Research paper

Microtubule associated protein 9 inhibits liver tumorigenesis by suppressing ERCC3

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ABSTRACT

Background: Chromosomal instability plays an important part in cancer, but its genetic basis in liver tumorigenesis remains largely unclear. We aimed to characterize the mechanistic significance and clinical implication of mitotic regulator microtubule-associated protein 9 (MAP9) in hepatocellular carcinoma (HCC).

Methods: The biological functions of MAP9 were determined by in vitro tumorigenicity assays. Systematic MAP9 knockout mouse (MAP9Δ/Δ) and hepatocyte-specific MAP9 knockout mouse (MAP9Δhep) were generated to confirm the role of MAP9 in HCC. The clinical impact of MAP9 was assessed in primary HCC tissue samples.

Findings: We found that MAP9 was frequently silenced in HCC tissue samples. The transcriptional silence of MAP9 in liver cancer cell lines and tissue samples was mediated by its promoter hypermethylation. MAP9 promoter hypermethylation or downregulation was associated with poor survival and recurrence in patients with HCC. Mechanistically, ectopic expression of MAP9 in LO2 and HepG2 cell lines impaired cell proliferation, colony formation, migration and invasion, and induced cell apoptosis and cycle arrest, whereas knockdown of MAP9 in Miha cell line showed the opposite effects. We found that MAP9Δhep mice spontaneously developed a liver hyperplastic nodule and MAP9Δhep accelerated diethylnitrosamine-induced HCC formation. The tumour suppressive effect of MAP9 in HCC was mediated by downregulating excision repair cross-complementation group 3 (ERCC3), a nucleotide excision repair gene. Restoration of ERCC3 expression possessed an oncogenic potency and abrogated the tumour suppressive effects of MAP9.

Interpretation: MAP9 is a novel tumour suppressor in HCC by inhibiting ERCC3 expression, and serves as a prognostic factor in HCC patients.

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In the present study, we identified that MAP9 was commonly silenced in liver cancer cell lines and tissue samples due to its promoter hypermethylation. MAP9 promoter hypermethylation harboured a robust association with poor survival and tumour recurrence in patients with HCC. Moreover, knockout of MAP9 (MAP9ΔΔ) in mice elicited spontaneous liver hyperplastic nodule formation and hepatocyte-specific MAP9 knockout facilitated diethylnitrosamine (DEN)-induced HCC. Mechanistically, loss of MAP9 expression promoted liver cancer cell growth by inducing apoptosis escape and cell cycle progression and reviving invasive potential through upregulating ERCC3 expression.

### 2.2. Tissue microarray and immunohistochemistry

Tissue microarray (TMA), which was generated from formalin-fixed and paraffin-embedded archived tissue samples of 22 patients with liver cancer prior to radiotherapy or chemotherapy, was purchased from Shanghai Outdo Biotech Co., LTD (Shanghai, China). Immunohistochemistry (IHC) for MAP9 was performed on paraffin sections of TMA using anti-MAP9 antibody (HPA037864, 1:200, Sigma-Aldrich). The extent of MAP9 cytoplasmic staining was evaluated by H-score, as previously described [14]. IHC analysis of Ki-67 levels was also conducted on paraffin slides of mouse liver and tumour tissues using anti-Ki-67 antibodies (Abcam, Cambridge, UK).

### 2.3. Data mining in the cancer genome atlas (TCGA)

In TCGA-liver cancer, the data regarding paired liver cancer samples (n = 50), and 260 cases of patients with liver cancer including intact overall survival and recurrence time and status, MAP9 or ERCC3 expression level (RNA-seq), MAP9 DNA methylation level (Illumina 450k Infinium methylation beadchip), age, gender, HBV/HCV infection, liver fibrosis, tumour differentiation, pathological stage, TNM staging, tumour size and distant metastases were downloaded by using the UCSC Xena browser (https://xenabrowser.net/heatmap/).

### 2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted using TRIzol and transcribed into cDNA using a High Capacity cDNA Kit (Applied Biosystems, Foster City, CA). For quantitative PCR analysis, aliquots of cDNA were amplified using SYBR® Premix Ex Taq™ II (Takara Bio Inc, Japan) on LightCycler® 480 Instrument (Roche Diagnostics, Switzerland). Each sample was tested in triplicate. 2−ΔΔCT method was employed to evaluate the fold change in gene expression level. 2−ΔCT method was employed to determine the relative expression levels of corresponding genes. The sequences of primers used were listed in Supplementary Table S1.

