Supplementary Methods

Cell Cultures and reagents (continued)

Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin at 37 ℃ with 5% CO_2. The CSF1R antagonist, pexidartinib (PLX3397), was purchased from Selleck Chemicals (Houston, TX, USA). Murine isotype controls and PD-L1-blocking antibody (clone 10F.9G2) were purchased from BioXcell (West Lebanon, NH, USA).

Co-culture assay

6-well transwell chambers with 0.4 μm porous polycarbonate membrane (One Riverfront Plaza Corning, NY, USA) were used with 5×10^5 HepG2, Hep3B, MHCC97H, HCCLM3 cells seeded in the lower chamber 24 hours before co-culture either 1×10^6 THP-1 cells treated with PMA added in the upper chamber. After 48 hours, HCC cells, macrophages and supernatant were collected separately for analysis.

Plasmids construction and transfection

Plasmid construction was performed according to standard procedures as previously described[1]. Lentivirus vectors pLKO.1 TRC and pWPI.1 were used for producing recombinant lentiviruses. For example, the over-expressed lentivirus plasmids of human OPN, tagged full-length cDNA encoding human OPN was amplified by PCR and cloned into pWPI.1 vector. The constructs were confirmed by DNA sequencing. Other promoter constructs were cloned in the same manner. The primers are presented in Supplementary Table 8. Lentivirus was produced and collected after plasmid transfection of 293T cells. Transduction was performed as methods previously described[1].

Animal studies (continued)

Induction of carcinogenesis

Fourteen-day-old *OPN* KO and WT mice administered 25 mg/kg diethylnitrosamine (DEN; Sigma Aldrich, St. Louis, MO, USA) via i.p. injection to induce hepatocarcinogenesis. Two weeks after DEN treatment, mice were administered 0.5 μl/g carbon tetrachloride (CCl_4) dissolved in corn oil via i.p. injection once per week for 20 weeks as previously described[2]. DEN/CCl_4-treated mice and their corresponding

controls injected with saline were sacrificed at 6, 9, and 12 months of age. Liver and lungs were removed and macroscopically examined for gross lesions. The number of tumors was counted. Lung and liver tumor tissues were excised and either snap-frozen in liquid nitrogen or fixed in 4% formalin and paraffin-embedded, and sectioned. The sections were used for hematoxylin and eosin (H&E) staining, immunohistochemical analyses (IHC), immunofluorescence staining, and other analysis. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the institutional review board of Department Of Laboratory Animal Science, Fudan University (Shanghai, China).

Subcutaneous and orthotopic models

All BALB/c mice and C57BL/6 mice (4- to 6-week-old male) were obtained from Shanghai Slac Laboratory Animal Co. and fed in a pathogen-free vivarium under standard conditions. To establish the subcutaneous HCC model, Hepa1-6-OPN cells or Hepa1-6-control cells (5×10^6 cells in 100 μL of phosphate buffer saline [PBS]) were injected into the right flanks of mice. Mice bearing tumors about 50 mm^3 in volume were divided in 4 groups (n = 6 mice/group). For treatment with antibody, 200 μg anti-PD-L1 antibody (10F.9G2, Bio X Cell, West Lebanon, NH, USA) or rat IgG (Bio X Cell) as control was injected intraperitoneally every three days. For drug treatment, mice were treated with daily oral doses 40 mg/kg PLX3397 **(**Selleck; Houston, TX, USA) for 4 weeks (5 days per week). Tumor was measured weekly with a caliper, and tumor volume was calculated by the formula: $a \times b^2/2$ (where a and b represent the largest and smallest tumor diameters)[3, 4].

For orthotopic study, tumors from subcutaneous H22-OPN were minced into 1-2 mm^3 cubes and transplanted to the livers of BALB/c mice under anesthesia with Pentobarbital Sodium (40 mg/kg; Sigma Aldrich, St. Louis, MO, USA). Two weeks after being implanted with cubes, mice were divided in 4 groups (n = 5 mice/group) and received the same treatments with subcutaneous HCC model.

Clinical samples, tissue microarray and immunohistochemistry

HCC tissues were collected from 20 patients at the authors' institutes to detect the numbers of TAMs by flow cytometry and OPN level by immunohistochemistry. We also analyzed the protein expression levels of OPN, PD-L1 and TAMs-related protein by immunohistochemical analysis of 186 HCC specimens. None of these HCC patients received any preoperative cancer treatment. The clinical samples were collected from

patients after obtaining informed consent in accordance with a protocol approved by the Ethics Boards of Fudan University (Shanghai, China).

Formalin-fixed and paraffin-embedded tissues were used to construct tissue microarray (TMA) as previously described[1]. We took two 4 μm diameter core biopsies from the donor blocks and transferred these to the recipient paraffin block at predefined array positions, and constructed 186 cases of TMA blocks in this study.

