleck), cetuximab (0.01-1 µg ml<sup>-1</sup>, Merck), TAPI-1 (10 µM, PerkinElmer), SP600125 (25 µM, Calbiochem), SB203580 (10 µM, Cell Signaling), and SC-514 (100 µM, Calbiochem). As the number of Kupffer cells that can be recovered from one mouse is very low, it was technically impossible to carry out western blot analysis for all indicated signalling molecules on a single batch of Kupffer cells. Thus, Kupffer cells from two livers of the same genotype were pooled together to obtain around 20-30 µg of protein lysate, which was sufficient for one western blot.

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The following antibodies were used: pEGFR (Tyr1068 CST no. 3777, clone D7A5, 1/1000; Tyr1173 CST no. 4407, clone 5A5, 1/1000), EGFR (CST no. 4267, clone D3B8, 1/500), pAKT (CST no. 9255, clone G9, 1/2000), JNK (CST no. 52, 1/1000), p38 (CST no. 9212, 1/1000), pIKKα/β (CST no. 2697, clone 16A6, 1/1000), IKKβ (CST no. 2370, clone 2C8, 1/1000), pNF-κB (CST no. 3033, clone 3H11, 1/1000), NF-κB (CST no. 3304, 1/1000), pStat3 (CST no. 9145, clone D3A7, 1/2000), Stat3 (sc-7179, 1/1000), actin (Sigma A2066, 1/1000) and α-tubulin (Sigma T9026, clone DM1A, 1/500).

ELISA and MTT assay. Supernatants from Kupffer cell cultures were collected at the indicated times after stimulation and ELISA kits were employed according to the manufacturer's instructions. ELISA for IL-6, IL-17A, IL-22, IL-23 (eBioscience), angiurephilin (R&D Systems), CCL2, IL-1β and IL-1α (BD Biosciences-Pharmingen) were carried out. MTT assay (EZ4U, Biomedica) was carried out to quantify the number of viable Kupffer cells in the wells. The cytokine value was normalized to the number of viable Kupffer cells.

RT-PCR. Total RNA from cultured hepatocytes, cultured Kupffer cells or livers and liver tumours was isolated with TRIzol Reagent (Invitrogen). Complementary DNA synthesis was carried out with a SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. qRT-PCR reactions were carried out using SYBR Green Mix (Applied Biosystems). Primers detecting IFN-β (5'-GGGCTCTAAGAAAGGAATGCT-3'), IL-1α (5'-CAGGTTCTACGCTTACGATGTT-3'), IL-1α (5'-TTGTTGAGCTATTTACAAAAAGGT-3'), IL-6 (5'-TTCCACAGTGCTGCTTG-3'), IL-6 (5'-TTCCACAGTGCTGCTTG-3'). PCRs were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: an initial incubation at 50 °C for 20 s and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 54 °C for 1 min. Relative quantification of RNA was calculated by the ΔΔ Ct method. Omission of cDNA was used as a negative control.

© 2014 Macmillan Publishers Limited. All rights reserved.
**Patient material and immunohistochemistry.** Human samples were collected following informed consent according to the established protocol approved by the Ethics Committee of the Eastern Hepatobiliary Surgery Hospital and the Medical University of Vienna. The 129 HCC patients of the Chinese cohort were randomly taken from the pool of HCC patients undergoing curative resection in the Eastern Hepatobiliary Surgery Hospital between January 2002 and June 2006. None of these HCC patients received preoperative antioxidant treatment. The normal liver tissues were collected from the distal normal liver sections of patients undergoing surgery for liver haemangiomia, who did not show any evidence of chronic liver disease.

The 108 HCC patients of the European cohort were randomly taken from a pool of patients who underwent orthotopic liver transplantation for HCC at the Medical University of Vienna as previously published. None of these HCC patients received preoperative antioxidant treatment. Immunohistochemistry and quantitative analyses (scoring) on all human tissues (Chinese and European cohorts) were carried out by the same laboratory (China) in a blinded manner following standard procedures.

