Journal of Gastrointestinal Cancer (2021) 52:1248–1265
https://doi.org/10.1007/s12029-021-00782-z

INVITED REVIEWS

Zebrafish Xenotransplantation Models for Studying Gene Function and Drug Treatment in Hepatocellular Carcinoma

Seniye Targen[1] · **Ozlen Konu**[1]

Accepted: 13 November 2021 / Published online: 15 January 2022

Abstract

Introduction Zebrafish is a promising model organism for human disease including hepatocellular cancer (HCC). Recently, zebrafish has emerged also as a host for xenograft studies of liver cancer cell lines and patient derived tumors of HCC. Zebrafish embryos enable drug screening and gene function studies of xenografted cells via ease of microinjection and visualization of tumor growth and metastasis.

Objectives In this review, we aimed to overview zebrafish HCC and liver cancer xenotransplantation studies focusing on 'gene functional analysis' and 'drug/chemical screening'.

Methods Herein, a comprehensive literature search was performed for liver and HCC xenografts in zebrafish on PubMed using different key words and filters for molecular modifications or drug exposure.

Results Our literature search revealed around 250 studies which were filtered and summarized in a table (Table 1) revealing comprehensive collection of experimental and technical details on microinjection, injected cell lines, molecular modifications of injected cells, types and doses of drug treatments as well as biological assessments.

Conclusion This review provides a platform for HCC and liver xenografts and highlights studies performed to understand gene functionality and drug efficacy in vivo in zebrafish.

Keywords Zebrafish · Gene function · HCC · Xenograft

Introduction

Establishment of zebrafish xenotransplantation models of both immortalized cell lines and patient-derived tissue has pioneered a new era in cancer biology research as thoroughly reviewed by leading scientific research groups [1–3]. In the present review, specifically the hepatocellular carcinoma (HCC) and liver cancer xenograft models will be addressed with a particular emphasis on immortalized cell injection applications including gene functionality studies, generation of drug screening platforms, and patient-derived xenograft applications.

To date, tens of different cancer types of xenografts have been successfully applied to the zebrafish model, and HCC and liver cancer cell injections account for a fair share of the pie [3]. The field is growing; for example in 2020, a comprehensive systematic review performed by our group identified around 200 zebrafish xenotransplantation focused articles (of which seven, belonged to studies only focused on hepatocellular cancer), which we integrated to ZenoFishDb v1.1, a searchable database housing xenotransplantation studies in zebrafish [3]. Since then our ongoing literature search and others [4] identified many more.

Majority of the zebrafish xenograft models established for liver cancer consist of immortalized cell line engraftments and have been used for assessing different aspects of carcinogenesis including tumor growth, tumor foci formation and proliferation [5–7]; cancer stem cell proliferation [8]; metastasis-related events such as migration, dissemination, extravasation, and intravasation [9–12]; angiogenesis [5, 13]; and tumor microenvironment [14] under different experimental settings, mostly in embryos but some using immunosuppressed adult fish [15, 16].

✉ Ozlen Konu
konu@fen.bilkent.edu.tr

[1] Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

Table 1 Zebrafish xenotransplantation models of hepatocellular carcinoma cells: a highlight on "HCC cell injections with genetic modifications" and "chemical testing"