### 2.5. Western blot analysis

Protein lysates from cell lines and tissues were prepared using protease inhibitor cocktail (Roche)—containing radioimmunoprecipitation assay (RIPA) buffer. Protein concentration was determined by the DC protein assay method of Bradford (Bio-Rad, Hercules, CA). Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were immunostained with primary antibodies at 4 °C overnight and secondary antibody at room temperature for 1 h. Proteins were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare). The antibodies used in this study were listed in Supplementary Table S2.

### 2.6. Bisulfite treatment of DNA and methylation-specific PCR (MSP) analysis

Genomic DNA was extracted from liver cancer samples and cell lines and then was subjected to bisulfite treatment using CpG Genome DNA Modification Kit (Chemicon, USA). The sequences of primers were indicated in Supplementary Table S1. The band expanded with MSP primers corresponding to the DNA methylation in the promoter...
region was marked as “M”. The band expanded with non-methyla-
tion specific primers was marked as “U”.

2.7. Bisulfite genomic sequencing (BGS)

The PCR products of bisulfite-treated DNA were cloned into the
PCR4-Topo vector (Life Technologies, Carlsbad, CA). 10 colonies were
randomly chosen and sequenced. Sequencing analysis was per-
formed by SeqScape software (Applied Biosystems, Foster City, CA)
and 11 CpG sites spanning in the promoter region of 224 bp were
assessed.

2.8. Demethylation treatment

1 x 10⁶ cells were placed in 100-mm dishes and grown for 24 h.
Cells were then treated with 10 μM of 5-aza-2-deoxycytidine
(5-Aza) (Sigma-Aldrich, St Louis, MO) for 48 h. Culture medium con-
taining 5-Aza was replenished every day.

2.9. Construction of gene expression plasmid

The full-length open reading frame sequence of MAP9 or ERCC3
was obtained by RT-PCR amplification of normal human liver cDNA.
The PCR aliquots were subcloned into the mammalian expression
vector pcDNA3.1 and then verified by DNA sequencing. Overexpression
of MAP9 or ERCC3 in LO2 or HepG2 cells was transfected with
pcDNA3.1, pcDNA3.1-MAP9 or ERCC3 plasmid using lipofectamine
2000 (Life Technologies). Stable transfections were selected for
2 weeks with G418 antibiotics.

2.10. RNA interference and transfection

Knockdown of MAP9 expression in Miha cell line was performed
by a siRNA targeting MAP9. Miha cell line were transfected with
20 nM MAP9 siRNA (siMAP9: 5'-CGU GUC ACG UTT CGU GUC
3'-CGU GUC ACG UTT) (Shanghai, GenePharma) or control siRNA
(siNC: 5'-UUC UCC GAA CGU GUC UTT AT-3') (Shanghai, GenePharma) using lipofectamine
2000. After transfection for 48 h., the cells were ready for further
experiments.

2.11. Cell viability assay

Cell viability was examined by the MTT (3-[4,5-dimethylthiaziol-2-
yl]-2,5 diphenyl tetrazolium bromide) assay (Promega, Fitchburg,
WI). The experiments were performed three times independently.
Results were shown as the means ± SD.

2.12. Colony formation assay

For overexpression assay, LO2 and HepG2 cells (2 x 10⁵ cells/well)
were transfected with MAP9, ERCC3 or empty vector and were plated
in a 6-well plate. For knockdown assay, Miha cells were transfected
with siMAP9 or siNC. After culturing for 7–10 days, cells were fixed
with methanol and stained with crystal violet solution. Colonies with
>50 cells per colony were counted. All experiments were conducted
three times in triplicates.

2.13. Cell apoptosis analysis

Cell apoptosis was determined by staining cells with Annexin V
and 7-amino-actinomycin D (7-AAD) (BD Biosciences) with subse-
quent flow cytometry analysis. Cell populations were counted as via-
bale (Annexin V-negative, 7-AAD-negative), early apoptotic (Annexin
V-positive, 7-AAD-negative), late apoptotic (Annexin V-positive,
7-AAD-positive), or necrotic (Annexin V-negative, 7-AAD-positive).
The experiments were conducted three times in triplicates. In addition,
terminal deoxynucleotidyl transferase-mediated nick-end labelling
(TUNEL) assay (Roche) was employed for apoptosis measurement of
liver or liver tumour biopsies in mice. Nuclei with clear brown stain-
ing were regarded as TUNEL-positive apoptotic cells. The apoptosis
index was calculated as the percentage of TUNEL-positive nuclei after
counting at least 1000 cells.