The slides were incubated with the following primary antibodies: anti-EGFR (CST no. 4267, clone D38B1, 1/50); anti-CD68 (macrophage marker, abcam ab9553, clone KPi, 1/200). Staining for EGFR in hepatocytes or Kupffer cells for both the European and Chinese patient cohorts was semi-quantitatively examined and blindly scored by two independent observers using the following scale: 0, negative staining (0–10% positive); +, weak signal (10–20% positive); ++, intermediate signal (20–50% positive); +++, strong signal (>50% positive), as previously described. There were only slight variations between the scoring results of the two pathologists, and in such cases the lower scoring was taken to generate Table 1. Statistical analysis. Mouse experiments. The mouse experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment. Sample size calculation. For tumour studies we considered 10 mice per group to detect a relevant difference in means of 1.5 within-group s.d. at a two-sided significance level of 0.05 and a power of 90%, which ensures 80% power in case of a 20% drop-out rate. For the EGFR (esp.//esp.) experiment six mice per group were considered, which ensures a 90% power to detect a difference in means of two s.d. at the same significance level of 0.05. Experiments in mice (various injections, ex vivo cell and tissue isolations and so on) were carried out as indicated in the figure legends. Quantifications on histological samples were carried out by counting/measuring microscopic fields (HPF where indicated) as indicated in the legends. Data are presented as mean ± s.e.m. Student's t-test for independent samples and unequal variances was used to assess statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001). Each tumour measurement contributed by one animal is the mean value over several liver sections. On the basis of the central limit theorem, we can assume a normal distribution of these animal-specific means even if the underlying variable is not perfectly normally distributed. All statistical analyses were carried out with the SPSS18.0 software. A two-sided p < 0.05 was considered statistically significant.

**Human material.** The statistical analyses were carried out by the respective Chinese and Viennese laboratories. The experiments were not randomized. Staining for EGFR in hepatocytes or Kupffer cells of human patient material for both the European and the Chinese patient cohorts was semi-quantitatively examined and blindly scored by two independent observers. OS in both patient cohorts was defined as the time between the dates of surgery and death or the last follow-up. DFS was defined as the time between the dates of surgery and recurrence. If recurrence was not diagnosed, patients were classified on the date of death or the last follow-up. Survival curves were calculated using the Kaplan–Meier method. Median survival times (OS) and their 95% confidence intervals were reported. The survival in the European cohort is low for a current transplant population because many patients were transplanted with tumours that were too large (outside the now accepted Milan criteria) as previously described. The log-rank test was used to assess the effects of patient variables on DFS and OS.

33. Clausen, B. E., Burkhardt, C., Reichl, W., Renkawitz, R. & Forster, I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8, 265–277 (1999).

34. Lichtenberger, B. M. *et al.* Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. *Cell* 140, 268–279 (2010).

35. Sibilia, M. *et al.* The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. *Cell* 102, 211–220 (2000).

36. Drobits, B. *et al.* Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *J. Clin. Invest.* 122, 575–585 (2012).

37. Smedsrod, B. & Perloff, H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. *J. Leukoc. Biol.* 36, 213–230 (1985).

38. Sieghart, W. *et al.* Osteopontin expression predicts overall survival after liver transplantation for hepatocellular carcinoma in patients beyond the Milan criteria. *J. Hepatol.* 54, 89–97 (2011).

39. Mazzaferrro, V. *et al.* Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N. Engl. J. Med.* 334, 693–699 (1996).

40. Wang, K. *et al.* Overexpression of aspartyl-(asparaginyl)-p-hydroxylase in hepatocellular carcinoma is associated with worse surgical outcome. *Hepatology* 52, 164–173 (2010).
EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation

Hanane Lanaya, Anuradha Natarajan, Karin Komposch, Liang Li, Nicole Amberg, Lei Chen, Stefanie K. Wculek, Martina Hammer, Rainer Zenz, Markus Peck-Radosavljevic, Wolfgang Sieghart, Michael Trauner, Hongyang Wang and Maria Sibilia

In the version of this file originally published online the figure legends were incorrect. These errors have been corrected in this file 29 October 2014.
**SUPPLEMENTARY INFORMATION**