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HLF	n/a	hMOF	(siRNA application)	Fluorescently labeled (DiI dye) HLF cells were transplanted into the perivitelline space of the Tg(fli1:eGFP) fish at 2 dpf and maintained at 34 °C. In one set-up, the si-hMOF modified HLF (test) or mock cells were used for transplantation. Number of tumor cells at caudal hematopoietic tissue (CHT) and their intravasative and metastatic ability were assessed at 24 hpi. In the second set-up, a mixture of si-hMOF HLF cells or mock cells (1:1 ratio) was used for transplantation. The number of tumor cells at CHT was assessed at 24 hpi and 48 hpi	Intravasation and metastasis were assessed. Silencing of hMOF inhibited HCC cell intravasation and metastasis in HCC zebrafish xenografts	Poté et al. [12]
HCC-LM6	n/a	MAT2B	(shRNA application)	HCC-LM6 shMAT2B or mock cells, 150–200 in number, were injected to yolk sac of 2 dpf wild-type fish and maintained at 33 °C. Relative migration range was analyzed for the test and control xenograft groups	Migration was assessed. MAT2B-silenced cell xenografts exhibited diminished tumor cell migration	Wu et al. [9]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HCC-LM3	Adrenosterone	HSD11β1	(shRNA application)	Fluorescently labeled (RFP) HCC-LM3 cells (HCC-LM3R) were injected into the duct of Cuvier of the Tg(kdrl:eGFP) fish at 2 dpf. Experimental designs included establishment of (a) HCC-LMR3R xenografts in the presence and absence of adrenosterone and (b) shHSD11β1 or mock targeted HCC-LM3R xenografts without drug exposure. Frequency of fish showing regional cell dissemination percentage, i.e., head, trunk, and end-tail, was then analyzed at 24 hpi	Metastatic dissemination was assessed. Inhibition of HSD11β1 via pharmacological (adrenosterone) or genetic ablation (gene silencing) suppressed metastatic dissemination of HCC cells in vivo	Nakayama et al. [10]
HepG2	n/a	eIF3i	(shRNA application)	App. fifty-hundred fluorescently labeled eIF3i shRNA-modified HepG2 cells or mock cells were injected to the perivitelline space of 2 dpf Tg(flk:mcherry) embryos. The embryos were kept at 33 °C for a week	Angiogenesis was assessed. eIF3i shRNA HepG2 cell-implanted embryos exhibited angiogenesis to much lesser extent, almost unobvious, when compared to control cell injections	Yuan et al. [35]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
Hep3B	DMJ (1-deoxymannojirimycin), 4-PBA (sodium 4-phenylbutyrate), TUDCA (tauroursodeoxycholic acid)	MAN1C1, MAN1A1	(Overexpression-MAN1C1, MAN1A1), (shRNA-MAN1A1)	Overall, approximately two hundred fluorescently labeled cells (CFSE/DiI dye) were injected to the yolk sac of 2 dpf fish. Drugs (inhibitors) 4-PBA (50 μM), TUDCA (500 μg/ml), and 1-DMJ (50 μM) were added at 1 dpi. Migratory and proliferative capability of tumor cells were assessed at 1 dpi and 3 dpi Set-up 1: MAN1C1 overexpressing Hep3B cells or mock cells Set-up 2: MAN1A1 overexpressing Hep3B cells or mock cells exposed to DMJ, 4-PBA, TUDCA, MAN1A1shRNA, or control	Proliferation and migration were assessed. For the first set-up, embryos with migration behavior percentage and CFSE increasing percentage were graphed and MAN1C1-overexpression led to decreased migratory and proliferative behavior of HCC cells in vivo. For the second set-up, embryos with proliferative behavior percentage and CFSE increasing percentage were graphed and proliferative capability of MAN1A1-Hep3B cells was inhibited by DMJ-1, 4-PBA, TUCDA, and MAN1A1 shRNA treatments	Tu et al. [17]
SNU-449	n/a	lncRNA HOTAIR	(Overexpression)	One hundred fluorescently labeled (DiI dye) SNU-449-HOTAIR overexpressing or mock cells were injected to the yolk sac of 2 dpf fish. The metastatic capacity of the cells was analyzed 4 dpi	Metastasis was assessed. Metastasis percentage was upregulated in HOTAIR overexpressing SNU-449 xenografts	Topel et al. [19]