2.14. Cell cycle analysis

LO2 and HepG2 cells that were transfected with MAP9 or empty
vector were plated in a 6-well plate, while Miha cells were trans-
fected with siMAP9 or siNC. After 48 h of transfection, the cells were
fixed in ice-cold 70% ethanol-phosphate-buffered saline for 24 h
before staining with 50 μg/ml propidium iodide (BD Biosciences,
Franklin Lakes, NJ). The cells were sorted by BD Accuri™ C6 (BD Bio-
sciences), and cell cycle distributions were analysed using the Mod-
FitLT 4.1 software (Verity Software House, Topsham, ME). All
experiments were conducted three times in triplicates.

2.15. Wound-healing assay

Cell migration was assessed using the wound-healing assay.
Briefly, LO2 and HepG2 cells transfected with pcDNA3.1-MAP9 or
pcDNA3.1-MAP9 were cultured in six-well plates. When the cells
reached 80% confluence, three scratch wounds in each well were
made using a P-200 pipette tip. Fresh medium supplemented with
reduced (5%) foetal bovine serum was then added, and the wound-
closure was observed for 72 h. Photographs were taken at 0, 24, 48
and 72 h, respectively.

2.16. Migration and invasion assays

For migration and invasion assays, migration transwell chambers
and matrigel-coated chambers (Becton Dickinson, Waltham, MA,
USA) were used. Briefly, 5 x 10⁵ cells were seeded into the upper
chamber in serum-free culture medium. The lower chamber was
filled with completed medium with 10% FBS. After 16 h for migration
assay and 24 h for invasion assay, cells that have migrated or invaded
through the membrane were stained with crystal violet and counted.

2.17. Immunofluorescence

Cells were seeded onto coverslips in a 6-well plate and transfect-
d with pcDNA3.1 or pcDNA3.1-MAP9. After transfection for 48 h, cells
were fixed with 4% paraformaldehyde and permeabilized with 0.2%
Triton X-100, blocked in 5% BSA in PBS, and incubated with anti-
MAP9 (1:200 dilution, sc-164,961, Santa Cruz) and anti-XBP (1:40
dilution, AB27317, Abcam) overnight at 4 °C, followed by IgG second-
ary antibody conjugated with Alexa Fluor 594 (1:400 dilution, Yea-
sen) in the dark for 1 h. Cells were then mounted with Prolong Gold
Antifade Mountant with DAPI (Life Technologies). Images were cap-
tured using a Carl Zeiss LSM 780 confocal laser scanning microscope
(Carl Zeiss AG).

2.18. RT2 profiler™ PCR array

Mouse Cancer PathwayFinder RT2 Profiler™ PCR array (PAMM-
033ZC, QIAGEN, USA) was utilized for quantitative PCR with the fol-
lowing cycling conditions: 10 min at 95 °C, 15 s at 95 °C, and 1 min
60 °C for 40 cycles with a final 4 °C hold. Five endogenous control
genes, namely, glucuronidase β, hypoxanthine guanine, heat-shock
protein 90, glyceraldehyde phosphate dehydrogenase, and β-actin,
were used for data normalization. Each replicate cycle threshold (CT)
was normalized to the average Ct value of five endogenous controls
per plate. Results were calculated using the 2^-ΔΔCT method. Heat
map was generated using the web-based program of RT2 Profiler™

J. Zhang et al. / EBioMedicine 53 (2020) 102701
PCR array data analysis. Variations in the tumour gene expression between MAP9 knockout mice and wild type mice are shown as a fold of increase or decrease.

