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## **The mitogen-activated protein kinase ERK5 regulates the development and growth of hepatocellular carcinoma**

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## **Abstract**

**Objective** The extracellular signal-regulated kinase 5 (ERK5 or BMK1) is involved in tumour development. The ERK5 gene may be amplified in hepatocellular carcinoma (HCC), but its biological role has not been clarified. In this study, we explored the role of ERK5 expression and activity in HCC in vitro and in vivo.

**Design** ERK5 expression was evaluated in human liver tissue. Cultured HepG2 and Huh-7 were studied after ERK5 knockdown by siRNA or in the presence of the specific pharmacological inhibitor, XMD8-92. The role of ERK5 in vivo was assessed using mouse Huh-7 xenografts.

**Results** In tissue specimens from patients with HCC, a higher percentage of cells with nuclear ERK5 expression was found both in HCC and in the surrounding cirrhotic tissue compared with normal liver tissue. Inhibition of ERK5 decreased HCC cell proliferation and increased the proportion of cells in G0/G1 phase. These effects were associated with increased expression of p27 and p15 and decreased CCND1. Treatment with XMD8-92 or ERK5 silencing prevented cell migration induced by epidermal growth factor or hypoxia and caused cytoskeletal remodelling. In mouse xenografts, the rate of tumour appearance and the size of tumours were significantly lower when Huh-7 was silenced for ERK5. Moreover, systemic treatment with XMD8-92 of mice with established HCC xenografts markedly reduced tumour growth and decreased the expression of the proto-oncogene c-Rel.

**Conclusions** ERK5 regulates the biology of HCC cells and modulates tumour development and growth in vivo. This pathway should be investigated as a possible therapeutic target in HCC.

## **Significance of this study**

### **What is already known on this subject?**

- Extracellular signal-regulated kinase 5 (ERK5), a member of the mitogen-activated protein kinase family, has been shown to be relevant for the appearance and progression of several types of cancer.
- The ERK5 gene has been previously reported to be amplified in hepatocellular carcinoma (HCC).
- Molecular targeted therapy of HCC is still suboptimal and additional targets are needed.

### **What are the new findings?**

- In patients with HCC, ERK5 is more abundantly expressed in the nucleus compared with normal liver tissue.
- Genetic or pharmacological inhibition of ERK5 results in reduced proliferation, migration and invasiveness of two HCC cell lines.
- Silencing of ERK5 reduces appearance and growth of HCC xenografts.
- A systemic pharmacological inhibitor of ERK5 blocks tumour growth in established HCC xenografts.

### **How might it impact on clinical practice in the foreseeable future?**

- The possibility to target this pathway with small molecule inhibitors, already in development, opens the possibility for a short-term translation to the clinic.

## Introduction

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy, accounting for about 80% of liver cancer worldwide.<sup>1</sup> Despite progress in the diagnosis and management of this tumour, its biology remains poorly understood, overall limiting the patient's outcome. The large majority of HCC develops in a chronically injured liver. Tumour microenvironment, inflammation, oxidative stress and hypoxia are believed to act in concert through various molecular events, to facilitate tumour initiation, progression and metastasis.<sup>2</sup> The recent approval of sorafenib for the treatment of advanced HCC demonstrates that a molecular targeted therapy is feasible for this malignancy.<sup>3</sup> However, the benefits are far from satisfactory, and additional therapeutic targets need to be identified.

The extracellular signal-regulated kinase 5 (ERK5), also referred to as big mitogen-activated kinase-1 (BMK1), is a member of the mitogen-activated protein kinase (MAPK) family.<sup>4-6</sup> Like for other members of this family, a kinase cascade leads to ERK5 activation via MEK5, a specific upstream kinase. ERK5 has been implicated in many cellular functions, including survival, proliferation and cytoskeletal remodelling (reviewed in ref. 7) and has been recently found to be involved in the pathogenesis of different types of cancer.<sup>8</sup> ERK5 is abundantly expressed in the liver, and the ERK5 gene has been shown to be amplified in human HCC cell lines and overexpressed in primary human HCC.<sup>9,10</sup> However, no information is currently available on the functional role of this kinase in HCC.

In this study, we have used different *in vitro* and *in vivo* approaches, together with genetic or pharmacological interference with ERK5 to investigate its significance in the context of HCC. The data reported herein indicate that ERK5 modulates several critical functions of HCC cells and inhibition of its activity blocks the development and growth of experimental HCC.

## Materials and methods

### Human liver tissue and immunohistochemistry

Liver specimens were obtained after written informed consent from each patient. The use of human material conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved for this study by the University of Torino Bioethical Committee. Immunostaining procedure was performed as described using a polyclonal antibody against ERK5 (see online supplementary table S1).<sup>11</sup> Serial slides were incubated with secondary antibody alone to check for specificity. For negative controls, the primary antibodies were replaced by an isotype-matched and concentration-matched irrelevant antibody.

Additional samples of frozen liver tissue (11 normal liver, 13 HCV-related cirrhosis with HCC) were lysed and used in western blot experiments to assess phosphoERK5 as described below.

### Cell lines and culture conditions

HepG2 and Huh-7 cells<sup>12,13</sup> were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. All cell culture reagents were from Euroclone (Pero, Italy). To evaluate the role of hypoxia, cells were seeded in normoxic conditions to obtain the desired subconfluence level (65%–70%) and then incubated in strictly controlled hypoxic conditions (3% O<sub>2</sub>) up to 24 h.

### **Measurement of cell proliferation**

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay, as previously described.<sup>14</sup>

### **Analysis of cell cycle**

In total, 80 000 cells/well were seeded in multiwell dishes and exposed to the appropriate conditions. After medium removal, 400  $\mu$ l of solution containing 50  $\mu$ g/mL propidium iodide, 0.1% w/v trisodium citrate and 0.1% NP40 was added. Samples were then incubated for 30 min at 4°C in the dark and nuclei analysed with an FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