Hep3B	Doxycycline	TAp73β	(Overexpression)	Doxycycline-inducible, TAp73β expressing Hep3B (Hep3B-TAp73β) cells were used for xenotransplantation. Three hundred fluorescently labeled (Vybrant DiI dye) Hep3B-TAp73β cells, which were previously treated with/without doxycycline for 72 h, were injected to the yolk sac of 2 dpf fish. Metastasic tumor percentage was graphed at 4 dpi	Metastasis was assessed. Metastatic tumor percentage was graphed at 4 dpi and doxycycline-induced TAp73β expression enhanced metastatic tumor percentage	Iscan et al. [18]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HuH7	R-Spo1 and Wnt3a recombinant proteins	LGR5	(Overexpression)	App. two hundred and fifty fluorescently labeled (DiI dye) LGR5 overexpressing Huh7 cells or mock cells were injected to yolk sac of 2 dpf fish. The xenografts were then exposed to R-Spo1 and Wnt3a recombinant proteins as treatment group or no treatment. The fish were then kept at 34 °C. The assessment ended at 5 dpi	Migration and dissemination of tumor cells were evaluated at 5 dpi and metastasis percentage was graphed. LGR5-overexpressing Huh7 cells exhibited enhanced metastasis percentage when compared to mock Huh7 cells	Akbari et al. [20]
HepG2	Compound Phyllanthus urinaria L. (CP)—a formula composed of different herb use which is used as a traditional Chinese medicine	HBx	(Overexpression)	Two hundred fluorescently labeled (DiI dye) HBx overexpressing cells were injected into the perivitelline space of 2 dpf fish. Then fish were exposed to different concentrations of CP (60 µg/ml or 120 µg/ml) or no CP (control). Tumor growth and metastasis were assessed by fluorescent microscopy imaging and ImageJ software analysis. Hematoxylin and eosin staining was also used for tumor growth assessment along with immunohistochemistry analysis for assessing caveolin-1, B-catenin, vimentin, and E-cadherin expression	Tumor growth and metastasis were assessed. Tumor growth and metastatic puncta were graphed. Overall, CP decreased tumor growth and metastasis in HCC-cell transplanted embryos. Caveolin-1, B-catenin, and vimentin protein expressions were downregulated whereas E-cadherin expression was upregulated in the tumor injected zebrafish	Huang et al. [21]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HepG2	n/a	CRIPTO	(Overexpression)	Initially, HepG2-CRIPTO cells (GFP+) (p1) derived from HEPG2-CRIPTO tumors established in immunodeficient mice. Then, fluorescently labeled HepG2-CRIPTO-p1 cells, HepG2-CRIPTO cells, or mock cells were used for injections. App. two hundred cells of HepG2-CRIPTO cells or mock cells were injected to the duct of Cuvier of 2 dpf fish. The fish were then kept at 33 °C. Tumor foci were assessed at 2 dpi	Migration and tumor foci formation were assessed. Foci count (number) was graphed for HepG2-CRIPTO-p1. HepG2-CRIPTO or mock-control groups. Both test groups exhibited increased foci formation at 2 dpi	Karkampouna et al. [6]
PLC5	ERK inhibitor III, β-catenin inhibitor (ICRT14)	RPIA	(Overexpression)	Two hundred fluorescently labeled (CFSE dye) RPIA-overexpressing PLC5 or mock cells were injected into the yolk sac of 2 dpf fish. The cells were treated with ERK inhibitor III, ICRT14 (β-catenin inhibitor), or both 24 h prior to injection. Then the fluorescence intensity was obtained at 1 dpi and 3 dpi	Proliferation was assessed. The CFSE changes' percentage and percentage of embryos were calculated for depicting proliferation inhibition and hepatoma cell proliferation, respectively. Overall, RPIA-PLC5 injected fish had higher proliferation rate when compared to control. ICRT14 (β-catenin inhibitor) had inhibitory effect on RPIA expressing PLC5 cell proliferation in xenograft model	Chou et al. [22]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
Hep3B	PTK787 (VEGFR tyrosine kinase inhibitor), WNK463 (pan WNK-kinase inhibitor), closantel (OSR1/SPAK kinase inhibitor)	n/a	n/a	Overall, app. two hundred Hep3B (RFP labeled) cells were injected to the yolk sac of Tg(fli1:eGFP) fish. Temperature was raised from 28 to 37 °C gradually, and fish were maintained at 37 °C. For the drug assessment part of the experimental set-ups, Hep3B injected transgenic embryos treated with either PTK787 (5 µM), WNK463 (2.5 µM), closantel (0.15 µM), or control were assessed for their proliferative capacity	Proliferation and angiogensis were assessed. Representative images were obtained at 1 dpi and 3 dpi. Cell proliferation area (percentage of embryos), cell proliferation intensity (percentage of embryos), percentage change (area), and percentage change (intensity) were graphed. Overall, tumor size was reduced with either of the treatments	Sie et al. [13]