2.19. MAP9 knockout (MAP9Δ/Δ) mouse model

MAP9 knockout (MAP9Δ/Δ) mice were generated by working with Beijing Biocytogen Co., Ltd (Beijing, China). A targeted vector was electroporated into embryonic stem cells with C57BL/6 background to generate a recombined MAP9 allele (fl-neo). Following selection and identification by PCR and southern blot, positive clones were injected into mouse blastocysts to generate chimerae mice, which were crossed with Rosa26-Flpe mice (B6.129S4-Gt (ROSA26) Sor tm1 (FlpIn-T2)Jynx) [15] to delete the eGFP and neomycin cassette to obtain the floxed allele (fl) mice with Flp recombinase transgene, and the offspring were further crossed with wild-type (WT) C57BL/6 mice to remove Flp recombinase transgene. To gain hepatocyte-specific depletion of MAP9 (MAP9Δ/Δhep), fl mice were crossed with hepatocyte-specific Alb-cre mice (Speer6-ps1 Tg (Alb-cre)21Mgn) [16] to obtain MAP9Δ/ΔAlb-Cre/Alb-Cre mice. MAP9 Δ/Δhep and its wild-type littermates were confirmed by PCR genotyping. All animal studies were conducted in accordance with guidelines and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

2.20. DEN-induced liver tumorigenesis in mice

To induce hepatocarcinogenesis, MAP9Δ/Δhep mice and WT mice were singly injected i.p. at 2 weeks of age with 5 mg/kg DEN (Sigma-Aldrich) and were sacrificed at the age of 30 weeks. For all experiments, only male mice were used.

2.21. Hematoxylin and eosin (H&E) staining

Mice livers were harvested and fixed in 4% paraformaldehyde, and preserved in optimal cutting temperature compound. The liver tissues were sliced in 5 μm sections and stained with H&E for the histological studies.

2.22. Statistical analysis

All the results were indicated as mean ± SD. Statistical analysis was conducted using GraphPad Prism version 6.0 (GraphPad Software) or Statistical Package for the Social Sciences (SPSS) (standard V.20.0) (IBM Corporation, New York, USA). The Pearson correlation coefficient was used to assess the correlation of MAP9 expression with its promoter methylation in the clinical samples. The Chi-square test or Fisher exact test was utilized for comparison of patient clinicopathologic characteristics and MAP9 expression or its promoter methylation level. Kaplan-Meier analysis and log-rank test were used to estimate the association of MAP9 methylation or its low expression with overall survival and tumour recurrence. Cox proportional hazards regression model was used to evaluate the prognostic value of MAP9 methylation or its low expression. Mann-Whitney U test or Student’s t-test was performed to compare the variables of two groups. P value < 0.05 were considered as statistical significance.

3. Results

3.1. MAP9 is commonly silenced in liver cancer cell lines and tissue samples

We examined MAP9 mRNA expression in 64 paired human HCC tissue samples and found that MAP9 mRNA levels were dramatically decreased in HCC tissues as compared with their adjacent non-tumour tissues, and downregulation of MAP9 mRNA was confirmed in 367 HCC tissues compared with 50 non-tumour tissues from TCGA dataset (Fig. 1a and Supplementary Fig. S1). Accordingly, MAP9 protein expression was found significantly reduced in HCC tissues (89.47±10.68) as compared with adjacent non-tumour tissues (131.00±4.71) by IHC analysis (Fig. 1b). Moreover, MAP9 was silenced in all 6 liver cancer cell lines (Huh6, Huh7, SNU-423, PLC5, SK-Hep1, and HepG2), but readily expressed in normal liver tissues as shown by RT-PCR and Western Blot analysis (Fig. 1c and c2). The immortalized hepatocyte cell line Mih showed MAP9 mRNA and protein expression, but LO2 was silenced (Fig. 1c1 and c2). We investigated the underlying mechanisms of the transcriptional silence of MAP9 by assessing its promoter methylation by methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS), and found an inverse correlation of MAP9 silence with its promoter hypermethylation in HCC cell lines and primary HCC tissues (Fig. 1c-e). The promoter methylation levels of MAP9 were significantly higher in HCC tissues than those in adjacent non-tumour tissues in our cohort (P < 0.0001, paired t-test) and TCGA cohort (P < 0.01, paired t-test), and showed a negative correlation with MAP9 mRNA expression in HCC tissues (Fig. 1f). De-methylation treatment by 5-Aza-20-deoxycytidine (5-Aza) could restore the expression of MAP9 in silenced HCC cell lines (Fig. 1g), suggesting that the transcriptional silence of MAP9 is mediated by its promoter hypermethylation in HCC.

