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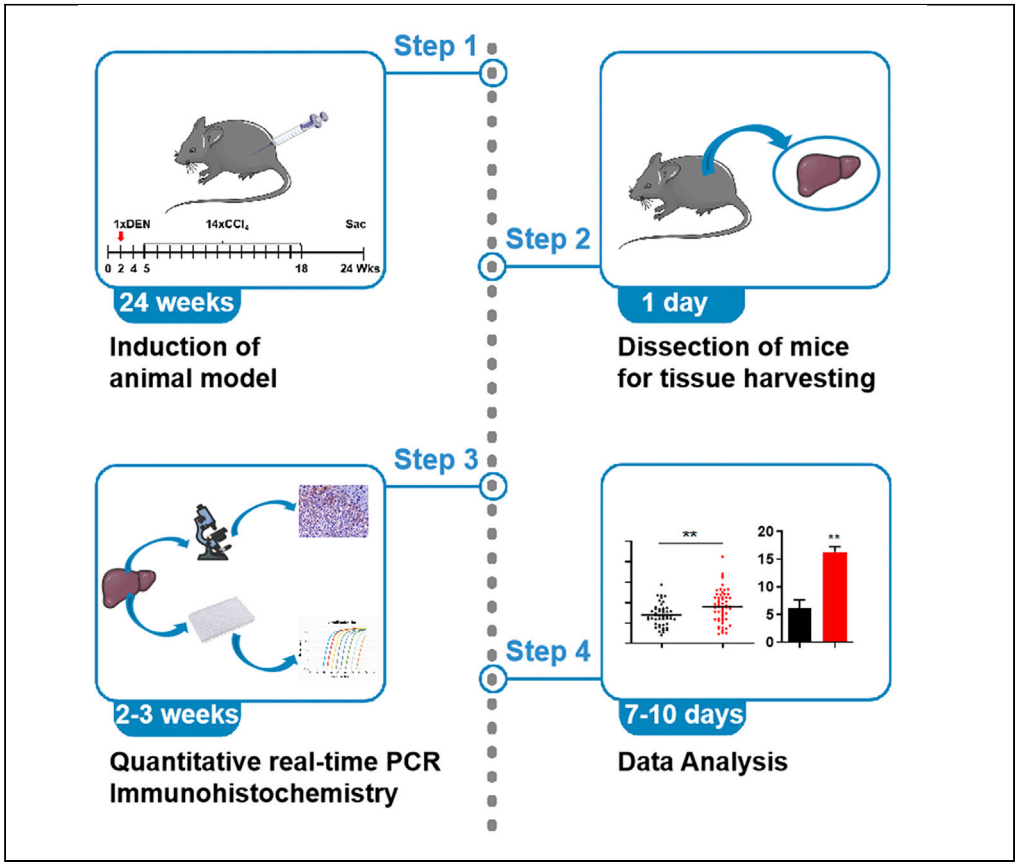
In vivo analysis of FBXO45-mediated fibrosis and liver tumorigenesis in a chemically induced mouse model of hepatocellular carcinoma



FBXO45, an E3 ubiquitin ligase highly expressed in liver tumors, is positively correlated with poor survival of hepatocellular carcinogenesis (HCC) patients, but whether FBXO45 drives HCC tumorigenesis remains largely unclear. Here, we describe a protocol that shortens the observation period for HCC tumorigenesis to assess the effects of FBXO45 in a DEN/CCl_4-induced HCC mouse model. We describe steps for chemical induction of HCC in FBXO45-overexpressing mice, followed by tissue collection and pathology assessment via quantitative real-time PCR, histology, and immunohistochemistry.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to chemically induce HCC in FBXO45-overexpressing mice

Procedure for the combined treatment of mice with DEN/CCl_4 to induce HCC

Assessment of cell morphology and gene and protein expression in liver tissues

Applicable to assess other genes of interest in chemically induced HCC mouse models

Zhang et al., STAR Protocols 4, 102124
March 17, 2023
https://doi.org/10.1016/j.xpro.2023.102124

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In vivo analysis of FBXO45-mediated fibrosis and liver tumorigenesis in a chemically induced mouse model of hepatocellular carcinoma

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https://doi.org/10.1016/j.xpro.2023.102124

SUMMARY

FBXO45, an E3 ubiquitin ligase highly expressed in liver tumors, is positively correlated with poor survival of hepatocellular carcinogenesis (HCC) patients, but whether FBXO45 drives HCC tumorigenesis remains largely unclear. Here, we describe a protocol that shortens the observation period for HCC tumorigenesis to assess the effects of FBXO45 in a DEN/CCl_4-induced HCC mouse model. We describe steps for chemical induction of HCC in FBXO45-overexpressing mice, followed by tissue collection and pathology assessment via quantitative real-time PCR, histology, and immunohistochemistry.