BEL-7402	JNK-IN-8—a pan-JNK inhibitor, JSH-23—a NF-κB inhibitor	n/a	n/a	App. two hundred fluorescently labeled BEL-7402 cells transplanted to the yolk sac of 2 dpf embryos. Then, at 2 dpi the fish were exposed to JNK-IN-8 (10 µM), JSH-23 (5 µM), and combination of drugs (JNK-IN-8 (10 µM) + JSH-23 (5 µM)) or control treatment	Tumor growth and dissemination were assessed. RFI tumor mass, inhibitory rate percentage, and number of disseminated foci from tumor mass were graphed. JNK-IN-8 and JSH-23 drugs exhibited synergistic effect and decreased tumor growth in vivo	Gao et al. [26]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HepG2	RU-A1 (BMI1 inhibitor)	n/a	n/a	Fluorescently labeled HepG2 cells (Luc-EGFP HepG2) to be transplanted had been exposed to RU-A1 (10 µM) for 72 h. Drug-exposed cells or mock cells were then injected to perivitelline space of 2 dpf casper embryos. The embryos were kept at 34 °C for 2 days and assessed for tumor formation. The fish were imaged at 2 dpf and 4 dpf	Tumor formation and survival analysis (Kaplan–Meier plots) were analyzed (day 0–day 10). Fluorescence intensity (% EGFP positive tumor area) was calculated; RU-A1 administration resulted in tumor growth inhibition. Percent survival was assessed for control, untreated, and RU-A1 treated fish. Untreated fish (bearing tumor cells) had lower survival rate when compared to RU-A1 treated fish (bearing tumor cells)	Bartucci et al. [8]
HepG2	Phenanthroimidazole derivative no. 6	n/a	n/a	Fluorescently labeled (DiI dye) HepG2 cells were injected into the perivitelline space of the Tg(fli1:eGFP) fish at 2 dpf. The fish were then treated with 0, 1, or 3 µM of drug no. 6 for 48 h. The proliferation multiple of tumor cells graph was generated by analyzing tumor cell fluorescence intensities at 48 hpi	Proliferation and growth inhibition were assessed. Phenanthroimidazole derivative no. 6 induced growth inhibition in HCC zebrafish xenografts	Zhang et al. [36]
HuH7	Vitamin K3 chloro derivative (VKT-2)	n/a	n/a	Two hundred fluorescently labeled (CM-DiI dye) Huh7 cells were injected to the yolk sac of 2 dpf fish. The inhibitory effect of VKT-2 (1 pM) was assessed by the means of fluorescence intensity and inhibitory rate percentage	Tumor growth was assessed. VKT-2 inhibited tumor growth in vivo	Dawood et al. [37]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HepJ5, Hep3B	Propyl gallate (PG)—synthetic antioxidant used for food preservation	n/a	n/a	App. two hundred fluorescently labeled (CFSE dye) HepJ5 or Hep3B cells were injected to yolk sac of 2 dpf fish. The fish were then treated with alternating doses of propyl gallate (PG) ranging from 0–40 µg/ml or vehicle control. Fluorescent cells were examined at 1 dpi and 3 dpi	Proliferation activity was graphed, as percentage for both cell lines. Overall, PG played role in suppressing cell proliferation in HCC xenografts in zebrafish	Wei et al. [24]
HuH7	Aloperine—quinolizidine-type alkaloid obtained from Sophora alopecuroides L. herb	n/a	n/a	Two hundred fluorescently labeled (CM-DiI dye) Huh7 cells were injected to yolk sac of 2 dpf fish. The fish were then exposed to different doses of aloperine (0.05 mM, 0.1 mM, 0.1 mM) or sorafenib (1 µM) (positive control). Upon treatment, they had been maintained at 28.5 °C for 72 h. Tumor formation was assessed at 72 hpi	Tumor formation and growth were assessed. Aloperine appeared to be inhibiting tumor formation in vivo	Liu et al. [23]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HuH7	Theabrownin (TB)—main bioactive constituent of tea, SP600125 (JNK inhibitor)	n/a	n/a	Two hundred fluorescently labeled (CM-DiI dye) Huh7 cells were injected to the yolk sac of 2 dpf fish. For one experimental design, at 3 dpf for 24 h, the fish were treated with theabrownin (16.7 μg/ml) or cisplatin (15 μg/ml) as treatment groups and a non-treated model existed as control. For another experimental design, at 3 dpf for 24 h, the fish were treated with theabrownin (TB; 16.7 μg/ml), SP600125 (20 pM), and theabrownin (16.7 μg/ml) + SP600125 (20 pM) and a non-treated group was included. For both designs, the fluorescence intensity and inhibitory rate percentages were calculated	Tumor growth was assessed. Theabrownin inhibited tumor growth in vivo. SP600125 diminished the inhibitory effect of TB in vivo	Xu et al. [27]