### **RNA interference and lentiviral vectors**

All siRNAs used were purchased from Dharmacon (Lafayette, USA). Transfection of human HCC cell lines was performed using the Amaxa nucleofection technology (Amaxa, Koln, Germany) as previously described, with 100 nM smart Pool siRNA specific for human ERK5 or non-targeting siRNA (sequence accession no. #NM\_002749).<sup>15</sup> Stable knockdown of ERK5 in Huh-7 cells was performed using lentiviral vectors.  $2 \times 10^6$  HEK293T cells were seeded in 100 mm diameter dishes in DMEM supplemented with 10% FBS and 2 mM glutamine without antibiotics (complete medium). After 24 h (40%–70% confluency) medium was replaced with fresh complete medium. The plasmid mixture was prepared as follows: 8  $\mu$ g lentiviral vectors encoding for shRNA (TRC1.5-pLKO.1-puro vector containing non-targeting sequence shRNA or targeting human MAPK7 NM\_139032, NM\_139034, NM\_002749, NM\_139033, clone ID: TRCN0000010275), 4  $\mu$ g pRSV-Rev, 4  $\mu$ g pMDLg/pRRE and 4  $\mu$ g pMDG.1-VSV and 150 mM NaCl (Polypus Transfection, Euroclone, Milan, Italy) to a final volume of 250  $\mu$ L. Transfection was performed using 40  $\mu$ L jetPEI reagent (Polypus Transfection, Euroclone, Milan, Italy) following the manufacturer's protocol. Twenty-four hours after transfection, the medium was replaced with fresh complete medium. The following day culture media from HEK293T was collected and fresh complete medium added to the cells. Harvested medium was centrifuged at 1500 rpm for 5 min and filtered through a 0.45  $\mu$ m filter and either directly added to Huh-7 cells or stored at  $-80^\circ\text{C}$  for later use. This procedure was repeated 1 day after. For infection, 400 000 Huh-7 cells were seeded in a 60 mm diameter dish at an optimal confluence of 50%. 2 mL/dish of virus supernatant were added in the presence of 5  $\mu$ g/mL polybrene. In some experiments, Huh-7 cells were infected using frozen medium containing lentivirus, following the same procedure. Infected Huh-7 cells were selected with 2  $\mu$ g/mL puromycin for at least 72 h.

### **Nuclear extract preparation**

Procedures for nuclear extracts preparation have been previously described.<sup>16</sup>

### **Western blot analysis**

Procedures for preparation of cell lysates, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and western blotting have been described elsewhere.<sup>17, 18</sup> Antibodies used are reported in online supplementary table S1.

### **Measurement of cell migration and invasion**

Subconfluent Huh-7 or HepG2 were serum-starved for 24 h and then washed, trypsinized and resuspended in serum-free medium containing 1% albumin at a concentration of  $3 \times 10^5$  cells/mL. Chemotaxis and chemoinvasion were measured in modified Boyden chamber equipped with 8  $\mu$ m pore filters (Millipore

Corp, Massachusetts, USA) and coated with rat tail collagen (20 µg/mL) (Collaborative Biomedical Products, Bedford, USA) or Matrigel (150 µg/mL) (BD Biosciences, Massachusetts, USA), to be used for chemotaxis and chemoinvasion assays, respectively.<sup>17</sup> At least 10 fields per filter were counted.

### **Immunofluorescence**

Cells were placed on glass coverslips after transfection and 72 h later immunofluorescence was performed as previously described<sup>15</sup> with the antibodies indicated in online supplementary table S1.

### **Xenograft model of HCC in mice**

In the first set of experiments, we used Huh-7 cells infected with lentiviral vectors encoding for ERK5 shRNA or non-targeting shRNA prepared as described above.  $8.5 \times 10^5$  cells were resuspended in DMEM and injected subcutaneously into the right flank of 6-week-old female athymic Nude Nu/nu mice (Harlan Laboratories, Indianapolis, Indiana, USA). Fifteen mice were injected with control cells and 16 with cells silenced for ERK5. Animal health was monitored daily and in case of appearance of a palpable tumour, its size was measured every 2–3 days with a calliper. Tumour volumes were calculated using the formula  $V = 4/3\pi (1/2 \text{ length} \times 1/2 \text{ width} \times 1/2 \text{ depth})$ .

In a second set of experiments,  $8.5 \times 10^5$  unmodified Huh-7 cells were resuspended in DMEM and injected subcutaneously as described above into 14 athymic Nude Nu/nu mice (Harlan Laboratories, Indianapolis, Indiana, USA). At appearance of a palpable tumour (2–3 mm diameter), mice were randomised into two groups. The XMD8-92 group was treated with XMD8-92 at the dose of 50 mg/kg twice daily, intraperitoneally. The control group received injections of the same volume of carrier solution (30% cyclodextrine) with the same frequency. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) and experiments were performed after permission of local authorities.

### **BrdU staining**

In vivo bromodeoxyuridine (BrdU) labelling was performed by intraperitoneal injection of 50 mg/kg BrdU (Sigma-Aldrich, Missouri, USA), 1 h prior to sacrifice. Tumour tissues were fixed overnight in 4% neutral buffered paraformaldehyde, paraffin embedded and cut into 3 µm sections. BrdU staining was performed according to the manufacturer's instructions (Dako, Germany). The number of positive cells in five microscopic fields were counted.

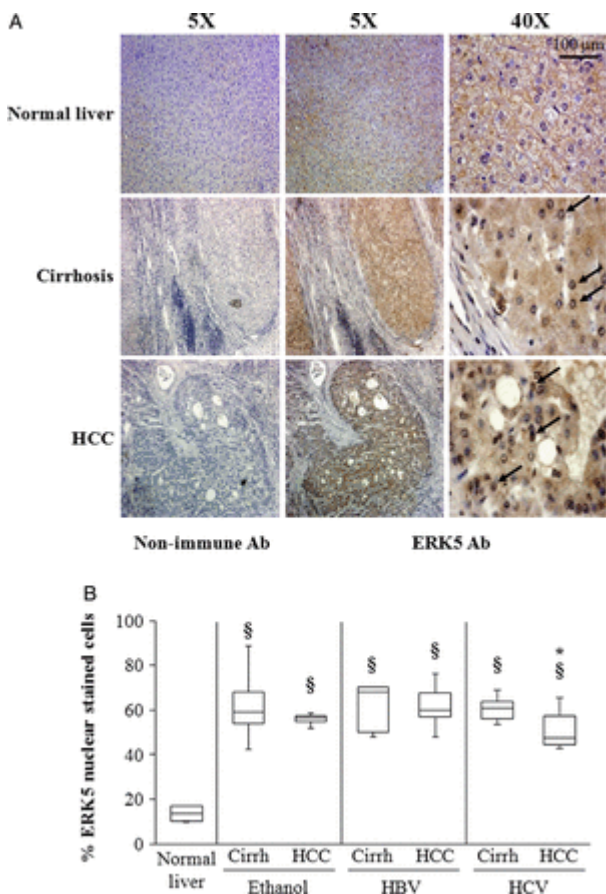
### **Statistical analysis**

Autoluminograms and immunofluorescence images are representative of at least three experiments with comparable results. All bar graphs show the combined results of three independent experiments. Statistical analysis of the data was performed by two-tailed Student's t test, analysis of variance or Fisher's exact test, as appropriate. p Values lower than 0.05 were considered significant.