For complete details on the use and execution of this protocol, please refer to Lin et al. (2021).[1]

BEFORE YOU BEGIN

The occurrence and development of Hepatocellular carcinogenesis (HCC) is a multi-step and multi-factorial process. In order to explore its etiology and seek effective therapeutic strategies, it is necessary to establish animal models that can accurately simulate the progression of HCC. The following content introduces a conditional transgenic knock-in mouse model to mimic the pathological process from hepatitis to liver fibrosis and finally to cirrhosis. The chemical induction method used was a single injection of N-Nitrosodiethylamine (DEN) at the age of 2 weeks, followed by 14 weekly injection of Carbon tetrachloride (CCl_4). We then detail the steps to isolate liver tissues for histological and gene expression analysis.

Preparation of animals

Timing: 26–35 weeks

1. Order FBXO45-ROSA26 knock-in mice aged 6–8 weeks old.

Note: The FBXO45 knock-in transgenic mice highly expressing FBXO45 at the ROSA26 locus were created by CRISPR/Cas9-mediated genome engineering from Cyagen Biosciences (Guang Zhou). To generate this mouse, fertilized mouse zygotes were co-injected with a mixture of Cas9 mRNA, gRNA to mouse ROSA26 gene, and a construct containing a FBXO45 cDNA. Injected zygotes were transferred into pseudo pregnant C57BL/6N female mice. Correctly targeted mice were determined by PCR and gene sequence.

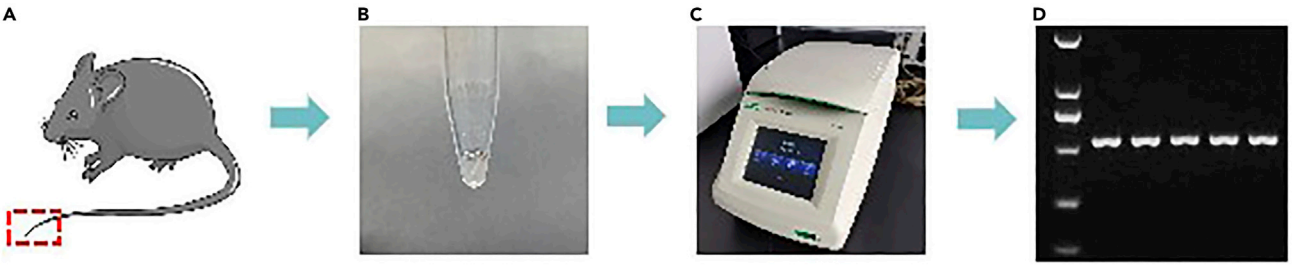

Figure 1. Steps of genotyping test for mouse tail
(A) Cut off 3–5 mm of mouse tail.
(B) Add tissue digest to extract DNA template.
(C) Run PCR for all samples according to manufacturer's instructions.
(D) Agarose gel electrophoresis of PCR products.

ROSA26 was first discovered by Friedrich and Soriano when they studied gene mutations in mouse embryonic stem cells (ESCs). The ROSA26 locus is known in the scientific community by the official name: gene trap ROSA 26 [Gt(ROSA)26Sor]. ROSA26 is a non-coding gene composed of three exons on mouse chromosome 6, a region where it is easy to insert genes. There are no known functional proteins encoded by the ROSA26 gene. Additionally, the ROSA26 locus makes it easy to perform homologous recombination (HR), maintains expression levels of the protein from gene constructs inserted into this region, and does not affect the expression or function of other endogenous genes. Given its wide expression across all cell types and developmental stages, the ROSA26 region is often used as a safe site for gene targeting in mouse models.

2. Keep animals under specific pathogen-free (SPF) conditions.
3. Mice need 3 days to a week of acclimatization to ensure their physiological and behavioral adaptation to the new environment before the experiment.

Preparation of genotyping

Timing: 8–10 weeks

4. Cross FBXO45-OE (over-expressed) mice with C57BL/6J mice and house them separately from the males in other cages when pregnant.
5. Aseptically excise a little bit tail from the pup (10–14 days after birth) for genotyping using Mouse Direct PCR Kit (Figure 1).
   a. Place the tails in a 1.5 mL sterile tube with 50 μL fresh tissue digest and incubate at 55°C for 15 min.

   Note: Be sure to completely submerge the tissue in the digestion solution.

   b. Incubate samples in a water/metal bath at 95°C for 5 min to inactivate protease activity.
   c. Centrifuge at 12,000 × *g* for 5 min.
   d. Collect the supernatant and use as PCR template.
   e. Primers are listed in the key resources table. Prepare the PCR reaction system on ice and the conditions of PCR reaction parameters can be referred to the following example.