HepG2	Arenobufagin (ArBu)—a natural bufadienolide constituent of toad venom, ingredient of Chan Su traditional Chinese medicine	n/a	n/a	App. eight hundred fluorescently labeled (CM-DiI dye) HepG2 injected to 2 dpf fish and then the fish were treated with different doses of ArBu (1.1 ng/ml or 3.3 ng/ml) or cisplatin (14.9 μg/ml) (positive control) for 3 days. Next, the fluorescence intensity and inhibitory rate percentage were addressed	Tumor growth and lipidomic analysis were performed. ArBu administration exhibited anti-tumor activity in vivo. ArBu regulated lipid homeostasis in zebrafish xenografts	Zhao et al. [25]
Hep3B	1β–OH–arenobufagin	n/a	n/a	App. two hundred fluorescently labeled (CM-DiI dye) Hep3B cells were injected to yolk sac of 2 dpf zebrafish. The embryos were treated with different doses of 1β–OH–arenobufagin (2.5, 5 or 10 μM) or sorafenib (positive control)	Tumor formation, p-mTOR (Ser 2448) expression, and apoptosis were assessed. 1β–OH–arenobufagin had anti-tumor effect in vivo	Deng et al. [38]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
Hep3B	Saringosterol acetate (SSA)—derived from brown algae, Hizikia fusiforme	n/a	n/a	Adult and embryo zebrafish xenografts were established. For the adult fish xenograft, different doses of SSA (2 µg/g or 5 µg/g) was injected once every three days. One week later, fluorescently labeled Hep3B cells (CM-DiI dye) (2×10^6 or 5×10^6 cells) were injected to the abdominal cavity of fish, ten injections for a month. For the embryonic xenograft, four hundred fluorescently labeled Hep3B cells were injected to the yolk sac of 2 dpf Tg(fli1:EGFP) fish. The SSA (12.5 µg/ml) were then administered and the embryos were incubated 31–33 °C for 48 h. Fluorescent microscopy was used for analyses	Tumor growth and metastasis were assessed. SSA suppressed tumor growth and metastasis in adult fish xenografts. Tumor growth was also inhibited by SSA in embryo injected xenografts	Kim et al. [16]
HepJ5, Hep3B	Methyl gallate (MG)—compound derived from gallic acid	n/a	n/a	App. two hundred fluorescently labeled HepJ5 or Hep3B cells were injected to yolk sac of 2 dpf zebrafish embryos. The embryos were then treated with 40 µg/ml MG or vehicle and the fluorescence intensity were assessed at 1 dpi and 3 dpi for assessing proliferation	Proliferation was assessed. Proliferation activity (1 dpi vs. 3 dpi) was shown in percentages. MG treatment was shown to be decreasing proliferative activity of HCC cells	Huang et al. [39]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
Bel-7402, Hep3B, and HuH7	Jiangan granules (JG)—drug combination composed of glycyrol, inermin, and bilobalide	n/a	n/a	App. two hundred fluorescently labeled Bel-7402, Hep3B, or HuH7 cells were injected to yolk sac of 2 dpf wild-type zebrafish embryos. The embryos were then kept at 35 °C until 3 dpf and then treated with JG granules 27.8, 83.3, or 250 μg/ml or cisplatin 15 μg/ml (positive control) or vehicle control. The fluorescence intensity was then used for assessing tumor growth inhibition	Tumor growth inhibition percentage was assessed and graphed. JG granules demonstrated inhibitory effect in vivo	Chen et al. [40]
HepG2	Polyphyllin VII (PP7), a steroidal saponin extracted from Paris polyphylla	n/a	n/a	Fluorescently labeled HepG2 cells (CM-DiI) were transplanted into the yolk sac of 2 dpf embryos. Next, the embryos were treated with varying concentrations of PP7 (0.13, 0.25, 0.50, and 0.75 μM). The assessments were obtained 3 days after following drug treatment, at 3 dpi, by employing a laser scanning confocal microscope	Tumor volume, dissemination, and metastasis were assessed. Tumor volume and number of disseminated foci from tumor mass were graphed. PP7 inhibited tumor growth and dissemination	Zhang et al. [41]