## Results

### Expression of ERK5 in human HCC

We first analysed the expression pattern of ERK5 in human tissue obtained from patients with cirrhosis and HCC, and compared it with the one in normal liver tissue. The characteristics of the patients studied are reported in online supplementary table S2. Aetiology of cirrhosis was alcoholic liver disease or chronic viral hepatitis (HCV or HBV-related). Using immunohistochemistry, staining for ERK5 was found in all samples, regardless of the presence of chronic injury and/or cancer (figure 1A). Since ERK5 nuclear localisation is associated with the expression of specific targets, we further investigated the subcellular distribution of ERK5. A cytoplasmic staining was evident in hepatocytes in all specimens. On the other hand, a marked increase in the percentage of cells showing nuclear localisation of ERK5 was found in both HCC and in the surrounding cirrhotic tissue obtained in the same patients (figure 1B). No differences were found comparing G1 (seven cases) and G2 (nine cases) grade HCC (data not shown). Higher nuclear ERK5 localisation was not associated with increased ERK5 phosphorylation at MEK5 consensus sites, as determined by western blotting (data not shown).



**Figure 1**

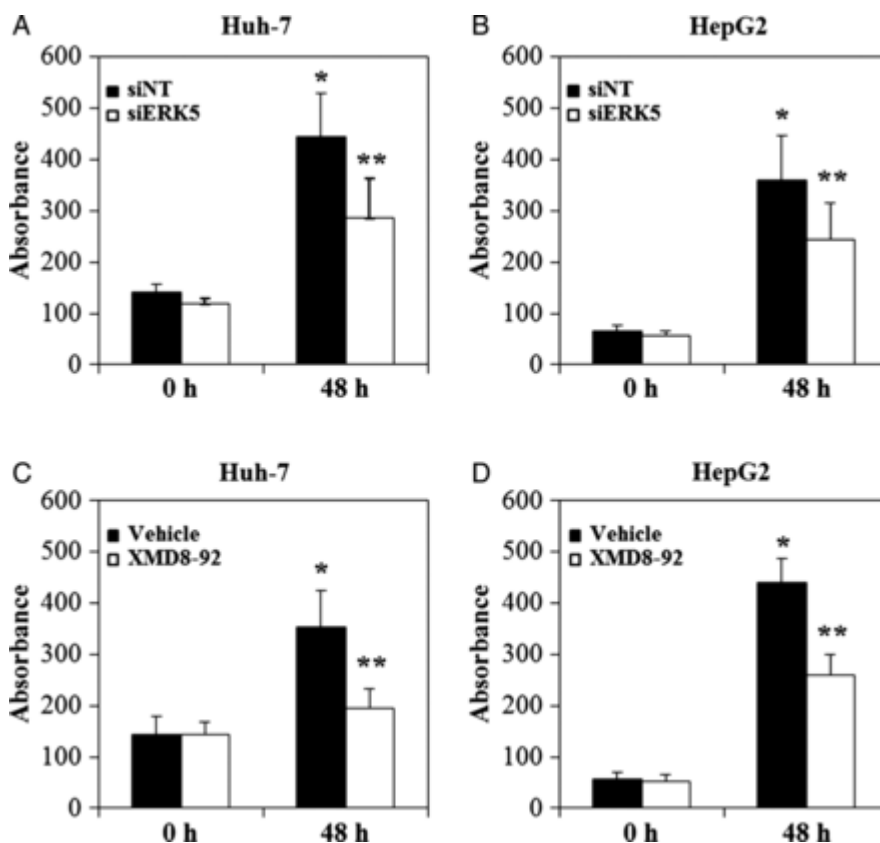
Extracellular signal-regulated kinase 5 (ERK5) expression and subcellular localisation in human liver tissue. (A) Representative immunohistochemistry analysis performed in normal liver tissue or in tissue from patients with cirrhosis and hepatocellular carcinoma (HCC). Immunostaining for ERK5 (ERK5 Ab) is indicated by brown colour. Examples of nuclear ERK5 staining are indicated by arrows. Serial slides were incubated with secondary antibody alone (non-immune Ab). Original magnification and scale bar as indicated. (B) Box plots showing the percentage of cells with nuclear localisation of ERK5 in normal liver tissue (n=4) or in patients with HCC arising in cirrhosis (ethanol group, n=4; HBV group, n=5; HCV group, n=7). \*p<0.05 vs cirrhosis within the HCV group; §p<0.005 versus normal.



## ERK5 modulates proliferation and cell cycle progression in HCC cells

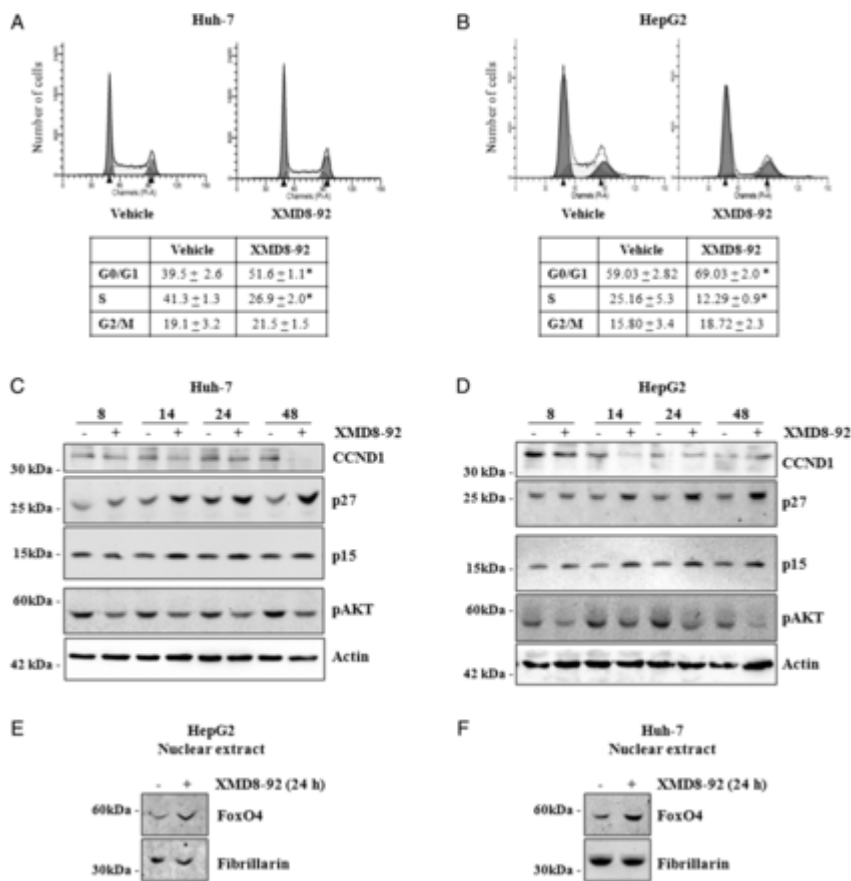
We used two well-characterised cell lines, namely Huh-7 and HepG2. In both these cell lines, nuclear ERK5 staining was detectable in complete culture medium and in conditions of serum starvation, thus recapitulating the observations made in human samples with HCC (see online supplementary figure S1). To establish the biological role of ERK5, we used two different and complementary experimental approaches (see online supplementary figure S2). Gene silencing with ERK5-specific siRNA resulted in an average 60% reduction in protein levels (see online supplementary figure S2A,B). Moreover, pharmacological inhibition of ERK5 kinase activity was carried out using XMD8-92.<sup>19</sup> This compound was able to prevent phosphorylation-dependent retardation of electrophoretic ERK5 migration, induced by agonists such as epidermal growth factor (EGF) (see online supplementary figure S2C). Moreover, the increase in ERK5 kinase activity induced by FBS was completely blocked by this compound (see online supplementary figure S2D).