3.2. MAP9 promoter hypermethylation is an independent predictor of poor prognosis in HCC patients

To assess the association of MAP9 methylation with the clinicopathological characteristics and prognosis of HCC patients, we measured the MAP9 promoter methylation status in 64 HCC patients from our cohort and 260 HCC patients from TCGA cohort. MAP9 hypermethylation was detected in 26.56% (17/64) of HCC tissues in our cohort as well as in 27.69% (72/260) of HCC tissues in TCGA cohort (Supplementary Table S3). As demonstrated by Kaplan-Meier analysis, HCC patients with MAP9 hypermethylation had a significantly shorter survival in both our cohort and TCGA cohort (P < 0.001, Fig. 2a). MAP9 hypermethylation was also associated with liver fibrosis, Tumour-Node-Metastasis (TNM) staging and distant metastasis in our cohort (Supplementary Table S3). By univariate Cox regression analysis, we found that MAP9 hypermethylation was associated with a poor survival in both our cohort (HR 15.729, 95%CI 6.248 to 39.597, P < 0.0001) and TCGA cohort (HR 2.136, 95%CI 1.396 to 3.267, P < 0.0001) (Fig. 2b). As expected, the TNM stage, metastasis and tumour size were also prognostic factors (Fig. 2b). After adjustment for potential confounding factors including age, gender and TNM stage, MAP9 hypermethylation in these two cohorts was found to be an independent prognostic factor of poor survival in HCC patients by multivariate Cox regression analysis (Fig. 2c). In addition, MAP9 hypermethylation was associated with tumour recurrence (Supplementary Fig. S2a). Univariable and Multivariate Cox regression analysis unveiled MAP9 hypermethylation as an independent prognostic factor of tumour recurrence in HCC patients (Supplementary Fig. S2b). Moreover, MAP9 low expression is a predictor of overall survival of HCC patients in both our cohort and TCGA cohort (Supplementary Table S4, 5) and tumour recurrence in TCGA cohort (Supplementary Table S6).

3.3. MAP9 inhibits liver cancer cell growth through inducing apoptosis and reducing cell cycle progression

We evaluated the functional role of MAP9 in HCC cell lines by generating two stably transfected cell lines (LO2 and HepG2) with MAP9 overexpression and siMAP9-transfected Mih cell line with loss of MAP9 function (Fig. 3a and Supplementary Fig. S3). The number of colonies formed in MAP9-transfected LO2 and HepG2 cell lines was substantially decreased as compared with the empty vector-
MAP9 is silenced by promoter hypermethylation in liver cancer cells and tissue samples. (a) MAP9 mRNA level was dramatically decreased in liver tumour tissues (T) as compared with the paired non-tumour tissues (N) in our and TCGA cohorts. All data are normalized to adjacent normal and paired t-test was used to analyse the statistical difference between adjacent normal and HCC group. \( n = 64 \) for adjacent normal and HCC groups in our cohort. \( P < 0.001 \) (paired t-test) vs. adjacent normal. \( n = 50 \) for adjacent normal and HCC groups in TCGA cohort. \( P < 0.01 \) (paired t-test) vs. adjacent normal. (b) Representative images of IHC staining of MAP9 protein expression in HCC tissues. Scale bar: 75 µm. All
transfected LO2 and HepG2 cell lines, while knockdown MAP9 promoted the colony formation ability in Miha cell line (Fig. 3b). In keeping with this, ectopic MAP9 expression suppressed cell viability in LO2 and HepG2 cell lines, while an inverse effect was observed in Miha cell line with MAP9 knockdown (Fig. 3c). We analysed the effects of MAP9 on cell apoptosis by flow cytometry. Restoration of MAP9 expression caused an increase in the number of early and late apoptotic cells in LO2 and HepG2 cell lines. On the other hand, MAP9 knockdown diminished cell apoptosis in Miha cell lines (Fig. 3d). Accordingly, MAP9 enhanced the protein levels of activated form of caspase-9, caspase-7, caspase-3, and PARP in LO2 and HepG2 cell lines, while an inversed effect was observed in Miha cell line with MAP9 knockdown (Fig. 3d).