PCR reaction master mix

Reagent	Amount
DNA template	1 μL
2 × M-PCR OPTI™ Mix	10 μL

(*Continued on next page*)

Continued	
Reagent	Amount
Forward primer (10 μM)	0.5 μL
Reverse primer (10 μM)	0.5 μL
ddH_2O	8 μL
Total	20 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	20 s	35 cycles
Annealing	60°C	30 s	
Extension	72°C	2 kb/min	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

f. After the PCR reaction, perform agarose gel electrophoresis to detect PCR product (Figure 2).

Note: Also cutting off toes or ears for marking to facilitate subsequent correspondence with test results. To prevent contamination of the DNA from pups, the surgical instruments should be wiped with alcohol cotton balls before using.

6. Select and rear the identified pups together at 4 weeks of age (3–5 per cage) for the next experiment. Only male mice were used in this study.[2]

Note: Mice should be crossed for at least 3 generations to obtain transgenic mice with exogenous gene single site integration and stable expression.

Institutional permissions

Mouse in this study were housed and maintained at the Animal Center of Army Military Medical University. All experiments procedures are performed based on the protocols by the Institutional Animal Care and Use Committees of Army Military Medical University.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-FBXO45 (1: 300)	Bioss	Cat#bs-13150R
Mouse anti-Ki67 (1: 800)	Millipore	Cat#FCMAB103AP
Chemicals, peptides, and recombinant proteins		
N-Nitrosodiethylamine(DEN)	Sigma	Cat#N0756
Carbon tetrachloride(CCl_4)	Macklin	Cat#C805332
Olive oil	Sangon Biotech	Cat#A502795
0.9% (w/v) sodium chloride	Southwest Pharmaceutical	N/A
10% neutral buffered formalin	Sigma	Cat#HT501128
RNAiso Plus reagent	Takara	Cat#9108
Isoflurane	Sigma	Cat#PHR2874
Chloroform	Nanjing Reagent	Cat#C0761520224
Isopropanol	Macklin	Cat#I811925

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DEPC water	Sangon Biotech	Cat#B501005-0500
Hematoxylin staining Solution	Biosharp	Cat#BL702B
Eosin staining solution	Biosharp	Cat#BL703B
Sodium citrate antigen retrieval solution (50×)	Solarbio	Cat#C1032
Hydrogen peroxide	Elabscience	Cat#E-IR-R115
Methanol	Macklin	Cat#M813895
Xylenes	Macklin	Cat#X820585
Ethyl alcohol	Macklin	Cat#E809061
Neutral balsam	Biosharp	Cat#BL704A
Paraffin	Aladdin	Cat#P100928
1×PBS buffer	BBI life sciences	Cat#E607008
Critical commercial assays		
Mouse Direct PCR Kit	Bimake	Cat#B40013
PrimeScript RT reagent Kit with gDNA Eraser	Takara	Cat#RR047
TB Green Premix Ex Taq II	Takara	Cat#RR820
DAB Color Development Kit (20×)	Zsgb-Bio	Cat#ZLI-9019
Immunohistochemistry Detection Kit	Zsgb-Bio	Cat#SP-9001
Experimental models: Organisms/strains		
FBXO45-OE; Wild type; 4-week-old male C57BL/6J mice	Cyagen Biosciences Inc.	N/A
Oligonucleotides		
FBXO45 genotyping forward primer	Sangon Biotech	GCTGGGAAGTGACGACCAGAG
FBXO45 genotyping reverse primer	Sangon Biotech	AGCCAGAAGTCAGATGCTCAAGG
Acta2 forward primer	Sangon Biotech	CCGCCATGTATGTGGCTATT
Acta2 reverse primer	Sangon Biotech	CAGTTGTACGTCCAGAGGCATA
Timp1 forward primer	Sangon Biotech	CTCGGACCTGGATGCTAAAA
Timp1 reverse primer	Sangon Biotech	ACTCTTCACTGCGGTTCTGG
Col1a1 forward primer	Sangon Biotech	TAAGGGTCCCCAATGGTGAGA
Col1a1 reverse primer	Sangon Biotech	GGGTCCCTCGACTCCTACAT
Col1a2 forward primer	Sangon Biotech	CCAGCGAAGAACTCATACAGC
Col1a2 reverse primer	Sangon Biotech	GGACACCCCTTCTACGTTGT
Pdgfb forward primer	Sangon Biotech	GGTGAGCAAGGTTGTAATGG
Pdgfb reverse primer	Sangon Biotech	GGAGGCAATGGACAGACAA
Pdgfrb forward primer	Sangon Biotech	TCCCACATTCCTTGCCCTTC
Pdgfrb reverse primer	Sangon Biotech	GCACAGGGTCCACGTAGATG
Il6 forward primer	Sangon Biotech	GAGCCCACCAAGAACGATAG
Il6 reverse primer	Sangon Biotech	TCATTTCCACGATTTCCCAG
Il1b forward primer	Sangon Biotech	GCTTCAGGCAGGCAGTATCA
Il1b reverse primer	Sangon Biotech	GACAGCACGAGGCTTTTTTG
Tnfa forward primer	Sangon Biotech	CAAGATGCTGGGACAGTGAC
Tnfa reverse primer	Sangon Biotech	AGGGAAGAATCTGGAAAGGT
Ccl2 forward primer	Sangon Biotech	AAAAACCTGGATCGGAACCAA
Ccl2 reverse primer	Sangon Biotech	CGGGTCAACTTCACATTCAAAG
Software and algorithms		
GraphPad Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
1 mL syringe	Huayon	Cat#21-3026
50 mL syringe	Huayon	Cat#21-3020
1.5 mL centrifuge tube	Axygen	Cat#MCT-150-C
50 mL centrifuge tube	Nest	Cat#602052
Dissecting scissors	Huayon	Cat#18-0110
Forceps	Huayon	Cat#18-1240
Oscillator	Kylin-Bell	Cat#VORTEX-6
Tissue embedding mold	Citotest	Cat#31050106W