Time-dependent increase in the number of HCC cells cultured in complete medium was significantly reduced upon ERK5 knockdown in Huh-7 or HepG2 cells (figure 2A, B). Similar results were obtained when the kinase activity of ERK5 was inhibited by XMD8-92 (figure 2C, D). The relevance of ERK5 in the control of proliferation in HCC cells was further investigated monitoring the effects of ERK5 inhibition on the cell cycle (figure 3). Treatment with XMD8-92 resulted in a reduction of the percentage of cells in the S phase and a concomitant increase in the proportion of cells in G0/G1 phase (figure 3A, B). In line with these observations, genetic inhibition of ERK5 also determined a modest, but significant increase in the percentage of cells in G0/G1 phase (see online supplementary figure S3).



**Figure 2**

Effect of extracellular signal-regulated kinase 5 (ERK5) genetic and pharmacological inhibition on the proliferation of hepatocellular carcinoma cell lines. Huh-7 (A) or HepG2 (B) cells were transfected with ERK5 specific (white columns) or non-targeting (black columns) siRNA. Twenty-four hours after transfection, cells were serum-starved for 24 h and incubated in the presence of 10% fetal bovine serum (FBS) for further 48 h. Huh-7 (C) or HepG2 (D) were serum-starved for 24 h and then incubated in 10% FBS and either vehicle (black columns) or 10  $\mu$ M XMD8-92 (white columns) for further 48 h. (A–D) MTT assay was performed after starvation (0 h) or at the end of treatments (48 h). Data are from three independent experiments performed in triplicate. \* $p$ <0.05 versus control at 0 h; \*\* $p$ <0.05 versus control at 48 h.



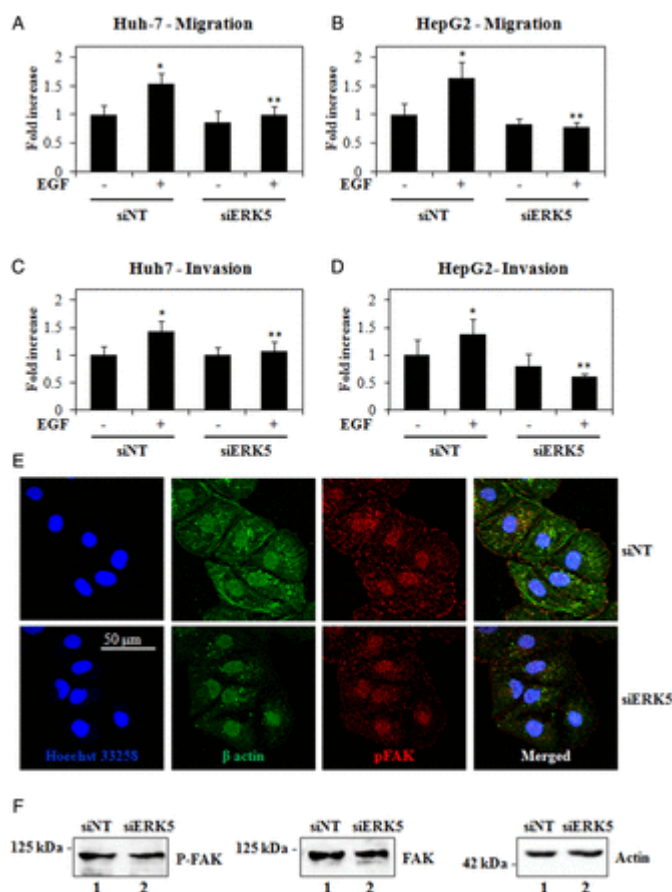
**Figure 3**

Effects of pharmacological extracellular signal-regulated kinase 5 (ERK5) inhibition on the cell cycle phase distribution and on expression of cell cycle regulators in hepatocellular carcinoma cell lines. Huh-7 (A) or HepG2 (B) cells were serum-starved for 24 h and then incubated in the presence or absence of 10  $\mu$ M XMD8-92 for 45 min. Ten per cent fetal bovine serum (FBS) was then added and cells incubated for an additional 48 h. Cell cycle phase distribution was determined by flow cytometry. Data below the graphs are mean  $\pm$  SEM from three independent experiments (\* $p$ <0.05 vs vehicle). Huh-7 (C) or HepG2 (D) cells were serum-starved for 24 h and then incubated in the presence or absence of 10  $\mu$ M XMD8-92 for 45 min. Ten per cent FBS was then added and cells incubated for the indicated times. Western blot analysis was performed using the indicated antibodies. Migration of molecular weight markers is indicated on the left. Nuclear extracts from Huh-7 (E) or HepG2 (F) cells treated as in panels C–D for 24 h were analysed by western blotting with the indicated antibodies.

ERK5 inhibition with XMD8-92 was associated with a decreased expression of cyclin D1 (figure 3C, D), primarily involved in the G1/S transition.<sup>20</sup> In contrast, no major changes in the expression of cyclin E, cyclin A or of the transcription factor E2F1 were observed (data not shown). Furthermore, we found a marked increase in the expression of the cyclin-dependent kinase inhibitors, p27 and p15 (figure 3C, D). These changes induced by the ERK5 inhibitor are likely responsible for the observed block of G1/S transition. On the other hand, no signs of apoptosis were detected even after prolonged ERK5 inhibition, as indicated by the lack of a pre-G0/G1 peak and of increased caspase 3 activation or annexin V staining (figure 3A, B, see online supplementary figures S3–4). We also found that ERK5 inhibition is associated with decreased phosphorylation of AKT (protein kinase B), together with an increased expression of nuclear FoxO4 (figure 3C, F). The AKT/FoxO pathway has been clearly shown to regulate expression of p27 and p15.<sup>21</sup>

### Involvement of ERK5 in cell migration and invasion in response to soluble mediators and hypoxia

To further explore the possible contribution of ERK5 to an aggressive phenotype of HCC cells, we investigated the effects of ERK5 silencing or inhibition of its activity, on the capacity of HCC cells to migrate or invade through a basement membrane-like matrix. ERK5 silencing reduced the ability of soluble mediators such as EGF to induce migration in both cell lines analysed (figure 4A, B). Moreover, agonist-induced increase in HCC cell chemoinvasion was significantly limited upon depletion of ERK5 (figure 4C, D). Of note, comparable results were obtained when the activity of ERK5 was pharmacologically inhibited with XMD8-92 (see online supplementary figure S3).