DOI: 10.1038/ncb3031

**Supplementary Figure 1** HCC induction and liver damage in mice and hepatocytes. (a) Tumor development was initiated in male mice of the indicated genotypes by DEN injection at 4 weeks of age (black arrow). At 8 weeks of age tumors were promoted by a diet complemented with phenobarbital (PB) until mice were sacrificed. Top: EGFRΔMx mice (EGFRΔMx; Alfp-Cre). Middle: EGFRΔMx mice (EGFRΔMx; Mx-Cre) that received 3 pIpC injections with 2 days intervals at 7 weeks of age to delete EGFR in the liver (grey arrow). Bottom: EGFRΔMx mice (EGFRΔMx; Mx-Cre) that received pIpC injections on day 9, 11, and 13 (grey arrow) after birth. (b) Southern Blot analysis showing EGFR deletion in EGFRΔMx (left) and EGFRΔhep (right, non-recombined EGFR allele from non-parenchymal cells) livers (f: floxed EGFR allele (6kb), Δ: Cre-deleted EGFR allele (3.9kb)). (c) Ki67-positive (left, EGFRΔMx: n=45 (6 mice); EGFRΔMx: n=48 (7 mice)) and TUNEL-positive cells (right, EGFRΔMx: n=56 (6 mice); EGFRΔMx: n=55 (6 mice)) in adjacent non-tumor tissue. n=HPF. (d) Representative H&E staining of livers 0, 36, 48, and 72 hours after DEN intoxication in vivo. Black arrows indicate necrotic areas. Scale bar: 500μm. (e-f) Alanine transaminase (ALT, 0hrs and 24hrs: n=5 mice per genotype, 48hrs: EGFRΔMx and EGFRΔhep: n=4, EGFRΔMx: n=5 mice) and Aspartate transaminase (AST, 0hrs: EGFRΔMx: n=3, EGFRΔhep: n=3, EGFRΔMx: n=4, 48hrs: EGFRΔMx: n=3, EGFRΔMx: n=4, 48hrs: EGFRΔMx: n=4, EGFRΔMx: n=3 mice) measured in serum 0, 24, and 48hrs after DEN intoxication. (g) Representative active caspase-3 staining (Alexa 488, green) and nuclei (DAPI, blue) of EGFRΔMx and EGFRΔhep liver sections 24 and 96 hours after DEN intoxication, showing increased apoptosis in EGFR-deficient livers after 96 hours. Scale bar indicates 500μm. (h) HMGB1 staining of cultured EGFRΔMx and EGFRΔhep hepatocytes 12 hours after DEN treatment in vitro. Nuclei (DAPI, blue), actin (Phalloidin, red), HMGB1 (Alexa 488, green). Scale bar indicates 100μm. (i) Active caspase-3 staining of cultured hepatocytes of EGFRΔMx and EGFRΔhep mice incubated with TNFα/CHX for 12 hours. Nuclei (DAPI, blue), actin (Phalloidin, red), active caspase-3 (Alexa 488, green). Scale bar indicates 50μm. (c) Data represent means±s.e.m. (e-f) Data represent means±s.d. Student’s t-test for independent samples and unequal variances was used to assess statistical significance (*p<0.05, **p<0.01, ***p<0.001). Original data are provided in Supplementary Table 1.
**Supplementary Figure 2** EGFR deletion in \( EGFR^{\Delta Mx^*} \) mice and cytokine production upon DEN injection. (a) ELISA showing IL-1\( \beta \) in the supernatant of primary hepatocyte cultures 4 hours after incubation with increasing amounts of DEN \textit{in vitro}. (primary hepatocyte isolates of \( EGFR^{ff} \) (n=3) and \( EGFR^{\Delta hep} \) (n=3)). (b) Release of IL-1\( \beta \) and IL-1\( \alpha \) to the supernatant of cultured primary hepatocytes of \( EGFR^{ff} \), \( EGFR^{\Delta hep} \), and \( EGFR^{\Delta Mx} \) mice after incubation with TNF\( \alpha \) quantified by ELISA. n.d. = not detectable. Result of two pooled independent experiments is shown. For each experiment hepatocytes isolated from 2 livers per genotype were pooled and analyzed as 4 technical replicates (primary hepatocytes of n=4 mice were analyzed in total for each genotype). (c) Western Blot analysis of EGFR in livers of \( EGFR^{ff} \) and \( EGFR^{\Delta Mx^*} \) mice. (d) Representative livers of \( EGFR^{ff} \) and \( EGFR^{\Delta Mx^*} \) mice 46 weeks after tumor initiation. Scale bar indicates 1cm. (e) Tumor mass (left, \( EGFR^{ff} \) (n=10) and \( EGFR^{\Delta Mx^*} \) (n=9)), area (middle, \( EGFR^{ff} \) (n=7) and \( EGFR^{\Delta Mx^*} \) (n=9)), and number (right, \( EGFR^{ff} \) (n=7) and \( EGFR^{\Delta Mx^*} \) (n=9)). Two pooled independent experiments. Data (a-b) represent mean±s.d. Data (e) represent mean±s.e.m. Student’s \( t \)-test for independent samples and unequal variances was used to assess statistical significance (*p<0.05, **p<0.01, ***p<0.001). Original data are provided in Supplementary Table 1.