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Slides	Citotest	Cat#188105W
Coverslip	Citotest	Cat#10212450C
Pap pen	Zsgb-Bio	Cat#Zsgb-Bio
Vernier caliper	Asoyoga	Cat#ASJ-424
Hemostatic cotton	N/A	N/A
Tissue homogenizer	Tiangen	Cat#OSE-Y30
Electronic analytical balance	Sartorius	Cat#BSA124S-CW
Microscope (BX41)	Olympus	N/A

MATERIALS AND EQUIPMENT

Reagent	Final concentration	Stock concentration	Amount
DEN	2.5 mg/mL in sodium chloride	100 mg/mL in sodium chloride	25 mg/kg body weight
CCl_4	20% in olive oil	98% in olive oil	0.5 mL/kg body weight

Note: Place solution in a light-proof environment and stored for no longer than 1 week at 4°C, and −20°C for 1 month.

⚠ CRITICAL: The reagent must be implemented in strict accordance with Regulations on the Control over Safety of Dangerous Chemicals and relevant regulations on the use, storage and disposal of hazardous materials.

STEP-BY-STEP METHOD DETAILS

DEN/CCl_4-induced HCC animal model

Timing: 24 weeks

This section describes the injection dose, administration route and treatment cycle for DEN combined with CCl_4 to induce HCC in mice.

1. Weigh each wild type (WT) and FBXO45-OE mice aged at 14 days.
2. Prepare DEN.
   a. Dilute the stock solution 40 times to obtain a working solution at a concentration of 2.5 mg/mL.

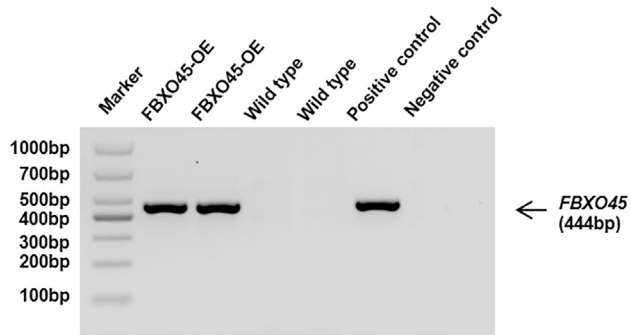

Figure 2. Genotyping results of FBXO45-OE mice by agarose electrophoresis
Obtained with permission and modified from Lin et al.[1]

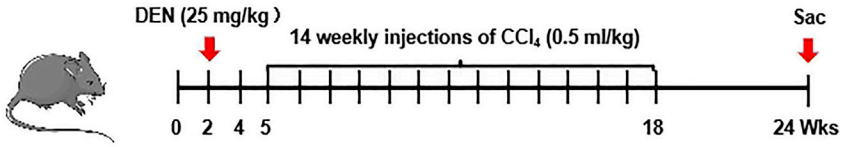

Figure 3. Experimental design in FBXO45-OE and WT mouse induced by DEN/CCl_4
Obtained with permission and modified from Lin et al.[1]

b. Calculate the dose of DEN based on the body weight of each mouse (25 mg/kg). For example: a 15 g mouse need to be injected with 150 μL of DEN working solution.

Note: DEN is highly toxic, which is irritating to the eyes, skin and mucous membranes, and may cause liver and kidney damage by ingestion, skin absorption and inhalation. Operators should wear protective glasses and gloves to avoid direct contact with DEN, and keep the operation site well ventilated.