Additionally, ectopic MAP9 expression caused an elevated accumulation of LO2 and HepG2 cells in G1 phase and a corresponding decrease in the S phase, while knockdown of MAP9 induced a decrease of G1 phase population but an increase of S phase population in Miha cell line (Fig. 3e). We consistently observed that G1 phase gatekeepers p27Kip1 and p21Cip1 expression levels were enhanced but G1-S transition promoters Cyclin D1 and CDK4 were reduced by MAP9 overexpression, while the opposite results were presented by MAP9 knockdown (Fig. 3e). Therefore, our results indicated that MAP9 favored the apoptosis and prohibited the cell cycle in liver cell lines.

3.4. MAP9 inhibits liver cell migration and invasion

The effects of MAP9 on cell migration and invasion were further assessed. The migration capabilities were noticeably reduced by MAP9 overexpression in LO2 and HepG2 cells, but increased by MAP9 knockdown (Fig. 3f).
knockdown of MAP9 in Miha cells (Fig. 4a) by wound-healing assay. The similar results were presented by Transwell migration assay (Fig. 4b). In addition, the invasive potential was dramatically weakened by MAP9 overexpression in LO2 and HepG2 cells, but reinforced by knockdown of MAP9 in Miha cells (Fig. 4c) by Transwell invasion assay.
3.5. Hepatocyte-specific knockout of MAP9 accelerates DEN-induced hepatocarcinogenesis

To confirm the functional significance of MAP9 in HCC formation, we constructed Map9 conditional hepatocyte-specific knockout mice (MAP9Δ/Δhep) (Fig. 5a). MAP9Δ/Δhep and WT mice administered with a single dose of DEN at 2 weeks of age. At 30 weeks of age, mice were sacrificed and the liver tissues were analysed. The absence of Map9 mRNA and protein in the liver tissues of MAP9Δ/Δ mice was confirmed by RT-PCR and Western blot analysis (Fig. 5b). MAP9Δ/Δhep mice developed significant more tumours (100%, 5/5) as compare with WT mice (40%, 4/10), accompanied with hepatocyte dysplasia, liver cell injury, necrosis and inflammatory cell infiltration (Fig. 5c). MAP9Δ/Δhep mice displayed the increased liver weight, tumour volume and tumour number (Fig. 5d). Liver tumours derived from MAP9Δ/Δhep mice exhibited a higher Ki-67 score (15.20 ± 3.44) but a lower apoptosis index (5.61 ± 0.77) compared with the WT mice (7.36 ± 1.65; 2.08 ± 0.36) (Fig. 5e). These indicated that hepatocyte-specific Map9 knockout promoted DEN-induced HCC formation.
3.6 Knockout of MAP9 causes a spontaneous liver hyperplastic nodule in mice

Having observed the effect of MAP9 Δ/Δ on DEN-induced hepatocarcinogenesis, we investigated whether MAP9 Δ/Δ could induce a spontaneous HCC formation. At 40 weeks of age, MAP9 Δ/Δ mice were sacrificed and the liver tissues were examined. MAP9 was confirmed to be deleted in liver tissues of MAP9 Δ/Δ mice (Fig. 6a). MAP9 Δ/Δ mice developed a spontaneous liver hyperplastic nodule, which was confirmed histologically as hepatocyte dysplasia with inflammatory cell infiltration (Fig. 6b). When compared with the WT mice, MAP9 Δ/Δ mice displayed an increased Ki-67 score (15.61 ± 0.71) but a decreased cell apoptosis index (2.58 ± 0.11) in liver tissues compared with the WT mice (10.50 ± 1.04; 6.17 ± 0.73) (Fig. 6c). There were no significant differences in body weight and liver weight between MAP9 Δ/Δ and WT mice (Supplementary Fig. S4).
3.7. ERCC3 is identified as a negative regulator of MAP9

At 12 weeks of age, MAP9 Δ/Δ and WT littermates were sacrificed and the liver tissues were analysed. MAP9 was confirmed to be deleted in liver tissues of MAP9 Δ/Δ mice, which showed decreased body weight and liver weight compared with the WT mice (Fig. 7a).