Immunohistochemical (IHC) staining was performed as described previously[1]. Briefly, following deparaffinization rehydrating and antigen retrieval, primary antibodies were applied to slides, incubated at 4°C overnight, and followed with secondary antibody incubation (Dako Cytomation, Glostrup, Denmark) at 37°C for 30 minutes. The primary antibodies and dilutions used are listed in Supplementary Table 9. Staining was carried out with DAB and counter-staining was performed with hematoxylin.

Evaluation of immunohistochemical scores

Scoring for OPN and PD-L1 staining was conducted based on the percentage of positive-staining cells: 0-5% scored 0, 6-25% scored 1, 26-50% scored 2, 51-75% scored 3 and more than 75% scored 4; and staining intensity: no staining scored 0, weakly staining scored 1, moderately staining scored 2 and strongly staining scored 3. The final score was calculated using the percentage score × staining intensity score as described previously[5].

Western blot assay

Western blot was performed as described previously[6]. Briefly, whole cell lysis was lysed in RIPA buffer containing protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, membranes were incubated with the primary antibody. The primary antibodies and dilutions used are listed in Supplementary Table 9. Primary antibodies were applied, followed by horseradish-peroxidase–conjugated secondary antibodies. Antibody binding was detected by enhanced chemiluminescence assays, and each band was detected with Image Acquisition using ImageQuant™ LAS 4000 (GE Healthcare Life Sciences, San Diego, CA, USA).

Immunofluorescence (IF)

Cells were fixed by 4% paraformaldehyde at room temperature for 15 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes, blocked with 1% BSA for 1h, and then stained using primary antibodies. The secondary antibodies used were anti-mouse Alexa Fluor 488 or 594 dye conjugate and/or anti-rabbit Alexa Fluor 488 or 594 dye conjugate (Life Technologies). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI blue) (Life Technologies). Finally, the slides were mounted and imaged under Nikon A1 confocal microscope (Nikon; Minato-ku, Tokyo, Japan).

qRT-PCR assays

Total RNA was extracted from cells and frozen samples by using Trizol reagent (Invitrogen, California, USA) and then reversely transcripted into cDNA. Real-time PCR was performed using SYBR Green PCR Master Mix (DBI® Bioscience, Ludwigshafen, Germany) and ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Results were normalized to β-actin for mRNA measurement. All the primer sequences used in this study were listed in Supplementary Table 8.

Human cytokine antibody array and ELISA

The CSF1 levels in the supernatant of co-culture systems were assessed using the Human Cytokine Antibody Array (AAH-CYT-3; RayBiotech®, Norcross, GA, USA) or Human CSF1 ELISA kit (ELH-MCSF-1; RayBiotech®), according to the manufacturer's directions.

Flow cytometry

Freshly resected HCC patient tumors or **mouse tumors of similar size (50mm^3)** were processed into single cell suspensions. Cells were stained with the antibodies or isotype antibody controls for 30 minutes at room temperature and washed by PBS for two times. Cells were resuspended with 300 μL PBS and analyzed by flow cytometry (Epics Altra, Beckman Coulter, USA).

Macrophage Migration Assays

THP-1 cells treated with PMA were added to the upper chamber of 8 μm pore transwell inserts (Corning, NY, USA) for 2 hours to attach to the membrane. The transwells were then moved to 24-well plates containing 600 μL supernatant of co-culture systems and

incubated at 37℃ for 24 hours. Cells were fixed in 4% paraformaldehyde and stained with 0.1% Crystal Violet. Migrated cells in the lower chamber were quantified using three random fields. This assay was repeated for three times.

Luciferase reporter assays

Luciferase activity was detected using the Dual Luciferase Assay System (Promega) according to manufacturer's instructions. The transfected cells were lysed in culture dishes containing a lysis buffer, and the resulting lysates were centrifuged at maximum speed for 1 minute in an Eppendorf microcentrifuge. Relative reporter luciferase activity was measured using a Modulus™ II Luminometer (Turner Biosystems, Madison, WI), and the transfection efficiencies were normalized according to the Renilla activity.

Chromatin Immunoprecipitation Assay (ChIP)

Cells were cross-linked in 40% paraformaldehyde at room temperature for 10 minutes. After washing with PBS, the cells were resuspended in buffer (50 mM Tris·HCl, pH 8.1, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride and 1% sodium dodecyl sulfate (SDS)). After centrifugation, the supernatants were incubated with specific antibodies or isotype control IgG overnight at 4℃. Antibody-DNA complexes were captured by the protein A/G beads. The pull-down DNA was purified and detected by qPCR.

Statistics analysis

All data are presented as the mean ± SEM. Statistical analyses were performed using SPSS 16.0 for Windows (IBM, Armonk, NY). The difference between groups was analyzed using Student *t*-test or one-way ANOVA analysis. The **association** of OPN, PD-L1 expression and TAMs infiltration with clinicopathological variables in HCC patients was evaluated by $\chi 2$ test or Fisher's exact test. Correlation analysis of OPN expression and TAM related genes in TCGA dataset was evaluated by Pearson correlation analysis. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. Risk factors independently related to morbidity and recurrence were tested by univariate and multivariate Cox regression analyses using a Cox proportional hazards model. A P value < 0.05 was considered statistically significant.

References

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