3. Load the 1 mL syringe with solution.

Note: Tap the syringe and press the plunger slowly to expel the air, then set the syringe aside and wait for injection.

4. Disinfect the abdomen surface of mice with 70% ethanol.
5. Gently Immobilize the mouse neck with index and thumb finger, while the pinky and ring finger are used to prevent them from moving.
6. Inject DEN solution or saline intraperitoneally at a 45° angle.

Note: Generally, only 1/3 depth of the needle can be inserted for avoiding damage to the organs.

Note: The injection volume is about 150–200 μL per mouse and prepare an extra 30 μL in case of any spillage.

7. Prepare CCl_4.
   a. CCl_4 should be diluted on the day of administration. Mix thoroughly in the ratio of CCl_4 stock solution: olive oil = 1: 4 to obtain a final concentration of 20%.
   b. Calculate the amount of CCl_4 at a final concentration of 0.5 mL/kg.

Note: CCl_4 is a typical liver toxin with mild irritation of mucous membranes and anesthetic effects on the central nervous system. If once in contact with the skin, take off contaminated clothing immediately and rinse with plenty of running water, and seek medical attention.

8. At 4 weeks of age (two weeks after DEN injection), administer CCl_4 or vehicle (olive oil) twice a week for additional 14 weeks.[3]

Note: Operation details are consistent with DEN injection.

9. Leave mice for 6 weeks after the last injection of CCl_4 (Figure 3).

Note: The health condition of mouse should be monitored daily after injection. If in poor status, give some supportive care such as melon and eggs.

Tissue sample collection

Timing: 1 day

This section describes how to harvest tissues and collect the different liver pieces for each experimental condition.

10. Weigh all the mice.
11. Anaesthetize the mice with isoflurane and sacrificed by cervical dislocation.

Optional: You can choose other anesthetics as chloral hydrate, Nembutal, etc, approved by the IACUC for human euthanasia.

12. Fix the mouse limbs on the dissecting board with pins.
13. Disinfect the abdomen surface (70% ethanol).
14. Lift the skin with forceps and then cutting along the mid-line to open the abdomen and chest using surgical scissors.

Note: In order not to affect the experimental operation and the observation of the experimental results, it is better to shave the abdominal fur of the mice.

15. Separate livers from mice quickly.

Optional: If hepatocytes analysis is planned, high quality liver perfusion is essential before liver tissue collection. Deliver the perfusion solution into inferior cava vein using the peristaltic pump to flush the inside of the liver for 10 min. All liver lobes should turn white as erythrocytes drain from the organ. Reference to published standard protocols.[4]

16. Rinse the liver with a 50 mL syringe filled with ice-cold 1×PBS.
17. Observe and count the number of macroscopically visible liver tumors, measure the largest diameter with vernier calipers.

⚠ CRITICAL: Tissues harvesting should be done in a relatively short period of time.

18. Take photos of the liver and tumor.

Note: A ruler should be placed underneath to accurately represent the size of the tumor.

19. Record data of each mouse about body weight, liver weight, number and size of tumors, occurrence of liver metastasis.
20. Resect different liver lobes for downstream analysis.
    a. For protein and RNA, store a piece of fresh tissues in liquid nitrogen.
    b. For histochemistry, immerse a slightly larger piece of tissue in 10% formalin for 24 h and then placed in 70% ethanol for 24 h before paraffin embedding.

Note: It is important to cut the same liver lobe from the same area in each tissue sample.

Optional: Collect other tissues such as spleen, kidney, and pancreas if required for your experiment.

21. Perform paraffin embedding and slide cutting 5 μm sections for subsequent histopathological assay.
22. Keep the rest of sample in liquid nitrogen for long-term storage.

RNA isolation and quantitative real-time PCR

Timing: 1–2 days

This section describes RNA extraction, cDNA synthesis and how to determine gene expression levels by qRT-PCR.

23. Place tissue sample into a 1.5 mL RNase-free and DNase-free EP tube.
24. Add 1 mL RNAiso Plus reagent and using a homogenizer to release the total RNA from the tissue.

Note: RNAiso Plus contains a strong denaturant and should be avoided in contact with skin and clothing.

25. Add 200 μL chloroform to the tube, and vortex for 10 s with an oscillator.

Note: Because chloroform is toxic, working in a fume hood when using.

26. Incubate the suspension for 15 min at room temperature (20°C–25°C) and centrifuge at 12,000 × *g* for 10 min at 4°C.
27. Aspirate the supernatant carefully into a new RNase-free and DNase-free EP tube.