We performed the hierarchical clustering analysis and identified about 30 aberrantly expressed genes in liver tissues from MAP9 Δ/Δ and WT mice by using a cancer pathway PCR array (Fig. 7b). Amongst them, several upregulated genes in MAP9 Δ/Δ group formed a gene co-expressed cluster, including Ercc3, Pmnx1, Foxo2, Ercc5, Lig4, B2m, Uqcrfs1, Xrcc4, Gadd45g, Ldha, Cdc20 and Serpinb2. Furthermore, Gene Set Enrichment Analysis (GSEA) was used to assess the enrichment of Kyoto encyclopedia of Genes and Genomes (KEGG) pathways. According to the Normalized Enrichment Score (NES) value and P value, a nucleotide excision repair (NER) pathway was identified to be most significantly associated with the phenotype of MAP9 deletion (Fig. 7c). Key genes in NER pathway had the core enrichment, such as Ercc3, Xrcc4, Ercc5, Lig4, of which Ercc3 exhibited the highest rank metric score and might act a critical role in liver pathogenesis in MAP9 Δ/Δ mice (Fig. 7c).

We further investigated the expression of ERCC3 and xeroderma pigmentosum complementation group B (XPB, the DNA helicase encoded by ERCC3) in MAP9 Δ/Δ mice and liver cells. MAP9 ablation increased the expression of ERCC3 mRNA and XPB protein levels in mouse liver tissues, whereas MAP9 overexpression reduced ERCC3 mRNA and XPB protein expression in HepG2 and LO2 cell lines (Fig. 7d). Immunofluorescence analysis showed that MAP9 overexpression was localized in the cytoplasm while XPB was localized in the nucleus in HepG2 and LO2 cell lines (Fig. 7e). We also found that ERCC3 expression was upregulated in HCC tissues as compared with adjacent non-tumour tissues and harboured a negative correlation with MAP9 expression in HCC samples (Supplementary Fig. S5). Moreover, ERCC3 was upregulated in 50 paired and 371 unpaired HCC tissues as compared with adjacent non-tumour tissues from TCGA cohort (Supplementary Fig. S6a). In contrary to MAP9 expression, the patients with ERCC3 high expression possessed a shorter survival (Supplementary Fig. S6b), as compared with those with ERCC3 low expression. Univariate and multivariate Cox regression analysis unveiled that ERCC3 high expression was an independent prognostic factor of poor survival in HCC patients (Supplementary Table S7).

3.8. ERCC3 counteracts the tumour suppressive effects of MAP9 in HCC cells

To evaluate whether MAP9 induces a tumour suppression through regulating ERCC3, we transfected ERCC3, MAP9, or co-transfected
ERCC3 and MAP9 in LO2 and HepG2 cell lines respectively. We found that ERCC3 overexpression promoted cell proliferation (Fig. 8a, b), increased the number of colonies (Fig. 8c) and boosted cell invasion (Fig. 8d) in LO2 and HepG2 cell lines. The inhibitory effects of MAP9 on cell proliferation, colony formation and cell invasion in LO2 and HepG2 cell lines were attenuated by ERCC3 (Fig. 8b/C0/d). These results collectively indicated that the tumour suppressive function of MAP9 in HCC was at least in part depending on the reduction of ERCC3 expression.

4. Discussion

In this study, we first demonstrated that MAP9 was frequently silenced in liver cancer cell lines and tissue samples due to its promoter hypermethylation. The clinical significance of MAP9 methylation or its low expression in patients with HCC was then assessed. MAP9 hypermethylation or its low expression (Fig. 2, Supplementary Fig. S2, Table S4-6) was an independent risk factor of poor survival and tumour recurrence in patients with HCC. The patients with MAP9 hypermethylation or low expression (Supplementary Fig. S7) conferred a shorter survival and a higher tumour recurrence. In light of the diversity in tumour proliferating status, aggressive potential and therapeutic outcomes, the prognosis of HCC patients varies. Some patients in early stage with MAP9 hypermethylation or its low expression were subjected to an unfavourable prognosis. Identification of more promising biomarkers may favour the assessment of the outcome of HCC patients. DNA promoter methylation has been reported as the potential predictors of HCC patients [3, 17]. This finding inferred MAP9 hypermethylation as a novel prognostic biomarker in patients with HCC.

The functional importance of MAP9 in HCC was investigated. Ectopic expression of MAP9 suppressed cell viability and colony formation, whereas knockdown of MAP9 showed an opposite effect in vitro. To confirm the tumour suppressive role of MAP9 in HCC, we generated MAP9 conditional knockout model. Hepatocyte-specific deletion of MAP9 in mice facilitated DEN-induced HCC progression. Moreover, MAP9 knockout mice developed spontaneously liver dysplastic nodule. These findings confirmed MAP9 was a novel tumour suppressor gene in HCC.