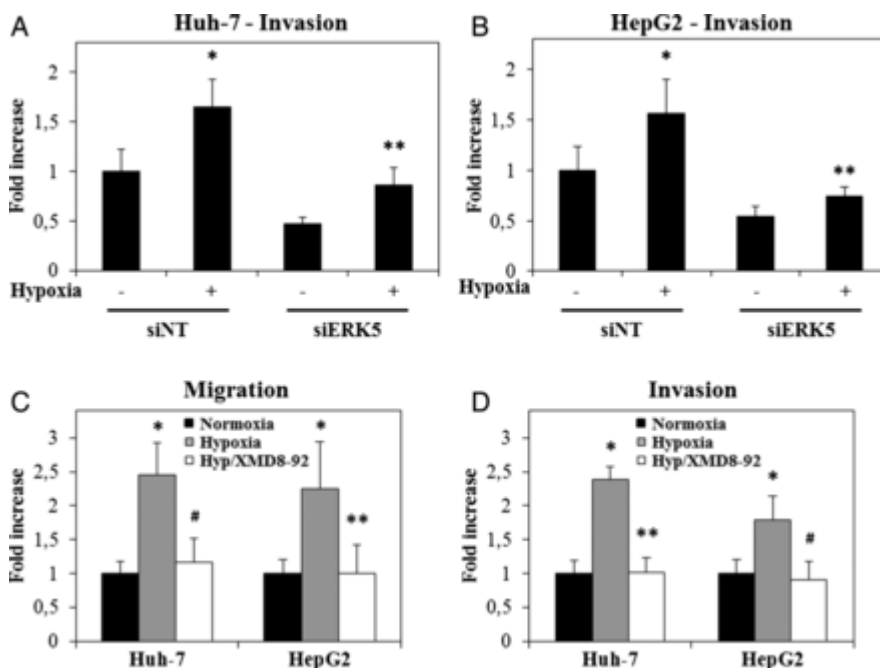
**Figure 4**

Effect of ERK5 genetic inhibition on Huh-7 and HepG2 motility and invasiveness. Huh-7 or HepG2 cells, as indicated, were transfected with non-targeting control siRNA (siNT) or with siRNA specific for ERK5 (siERK5). After 48 h, cells were washed and serum-starved for additional 24 h and detached from the culture dish. Migration (A and B) or invasiveness (C and D) in the presence

or absence of 100 ng/mL EGF was measured in modified Boyden chambers. Bar graphs represent the mean±SEM of three independent experiments. \* $p < 0.05$  versus unstimulated cells transfected with siNT; \*\* $p < 0.05$  versus EGF-treated cells transfected with siNT. Cells transfected as above, and after 72 h in complete medium were subjected to immunofluorescence (E) or western blotting (F) using the indicated antibodies. In (E), nuclei were stained with Hoechst 33258. ERK5, extracellular signal-regulated kinase 5; EGF, epidermal growth factor; FAK, focal adhesion kinase.

Cell migration requires a coordinated structural change of the cytoskeleton and highly dynamic reorganisation of focal contacts. We determined whether the reduced motility observed in ERK5 silenced cells was associated with cytoskeletal remodelling. Immunofluorescence staining of Huh-7 cells showed that after transfection of non-targeting siRNA, cells exhibited an organised microfilament network with long-actin fibres throughout the cell body, with focal contacts located preferentially at the periphery of the cell. Following ERK5 depletion by siRNA, a marked disorganisation of actin fibres and redistribution of focal contacts to the cell body was observed, as demonstrated by staining for phosphorylated focal adhesion kinase (FAK) (figure 4E). These evident changes induced by ERK5 silencing are compatible with a less motile phenotype. No changes in the total amount of phosphorylated or total FAK or  $\beta$ -actin were observed, indicating that ERK5 mainly affected redistribution of involved proteins (figure 4F).

Next, we investigated the role of ERK5 in modulating the invasive phenotype of HCC cells in response to hypoxia, a feature of chronic liver disease known to affect the behaviour of these cells.<sup>22, 23</sup> Hypoxia induced an evident stimulation of chemoinvasion (figure 5) in Huh-7 or HepG2 cells, as previously reported.<sup>23</sup> ERK5 knockdown by siRNA markedly and significantly inhibited the effects of hypoxia (figure 5A, B). To strengthen the evidence supporting an involvement of ERK5 in this important feature of HCC cells, migration and chemoinvasion were analysed in the presence of XMD8-92. Pharmacological inhibition of ERK5 activity completely prevented hypoxia-induced migration and invasiveness in both cell lines (figure 5C, D).



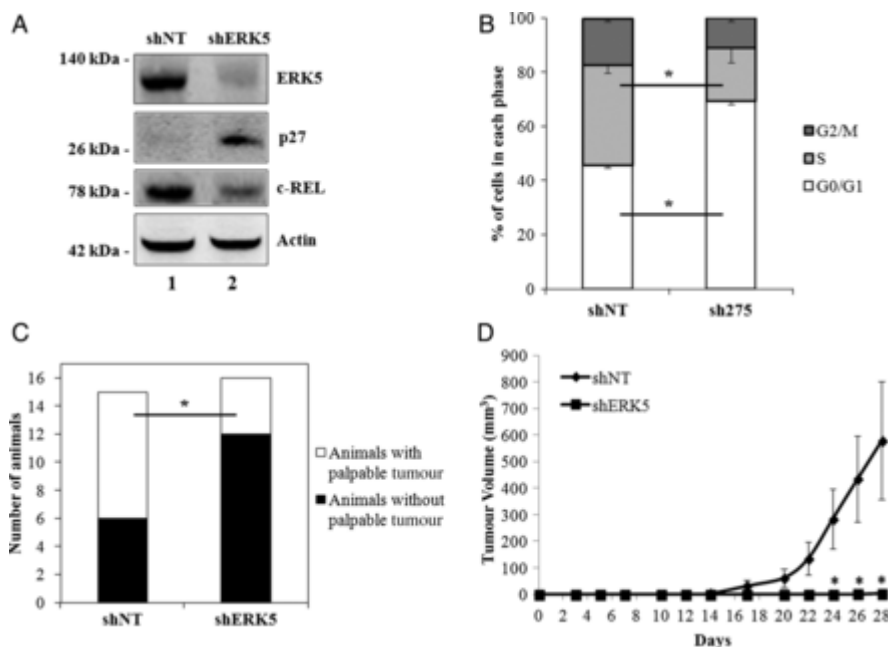
**Figure 5**

Effect of extracellular signal-regulated kinase 5 (ERK5) inhibition on hypoxia-stimulated cell motility. Cultured Huh-7 (A) or HepG2 (B) cells were transfected with non-targeting control siRNA (siNT) or with siRNA specific for ERK5 (siERK5). After 48 h cells were deprived of serum for 24 h and then detached from the culture dish. Invasiveness in condition of normoxia or hypoxia (3% O<sub>2</sub>) was measured in modified Boyden chambers. \* $p < 0.05$  versus normoxic condition, \*\* $p < 0.05$  versus hypoxic condition/siNT. (C, D) Cultured Huh-7 or HepG2 were serum-starved for 24 h and treated with vehicle or 10  $\mu$ M XMD8-92 for 1 h. Cells were then

detached from the culture dish, and migration (C) or invasiveness (D) in condition of normoxia (black columns), hypoxia (3% O<sub>2</sub>, grey columns) or hypoxia in the presence of 10 μM XMD8-92 (white columns) was measured in modified Boyden chambers., \*p<0.05 versus normoxia; \*\*p<0.05 or #p<0.01 versus hypoxic condition/vehicle.