Supplementary Figure 3 EGFR expression in tumor cells and Kupffer cells of human HCCs. (a) Representative Immunohistochemistry showing EGFR expression in tumor cells/hepatocytes of HCC. Scoring (0, +, ++, ++++) was performed according to the scale described below resulting in the generation of Table 1a. Scale bar indicates 50µm. (b–e) OS (b,d) and DFS (c,e) of HCC patients of the Chinese (b,c: 129 patients (n=70 negative for EGFR; n=59 positive for EGFR)) and European cohort (d,e: 108 patients (n=69 negative for EGFR; n=39 positive for EGFR)) with or without EGFR expression in hepatocytes. (f) Representative immunohistochemistry showing EGFR and CD68 staining (0, +, ++, ++++) in liver macrophages of human HCC. Scoring (0, +, ++, ++++) was performed according to the scale described below resulting in the generation of Table 1b. Scale bar indicates 50µm. (g) Representative immunofluorescent EGFR and CD68 co-staining in fresh frozen human HCC tissue (n=12). Nuclei (DAPI, blue), CD68 (Alexa 488, green) and EGFR (Alexa 594, red), merged (bottom right). White arrows indicate double positive cells. Scale bar indicates 50µm. **Scoring system:** 0=negative staining (0%–10% positive), 1=weak signal (10%–20% positive), 2=intermediate signal (20%–50% positive) and 3=strong signal (>50% positive) as previously described. Log-rank test was used to assess statistical significance.
Supplementary Figure 4 IL-6 production by Kupffer cells after various stimuli and inhibitor treatments. (a-b) ELISA quantifying IL-1β-induced IL-6 secretion by isolated Kupffer cells after preincubation with increasing amounts of the EGFR inhibitors Cetuximab (a) or BIBW2992 (Afatinib) (b). (n=2 primary Kupffer cell isolates). (c) IL-6 secretion by isolated Kupffer cells following stimulation with polyIC (20μg/ml), Imiquimod (12μg/ml) and LPS (100ng/ml). - = unstimulated. (n=2 primary Kupffer cell isolates). (d) ELISA quantifying IL-1β- or EGF-induced IL-17A, IL-22 and IL-23 secretion by isolated Kupffer cells (n=2 primary Kupffer cell isolates). (e-f) Representative Western Blot showing activation of the indicated proteins after 15 minutes stimulation with IL-1β (e) or EGF (f) in the presence of the respective inhibitors. Note: Each lane contains proteins isolated from pooling Kupffer cells of 3 different livers. Because the amount of proteins obtained from Kupffer cells from 3 pooled livers was not sufficient to perform Western blot analysis for all indicated proteins and treatments, 2 different isolates and Western blots for each series of treatment (EGF+ inhibitors and IL-1β + inhibitors) had to be performed. (e) Blot 1: IL-1β stimulated EGFRff and EGFRMx + inhibitors and expression of EGFR and JNK. Blot 2: IL-1β + inhibitors and expression of EGFR and JNK. Blot 2: IL-1β stimulated EGFRff and EGFRMx + inhibitors and expression of EGFR and JNK. Blot 3: Blot 1: 15′ EGFRff and EGFRMx + inhibitors and expression of p38, IKK, NF-κB, Stat3. (f) Blot 1: 15′ EGFRff and EGFRMx + inhibitors and expression of EGFR and JNK. Blot 2: EGFRff and EGFRMx + inhibitors and expression of p38, IKK, NF-κB, Stat3. The results were confirmed in a second set of isolates and Westerns. Original data are provided in Supplementary Table 1.
Supplementary Figure 5 Model of EGFR signaling in hepatocytes and Kupffer cells during HCC formation. EGFR signaling is hepatoprotective during DEN-induced liver damage as in the absence of EGFR, hepatocytes undergo more necrosis and apoptosis thus leading to increased IL-1β production and release. IL-1β stimulation of Kupffer cells in turn leads to release of IL-6, which is required for compensatory proliferation and repair of damaged hepatocytes. IL-1β-induced IL-6 production occurs in a bimodal way involving the activation of the IL-1R/MyD88 pathway to first induce EGFR ligands and ADAM17 expression with subsequent EGFR transactivation required for IL-6 production via JNK, p38 and IKK.
**Supplementary Figure 6** Uncropped images of western blots and PCR analysis. The uncropped films and gel photographs (Figure 3c) showing the Western blots and PCR analysis (Figure 3c) displayed in the main figures. Boxed areas indicate the cropped regions displayed in the respective figures.
Supplementary Table 1 Source data for experiments. The source data for experiments in panels 1d, 1e, 1i, 1k, 1l, 1m, 1n, 1o, 2a, 2b, 2e, 3f, 3g, 5a, 5b, 5c, 5e, 5f, 5g, 5h and 5i, and supplementary figures 1c, 1e, 1f, 2a, 2b, 2e, 4a, 4b, 4c and 4d are provided in this table.