The mechanisms of MAP9 as a tumour suppressor gene in HCC was investigated. The tumour suppressive function of MAP9 was mediated by inducing cell apoptosis and suppressing cell proliferation and cell cycle progression. MAP9 induced cell apoptosis by activating intrinsic and extrinsic apoptosis pathway, causing activation in caspase-9 and its downstream effectors caspase-3, caspase-7 and PARP. MAP9 blocked cell-cycle transition at G1-S phase, associated with decreased expression of cyclin D1 and CDK4 as well as increased expression of p27kip1 and p21cip1. Cyclin D1/CDK4 complex is engaged in control of cell proliferation during G1 phase, and p21cip1 and p27kip1 can inhibit its activity [18]. MAP9 mediated cell
apoptosis, cell proliferation and cell cycle progression in HCC cell lines were confirmed in a MAP9 knockout mouse model. Moreover, MAP9 inhibited the migration and invasion potential of HCC cells. Tumour metastasis is a predominant cause of cancer-related deaths [19]. This result further confirmed the role of MAP9 as a tumour suppressor gene in HCC.

To gain insights into the mechanisms by which MAP9 functioned as a tumour suppressor in HCC, we applied a gene expression profiling assay in liver tissues from MAP9 knockout and WT mice. We identified that NER pathway as one of the critical pathways was involved in MAP9 functional regulation in liver. NER, the most flexible of the DNA repair pathways in response to the multiformity of DNA lesions, executes the excision of the damaged DNA fragment and synthesis of a new DNA strand portion [20]. Deregulation of NER signalling has been reported in a variety of malignancies [21-24]. Here we demonstrated that NER signalling was involved in the liver pathogenesis in MAP9 knockout mice. We further revealed that ERCC3 was a core-enriched gene in NER pathway regulated by MAP9 in HCCs. Enhanced ERCC3/XPB expression was demonstrated in liver tissues of MAP9Δ/Δ mice. We discovered that ERCC3 harboured an increased expression, depicted a negative correlation with MAP9 expression and acted as an independent prognostic factor of poor survival in HCC patients. Besides, appreciable decrease in ERCC3/XPB expression or nuclear aggregation was caused by MAP9, but a substantial increase was shown in liver tumours in MAP9Δ/Δ mice. Re-expression of ERCC3 promoted cell growth and invasion, and abrogated the tumour suppressive effects of MAP9. These data collectively indicated that ERCC3 was required for MAP9 deficiency induced HCC. As a core gene in NER pathway, ERCC3 may influence the aggressive phenotype of liver cancer cells through DNA damage. We have recently reported that MAP9 acted as a microtubule stabilizer and MAP9 loss caused chromosomal instability especially in MAP9Δ/C0/C0 p53+/C0 mice [25]. Others have reported that MAP9 directly interacts with and stabilizes p53 [12], which could regulate NER pathway and contribute to DNA repair [26]. A direct interaction of p53 with ERCC3 was demonstrated in hepatitis C virus infection [27]. Therefore, MAP9 may regulate ERCC3 in NER pathway through upregulating p53. Further studies need to be conducted to confirm the exact mechanistic interplay of MAP9 on ERCC3 regulation.

Fig. 8. Ectopic expression of ERCC3 reverses the anti-tumour effects of MAP9 in HCC cells. (a) Overexpression of ERCC3 in LO2 and HepG2 cell lines was determined by RT-PCR. (b-d) MTT, colony formation and Transwell assays were used to assess the cell viability, colony number and cell invasive potential after co-transfection with ERCC3 and MAP9 plasmids in LO2 and HepG2 cell lines. All data are normalized to the control. n = 3 for each group, *P < 0.05 and **P < 0.01 (One-way ANOVA) vs. control.
In conclusion, we identified for the first time that MAP9 is a novel tumour suppressor gene in HCC. MAP9 exerts a tumour suppressive function by downregulating ERCC3/XBP expression and serves as an independent prognostic factor in HCC patients.

Declaration of Competing Interest

All authors declare no conflicts of interests.

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Author’s contribution

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Performed the research and data collection: JZ, JZH, YQZ, LYZ, CGL, YFZ and HW.

Data analysis and interpretation: JZ, JZH, YQZ, XZ, LYZ and HW.

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All authors read and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102701.

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