### ERK5 silencing limits tumour development in vivo

Based on the strong in vitro data indicating the involvement of ERK5 in the regulation of functions of HCC cells, relevant for tumour progression, we evaluated the possible impact of this pathway in an in vivo model of HCC. To do this, we established Huh-7 cells with long-term ERK5 silencing using lentiviral vectors encoding for ERK5-targeting shRNA (figure 6A). This strategy was found to effectively silence ERK5 for as long as 40 days in culture even in the absence of puromycin, previously used to select infected cells (data not shown). ERK5 knockdown was associated with an increase in p27 expression, in keeping with the previously shown effects of XMD8-92 (figure 3C, D). Moreover, screening for possible oncogenic targets of ERK5 in HCC cells showed a marked inhibition of c-Rel expression in cells silenced for ERK5 (figure 6A), thus identifying a novel target for this pathway. ERK5 silencing with shRNA determined a marked increase in the percentage of cells in G0/G1 phase, in keeping with data obtained with siRNA (figure 3A, B). Twenty-eight days after subcutaneous injection of Huh-7 cells, 60% of mice in the control group had a palpable tumour compared with only 25% of mice injected with ERK5-silenced Huh-7 (figure 6C). Remarkably, the size of tumours developing in mice receiving ERK5-silenced cells remained substantially stable (mean volume 4.7±1.6 mm<sup>3</sup> at the end of the experiment), whereas the mean volume was nearly 100-fold higher in mice injected with mock-infected cells (figure 6D).

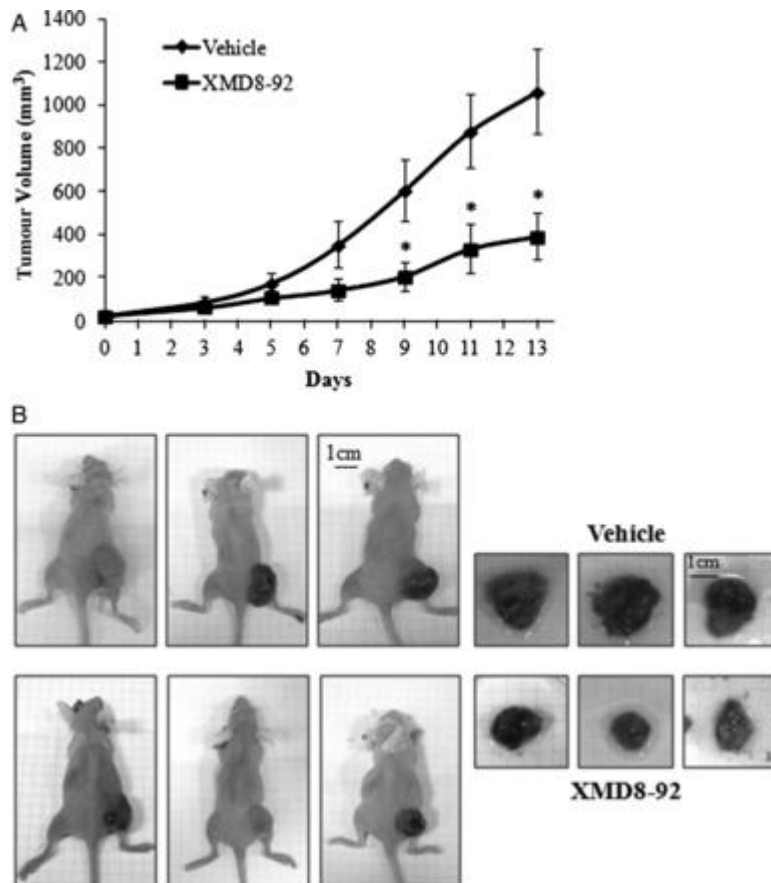


**Figure 6**

Effect of extracellular signal-regulated kinase 5 (ERK5) silencing on Huh-7 xenograft tumour formation. (A) Huh-7 cells were infected with lentiviral vectors expressing shRNA against ERK5 (shERK5) or non-targeting shRNA (shNT). Five days post-transduction and 3 days after selection with puromycin, cells were harvested, lysed and analysed by western blotting with the indicated antibodies. (B) Cells treated as indicated above were serum-starved for 24 h and then incubated in complete medium for 48 h. Cell cycle phase distribution was determined by flow cytometry. Data in graph are mean±SEM from three independent experiments (\*p<0.05 vs shNT). (C) Rate of tumour development after injection of cells infected with shNT or shERK5. Data are presented as the number of animals with (white portion) or without (black portion) the appearance of a palpable tumour. (\*p<0.05 vs shNT group). (D) Tumour volumes were measured in mice injected with shNT or shERK5 Huh-7 cells, as indicated above. Tumour volumes are expressed as mean±SEM (n=10 in the shNT group and n=7 in the shERK5 group).

## An antagonist of ERK5 activity reduces tumour growth and limits expression of oncogenic genes

We next explored the effects of XMD8-92 on the growth of established HCC xenografts. After appearance of a palpable tumour, nude mice bearing Huh-7 xenografts were randomised to receive XMD8-92 or vehicle for 13 days. Significant differences in tumour volumes between XMD8-92-treated and control groups were observed starting from day 9 after the initiation of treatment (figure 7A, B). At the end of treatment, XMD8-92 significantly inhibited tumour growth, with a 60% reduction in final volume compared with control mice. No significant differences in body weight were observed in the two groups of mice and no toxicity was detected (data not shown).