⚠ CRITICAL: There are 3 layers. The middle layer is semi-solid containing DNA, and the bottom layer is a red-colored organic solvent containing proteins, polysaccharides, fatty acid, cell debris, and a small amount of DNA. Upper aqueous layer containing RNA. Do not aspirate to the lower layer, otherwise it will easily cause DNA/protein contamination and affects subsequent experiments.

28. Add an equal volume of pre-cooled isopropanol, reverse up and down several times.
29. Centrifuge at 12,000 × *g* for 10 min at 4°C to precipitate the RNA.

Note: Store some isopropanol in the freezer 24 h before the RNA extraction.

30. To remove impurities from the RNA, add 1 mL DEPC water with 70% ethanol to the precipitate.

Pause point: The purified RNA should avoid repeated freeze-thawing and can be stored at −80°C for six months.

31. Synthesize cDNAs by the PrimeScript RT Reagent Kit with gDNA Eraser (https://www.takarabiomed.com.cn/DownLoad/RR047A.pdf) following the manufacturer's instructions.
32. Perform Relative fold-change in the expression of indicated genes by qRT-PCR using TB Green Premix Ex Taq II (https://www.takarabiomed.com.cn/DownLoad/RR820A.pdf).

Note: Calculate results based on the Ct value using the $2^{-\Delta\Delta Ct}$ method. We use GAPDH as a stable housekeeping gene; Each reaction takes 3 technical replicates and should show minimal changes.

qPCR reaction master mix	
Reagent	Amount
DNA template	2 μL
TB Green Premix EX Taq II	12.5 μL
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
ddH_2O	8.5 μL
Total	25 μL

qPCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	30 s	1
Denaturation	95°C	5 s	40 cycles
Annealing and extension	58°C–62°C	1 min	
Melt Curve Stage	95°C	10 s	1
	65°C	5 s	1
	95°C	10 s	1

Hematoxylin and eosin staining (H&E staining)

Timing: 1 day

This section describes the morphological and structural pathological changes of liver tissues in WT and FBXO45-OE mice.

33. Place the slides on a sectioning rack and bake in an oven at 65°C for 2–3 h to fully melt the paraffin wax.

Note: The baking time should not be too short as it is difficult to completely melt the paraffin, which may lead to uneven coloring of the sections; nor should it be too long as it may inactivate the antigens in the tissue and lead to false negative results.

34. Quickly dip the slides into 3 baths of xylene to deparaffinize 5 min each.
35. Transfer the slides to 100% anhydrous ethanol twice in sequence for 2 min each time.
36. Hydrate slides with 95% ethanol, 85% ethanol 75% ethanol and distilled water in a concentration gradient.

Note: Each bath for at least 3 min. And having 2 baths for each condition is better.

Note: Reagents must be placed in the right order when hydrating.

37. Add an appropriate amount of hematoxylin to the section for about 30 s, terminate the staining with distilled water.
38. Wash off the excess hematoxylin with tap water 15 min.
39. Drop the Eosin on the slides for about 15 s after the moisture is moderately removed with drying, terminate the staining with distilled water.
40. Wash off the excess eosin.
41. Transfer the slides sequentially to 3 concentration gradients of 70%, 80% and 95% ethanol solution for 2 min each time.
42. Put the slides into xylene and let it stand at room temperature (20°C–25°C) for about 5 min.
43. Drop a small amount of neutral balsam on the tissue, cover it with a coverslip.
44. Leave dry for at least 2 h to observe.

Note: Slowly press down the coverslip from one side to avoid air bubbles.

Immunohistochemistry staining assay (IHC)

Timing: 1 day

This section describes the quantification of the protein by antibody binding to the target antigen in the tissue.

45. Following the above steps 33–36, deparaffinize, rehydrate the tissue slides.
46. Boil the slides in 10 mM sodium citrate with pressure cooker at high pressure for 2.5 min.
47. Natural cooling to room temperature (20°C–25°C), remove and wash the slides with PBS or distilled water for 5 min 3 times.

Note: If the slides were removed from the autoclave into PBS immediately, the large temperature difference may cause the tissue to fall off completely or wrinkle.

48. Using the pap pen to draw a circle of appropriate size around the tissue.
49. Block in 3% hydrogen peroxide (diluted in distilled water) for 15 min in a wet box at room temperature (20°C–25°C).

Note: The fluid should be over the edge of the tissue to adequately block endogenous peroxidase.