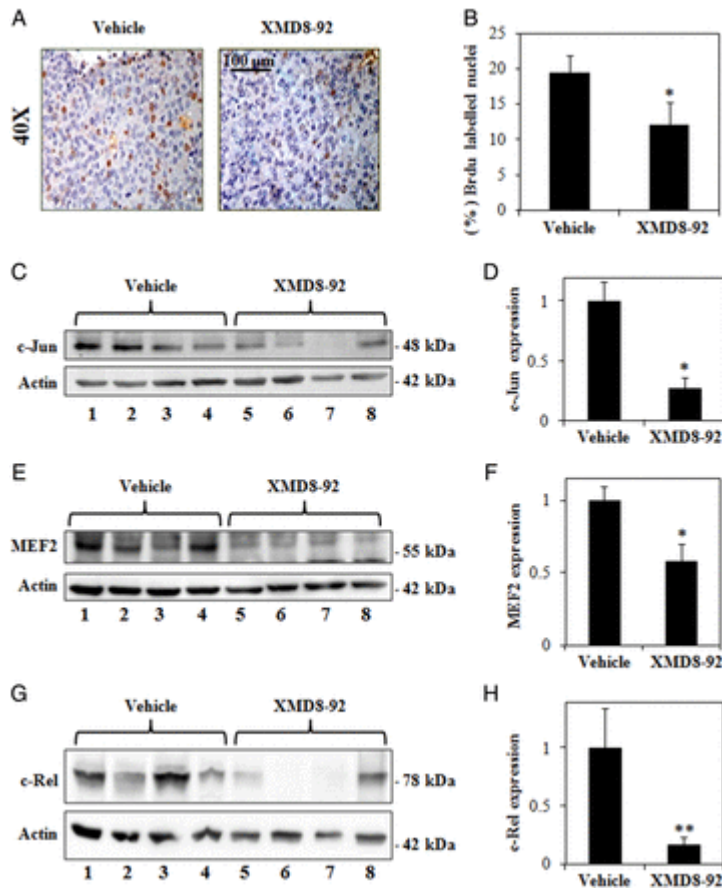


**Figure 7**

XMD8-92 reduces the growth of Huh-7 xenografts in nude mice. Nude mice were injected with unmodified Huh-7 and followed until development of a palpable tumour. Mice were then randomised to receive 50 mg/kg XMD8-92 or its carrier solution twice daily, intraperitoneally. (A) Tumour volumes are expressed as mean $\pm$ SEM (n=7 in each group). \*p<0.05 versus vehicle. (B) Representative mice from each group showing tumours at the end of the experiment.

To assess whether the lower tumour volume was associated with reduced cell proliferation in XMD8-92-treated HCC xenografts, we measured BrdU uptake in vivo. The number of BrdU-positive cells was significantly lower in mice treated with XMD8-92 than in vehicle-treated animals (figure 8A, B). These findings indicate that ERK5 inhibition is associated with reduced proliferation of HCC cells in vivo. Analysis of xenografts obtained from XMD8-92-treated mice also showed that the expression of c-Jun, a proto-oncogene essential for hepatocyte proliferation,<sup>24</sup> was markedly reduced compared with control samples (figure 8C, D). Moreover, we found a decreased expression of MEF2 (figure 8E, F), belonging to a family of transcription factors with important roles in early gene responses to stimulation with growth factors and stressors.<sup>25</sup> In addition, reduced MEF2 expression is a direct proof of the effective inhibition of ERK5 activity

in tumour xenografts. In fact, MEF2 is a direct target of ERK5 kinase activity and the resulting phosphorylation is required for c-Jun induction.<sup>26</sup> In line with the in vitro data (figure 6A), XMD8-92 treatment strongly decreased the expression of the transcription factor c-Rel (figure 8E), the deregulated activity of which has been associated with oncogenic cell growth.<sup>27</sup> In contrast, the levels of apoptosis were not significantly different comparing mice treated with XMD8-92 or vehicle, as indicated by caspase 3 or poly(ADP-ribose) polymerase cleavage (not shown).



**Figure 8**

Effects of XMD8-92 on proliferation and protein expression in Huh-7-xenografted cells. Data were obtained at the end of the experiment described in figure 7. (A) Representative bromodeoxyuridine (BrdU) labelling in sections obtained from Huh-7 tumour xenografts in mice treated with XMD8-92 or vehicle. The line mark corresponds to 100  $\mu$ m. (B) Quantification of BrdU-positive cells from the experiment reported in A (Mean $\pm$ SEM) (\* $p$ <0.05 vs vehicle;  $n$ =5 in each group). (C, E and G) Tumours were lysed and analysed by western blotting with the indicated antibodies. (D, F and H) Relative protein expression was determined by densitometric analysis. (\* $p$ <0.05, \*\* $p$ <0.05 vs vehicle,  $n$ =7 in each group).

## Discussion

In spite of remarkable advances in the diagnosis and treatment of HCC, this tumour remains one of the most difficult to treat, and the prognosis of the patients remains poor in a large proportion of cases. Several factors contribute to this dismal outlook, including that HCC develops in the large majority of cases on the background of chronic liver disease, especially cirrhosis, which limits the applicability of surgery and other ablative techniques. In addition, information on possible molecular targets in HCC has not yet translated into effective treatments and only sorafenib, a multikinase inhibitor, is currently approved for the

treatment of advanced HCC. Based on these considerations, there is an urgent need to identify novel molecular targets, possibly with the potential to be translated into clinical practice.

In this study, we provide compelling *in vitro* and *in vivo* evidence indicating that the MAPK ERK5 regulates the biology of HCC. ERK5 is an oncogene and is expressed at high levels in the liver. Moreover, the *MAPK7* gene, encoding for ERK5, was found to be amplified in human HCC specimens and cell lines.<sup>9,10</sup> Nonetheless, limited functional information on its possible role in HCC has been reported. Using two well-established cellular models, our *in vitro* data indicate that inhibition of the ERK5 pathway reduces the proliferation of Huh-7 and HepG2 cells, and that ERK5 activity is necessary for progression in the cell cycle. In addition, ERK5 was critical for the regulation of motility and chemoinvasion in both HCC cell lines, indicating a possible role in controlling local spread and metastatic potential. These functional data were accompanied by the observation that knockdown of ERK5 or inhibition of its enzymatic activity are associated with cytoskeletal remodelling and disruption of focal adhesions, in a fashion that is compatible with a less motile phenotype. Indeed, ERK5 genetic inhibition determined a redistribution of the phosphorylated form of FAK, normally located at focal adhesion sites, from the periphery to the central body of the cell. The involvement of ERK5 in cell motility has been attributed to downstream effects on the FAK pathway, which has a well-established role in cell migration and cytoskeletal remodelling. In most, but not all experimental systems,<sup>15,28</sup> enhanced FAK signalling promotes cell motility, whereas inhibition of FAK signalling through a variety of approaches impairs cell migration (reviewed in refs. 29, 30). A direct link between ERK5 and FAK has been demonstrated by the observation that ERK5 phosphorylates FAK in S910, thus promoting cell migration.<sup>31,32</sup> On the other side, ectopic expression of dominant negative ERK5 decreased FAK Y397 phosphorylation, which is necessary for Src recruiting and phosphorylation, cell adhesion and haptotactic motility on vitronectin.<sup>33</sup>

Hypoxia is a common hallmark of solid cancers and is associated with treatment failure and poor prognosis.<sup>34</sup> On the other hand, hypoxia is a physiological condition in the liver and a common occurrence in the setting of chronic injury. Several studies have shown that hypoxia can induce a mesenchyme-like phenotype in HCC cells, particularly with the acquisition of a more motile phenotype.<sup>23</sup> ERK5 phosphorylation is increased by hypoxia<sup>35</sup> and ERK5 regulates the expression of hypoxia-inducible factor-1 $\alpha$ , indicating a complex cross-talk in response to hypoxia.<sup>36</sup> ERK5 is also implicated in the epithelial to mesenchymal transition in hepatocytes in response to transforming growth factor- $\beta$ .<sup>37</sup> The identification of a role of ERK5 in hypoxia-induced motility is a novel finding that indicates the regulation of a particularly relevant mechanism of progression in solid tumour local invasion and progression.

*In vitro* experiments have also allowed us to obtain novel information on downstream targets of ERK5, possibly relevant to the biology of HCC. ERK5 inhibition blocked transition from G0/G1 to the S phase and this was associated with decreased expression of cyclin D1, a well-known target of the ERK5 pathway.<sup>38</sup> On the other hand, treatment with XMD8-92 increased the expression of p27, a cell cycle inhibitor recently reported to be regulated by ERK5.<sup>39,40</sup> Moreover, we found that ERK5 inhibition determined an increase in the expression of p15, another negative regulator of the transition from G0/G1 to S phase,<sup>41</sup> not previously connected to the ERK5 pathway. A possible mechanism linking ERK5 inhibition with increased expression of p27 and p15 is represented by the observed reduction of AKT phosphorylation, and the resulting increased expression of nuclear FoxO4. Indeed, the AKT/FoxO pathway has been shown in other systems to regulate p27 and p15 expression.<sup>21</sup> Moreover, these data confirm the existence of a link between ERK5 and the FoxO family previously found in other cellular systems.<sup>42</sup>



We also found that c-Rel, a transcription factor of the nuclear factor (NF)- $\kappa$ B family, is downregulated following ERK5 inhibition. Previous studies identified a role of ERK5 in the positive regulation of NF- $\kappa$ B activity.<sup>43,44</sup> However, our results provide the first evidence of a specific link between ERK5 and c-Rel. c-Rel regulates many biological processes including cell proliferation in liver cells,<sup>45</sup> and a high c-Rel expression has been linked to HCV-induced HCC.<sup>46</sup> Thus, downregulation of c-Rel identifies an additional molecular mechanism by which the ERK5 pathway could interfere with HCC cell proliferation.

We evaluated the expression pattern of ERK5 in human specimens collected from patients undergoing resection or transplantation for HCC. These data confirmed the abundant cytoplasmic expression of ERK5 in normal liver and in conditions of injury, as we examined HCC and the neighbouring non-neoplastic tissue from the same patients, where cirrhosis was constantly present. Remarkably, nuclear localisation of ERK5 was significantly higher in both HCC and cirrhotic tissue compared with normal liver. The presence of nuclear ERK5 overexpression is strongly associated with poor prognosis in patients with prostate cancer.<sup>47</sup> Unlike other members of the MAPK family, ERK5 phosphorylation by MEK5 is not a prerequisite for its nuclear entry.<sup>48</sup> Accordingly, phosphorylation of ERK5 on the MEK5 consensus site was not increased comparing diseased (either HCC or cirrhosis) versus normal tissue or HCC versus cirrhotic tissue, indicating that the presence of ERK5 in the nucleus in this context is not related to increased MEK5-dependent phosphorylation. It should be considered that ERK5 may be phosphorylated by MEK5-independent pathways.<sup>49</sup> Regardless of the mechanism, the presence of ERK5 within the nucleus is linked to transcriptional activation of genes necessary to cell proliferation via direct interaction with regulatory elements of target genes or by phosphorylation of transcription factors through ERK5 kinase activity. Taken together, these results indicate that the ERK5 pathway should be targeted at the level of ERK5 kinase activity, for example, with compounds such as XMD8-92.

An intriguing finding is that nuclear staining for ERK5 was similar comparing HCC and the surrounding cirrhotic tissue. Based on these results and on the fact that cirrhosis is a strong preneoplastic condition, we may speculate that ERK5 nuclear localisation is an early event in the series of processes leading to appearance of HCC in cirrhosis and that ERK5 activation could eventually be targeted to prevent the occurrence or recurrence of HCC. In this context, the possible presence or absence of nuclear ERK5 in defined preneoplastic states such as dysplastic nodules needs to be addressed in future studies. Along these lines, gene signature in surrounding cirrhotic tissues was found to be the strongest predictor of recurrence in a series of patients undergoing resection for HCC.<sup>50</sup>

As the *in vitro* biological actions of HCC cells only partially recapitulate the behaviour of cancer *in vivo*, we also tested the effects of interference with ERK5 on tumour development in a mouse model of HCC xenograft, using, also in this case, both a genetic and a pharmacological approach. Mice injected with cells silenced for ERK5 with shRNA developed tumours less frequently and their volume was markedly and significantly lower than the one measured in mice receiving mock-infected cells. These results were nicely complemented by those obtained administering the ERK5 inhibitor XMD8-92 to mice harbouring small tumours, the eventual growth of which was markedly limited by drug treatment. It should be emphasised that while genetic and pharmacological interference with ERK5 are clearly concordant in indicating a role of this kinase, their significance at the molecular level is not completely overlapping. In fact, ERK5 regulates a number of genes by direct interaction, independent of its kinase activity. Therefore, downregulation of the whole molecule may lead to a cellular reprogramming at least partially different from the one obtained by kinase inhibition. Future studies are needed to obtain additional information on this intriguing aspect of ERK5 biology. Taken together, data from this study strongly indicate that ERK5 is implicated in the biology of HCC cells *in vivo* and *in vitro*. Of particular interest is the fact that most of these data were obtained

using a specific pharmacological inhibitor, and comparable with genetic interference, indicating the potential translatability of this study to a clinical setting. As targeted therapy for HCC is still in its infancy, identification of novel targets, including ERK5, is of pivotal relevance for the eventual development of new therapeutic strategies.

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## Footnotes

- ER and GDM contributed equally.
- Contributors ER and GDM contributed equally to the work. ER, GDM and FM conceived and designed experiments. ER, GDM, IT, NN, CP, SC and EV carried out experiments and acquisition of data analysis/interpretation of results. ER, GDM and FM wrote the manuscript. AP, PDS and MP revised the manuscript. XD and NSG provided reagents and contributed to planning animal experimentation. ED evaluated and provided human specimens.
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- Competing interests Dana Farber Cancer Institute, the employer of NSG, holds the right of the compound XMD8-92, used in the present study. No other competing interests.
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