50. Discard blocking solution and wash with PBS for 5 min 3 times.
51. Incubate with 5% goat serum (diluted in PBS) for 30 min at room temperature (20°C–25°C).
52. Add specific antibodies (diluted in PBS): anti-FBXO45 (1:300) and anti-Ki67 (1:800) to the tissue slides at 4°C overnight.
53. Wash in PBS 3 times for 5 min.
54. Incubate with secondary antibody (Immunohistochemistry Detection Kit) at room temperature (20°C–25°C) for 60 min.
55. Repeat step 53.
56. Incubate with horseradish peroxidase (Immunohistochemistry Detection Kit) at room temperature (20°C–25°C) for 30 min.
57. Repeat step 53.
58. Add 1–2 drops of DAB to the slides.

Note: DAB has a certain degree of toxicity and must be operated with good protective measures.

Note: It is best to observe the degree of color development under a microscope to determine the moment of termination.

59. Repeat step 53.
60. Subsequently, counterstain the slides with hematoxylin, and rinse with tap water 3 times for 5 min each time.
61. Repeat steps 41–44.
62. Photograph under bright field with magnification at x400.

Note: Determine the staining intensity using the Spot Denso function of the AlphaEaseFC software by selecting same object area between tissues from FBXO45-OE and WT mice.

EXPECTED OUTCOMES

Under DEN and CCl_4 treatment condition, high FBXO45 expression drives hepatocarcinogenesis in mice,[1] indicated by more tumors and higher liver weight in FBXO45-OE mice compared with WT mice(Figure 4). Consistent with this finding, the fibrosis- and inflammation- related genes, and cell proliferation biomarker Ki67 were dramatically increased expression in the liver of FBXO45-OE mice compared with that in WT mice (Figures 5 and 6). To monitor the pathological changes during DEN/CCl_4 treatment, liver tissues were collected at 6^{th}, 14^{th} week of CCl_4 treatment and 14^{th} week of CCl_4 treatment plus 6 weeks observation. The H&E staining results showed that FBXO45-OE mice at 6^{th} week of CCl_4 treatment group exhibited more extensive inflammatory cell infiltration than any

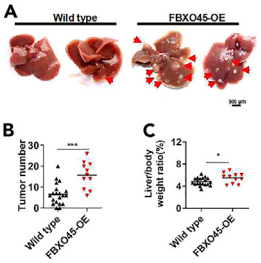

Figure 4. Representative image and statistical charts of livers

(A) Liver Photographs from FBXO45-OE (n = 11) and wild-type mice (n = 20). Scale bar, 500 μm.

(B) Tumor number ratio.

(C) The liver/body weight ratio. Data are represented as the mean ± SEM, *p < 0.05, ***p < 0.001. Obtained with permission and modified from Lin et al.[1]

other groups. Interestingly, H&E staining revealed a disorganized cell arrangement and significant heterogeneity with a clumped distribution in the liver tissues of FBXO45-OE mice at 14th week of CCl_4 treatment plus 6 weeks observation group compared with WT mice group (Figure 7). Taken together, these results suggest that FBXO45 accelerates inflammation and fibrosis during liver tumorigenesis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparisons between two groups were performed with a two-tailed unpaired t-test. When more than two groups were compared, one-way analysis of variance with Tukey post hoc test was used when data passed Shapiro-Wilk normality test. Differences are considered statistically significant at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

LIMITATIONS

There are still several limitations for this model. The combined induction requires CCl_4 injections at regular intervals, which makes the modeling process more cumbersome and cannot guarantee the absolute same dose and similar experimental environment as the previous injection. The formation of hepatocellular carcinoma is a dynamic and continuous process, and the application of interference measures will affect many indicators in mice, which is especially unfavorable to the experiments that require continuous weighing, monitoring of survival curves, and detection of biochemical parameters of serum.

TROUBLESHOOTING

Problem 1

Lower reproductive capacity in mice (step 6 of before you begin).

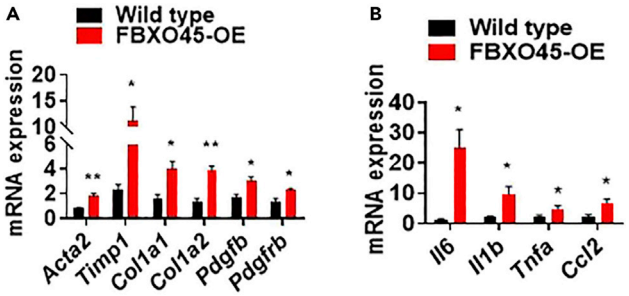

Figure 5. Real-time PCR analysis

(A) Transcript levels for the fibrosis-related genes (Acta2, Timp1, Col1a1, Col1a2, Pdgfb, and Pdgfrb).

(B) Transcript levels for the inflammatory markers (Il6, Il1b, Tnfa, and Ccl2). Data are represented as the mean ± SEM, *p < 0.05, **p < 0.01. Obtained with permission and modified from Lin et al.[1]

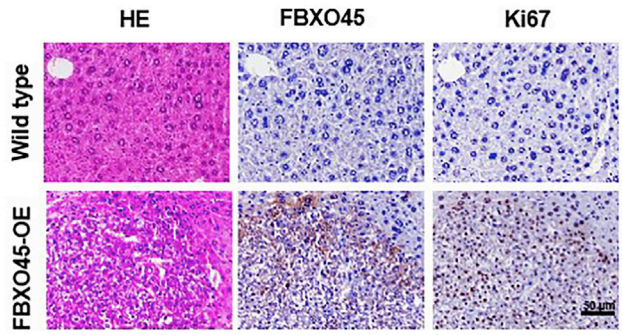

Figure 6. Representative IHC images of FBXO45/Ki67 signals in liver tissue samples
Scale bar, 50 μm. Obtained with permission and modified from Lin et al.[1]

Potential solution

Try to select females in estrus for breeding, and use 2:1 or 1:1 male-to-female ratio to increase pregnancy rate.

Problem 2

Mouse dies quickly after DEN injection (step 6 of step-by-step method details).

Potential solution

There are two possible reasons that lead to an early death after DEN injection.

Reason 1: due to the acute hepatotoxicity of DEN, it is likely to cause premature death and higher mortality in pups at the early stage of modeling. You can change the way of administration, such as gavage[5] and adding drugs to drinking water and feeds.[6] Always pay attention to direct contact with DEN during operation.

Reason 2: Air embolism can be caused by air entering the circulation. Therefore, it is critical to make sure suspension was absolutely air-free inside the syringe before injecting mice.

Problem 3

No tumorigenesis observed after 24 weeks of DEN/CCl_4 injection (step 17 of step-by-step method details).

Potential solution

In treated with both DEN and CCl_4, all animals developed hepatic adenomas at 22 weeks of age, 50% of which exhibit HCC.[7] If no significant tumors were observed after injection, it is necessary to switch to a mouse strain with higher susceptibility.[8] Have mouse strain positive control

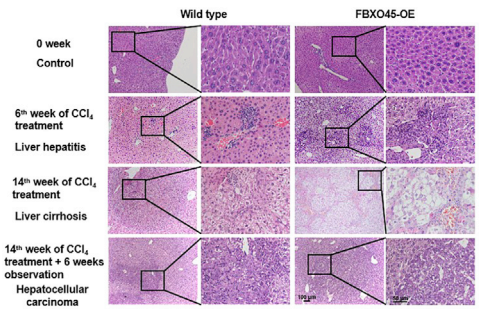

Figure 7. Effect of FBXO45 on the histological changes of liver tissues in DEN/CCl4-treated mice
Representativeimages of H&E staining showing the sequential progression of hepatitis, cirrhosis, and hepatocellular carcinoma in FBXO45-OE mice. Scale bars, 100 μm or 50 μm.

(C57BL/6J) if people use other mouse strains. Fibrosis should be evaluated to validate the effect of the CCl_4. As this protocol only focuses on C57BL/6J mice, people may use other mouse strains, but it might take longer in different animal facilities (microbiota). In addition, be sure the reagents are fresh and up to date prior to use.

Problem 4

Differences in CT values between technical replicates (step 32 of step-by-step method details).

Potential solution

In addition to improving the accuracy of spiking and correcting the fixed parameters of the instrument, it is necessary to optimize the experimental conditions as much as possible to achieve the best amplification efficiency of the reaction system.

Problem 5

Non-specific signal after staining of tissue sections (step 62 of step-by-step method details).

Potential solution

- Do not allow tissues to dry out between steps. Make sure that adequate coverage of tissue during antibody incubation and washing.
- Optimize antibody potency, including concentration, incubation time and temperature; change the wash buffer factors (e.g., by adding bovine serum albumin or fetal bovine serum to the buffer; diluting buffer from 1× to 0.5×).
- Reduce background staining by extending inactivation time and increasing inactivator concentration. Change the saturation (other industrial reagents, BSA 2%).
- Antigen retrieval pH or tools (pressure cooker, water bath, microwave).
- Different timing: antibody 1 h at room temperature (20°C–25°C) or overnight at 4°C.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chuan-Ming Xie (cmxie@tmmu.edu.cn).

Materials availability

No new materials were generated in this study.

Data and code availability

No new datasets or code was generated in this study.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (32071294) and the Introduction of Special Funds for Talents (4174C6) from the Third Military Medical University (Army Medical University) to C-.M.X.

AUTHOR CONTRIBUTIONS

C-.M.X. designed the experiments, L.F. performed mice breeding and carcinogen induction, X-.T.L. contributed to IHC staining and pathological analysis, J.Z. performed qRT-PCR and interpreted the data, and J.Z. and C-.M.X. drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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