HuH7	Busulfan	n/a	n/a	Establishment of adult fish transplantation for Huh7 cells was designed. Two days prior to transplantation, busulfan (20mg/kg body weight) was administered by i.p. injection to casper fish. Then, fluorescently labeled 1×10^5 HuH7 cells were injected to the fish trunk (near dorsal aorta). After recovery, the fish were maintained at 34 ºC. The fish were then imaged at 3, 7, and 15 dpi for monitoring tumor grafts and progression. Fish were euthanized at 15 dpi	Survival analysis (Kaplan–Meier plots) and tumor development were assessed. Approximately 30% of HuH7-transplanted adult fish died by 15 dpi, and overall survival accounted for app. 71%. Tumor was observed at 15 dpi at the peritoneal cavity	Khan et al. [15]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
Bel-7402	Recombinant human disintegrin domain of A disintegrin and metalloproteinase 15 (rhddADAM15)	n/a	n/a	App. eight hundred fluorescently labeledBel-7402 cells (CM-DiI dye) were injected into the yolk sac of 2 dpf fish embryos. After 1 day, rhddADAM15 (1, 2.5, 5, or 7 pM) or batimastat (490 pM) (positive control) was also injected to the yolk sac The embryos were imaged before and after 2 days of drug administration. Growth inhibition rate and metastasis were assessed	Proliferation, tumor growth, and metastasis were assessed. Total fluorescence intensity and total fluorescence area were graphed. rhddADAM15 exerted anti-proliferative effect in vivo	Hou et al. [42]
HepG2, Bel-7402	Adriamycin, gemcitabine, hydroxyurea, cis-platinum, 5-FU, furanodiene (natural terpenoid derived from Rhizoma Curcumae herb)	n/a	n/a	Fluorescently labeled HepG2 or Bel-7402 cells (CM-DiI dye) were injected to the yolk sac of 2 dpf fish embryos. After recovery, fish were maintained at 35 ℃ HepG2 cell transplanted xenografts were exposed to adriamycin (0.5, 1, or 2 ng), gemcitabine (5, 10, or 20 ng), hydroxyurea (250, 500, or 1000 μg/ml), cis-platinum (0.5, 1, or 2 ng), or 5-FU (65, 130, or 260 μg/ml) Bel-7402 xenografts were exposed to 5-FU (333 μM), furanodiene (1.4 μM), or furanodiene + 5-FU combined (1.4 + 333 μM)	Tumor growth was assessed. Cancer inhibition percentage was graphed. Overall, medium and higher doses of adriamycin (1 or 2 ng), gemcitabine (5, 10, or 20 ng), hydroxyurea (250, 500, or 1000 μg/ml), cis-platinum (0.5, 1, or 2 ng), or 5-FU (130 or 260 μg/ml) exhibited cancer inhibition in HepG2 xenografts Furanodiene exhibited synergistic effect when administered together with 5-FU and exhibited anti-HCC behavior in vivo	Zhu et al. [43]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HepG2, SNU-449	n/a	TXNIP	(Overexpression, siRNA)	Two-three hundred fluorescently labelled (DiO dye) HepG2 (TXNIP overexpressing or Mock) or SNU-449 (TXNIP-silenced or Mock) cells were injected into the yolk sac of 2 dpf fish. Embryos were maintained at 35 °C. Metastasis was assessed at 3 dpi	Metastasis was assessed. Metastatic fish percentage was graphed. HepG2 xenografts: TXNIP overexpression was associated with enhanced metastatic ability. SNU-449 xenografts: TXNIP silencing resulted in reduced metastatic fish percentage	Gunes et al. [44]
HuH7	11-{[3-(Dimethylamino) propoxy]imino}-N-[3-(dimethylamino) propyl]- 11H-indeno[1,2-b] quinoxaline-6- carboxamide hydrochloride (10a)	n/a	n/a	App. one hundred fluorescently labelled HuH7 cells were injected into the yolk sac of 2 dpf fish. The fish were then treated with different doses of 10a (0.1, 0.5 or 2 µg/ml) or vehicle control	Tumor growth and survival rate percentage of xenografted fish were assessed. 10a exhibited anti-tumorigenic effect in vivo. Survival rate did not change	Tseng et al. [45]
JHH6	Bortezomib	n/a	n/a	App. five hundred fluorescently labelled (DiI dye) JHH6 cells were injected to the yolk sac of 2 dpf fish. After recovery, the fish were maintained at 34 °C. Fish were imaged at 1 and 3 dpi JHH6 injected fish were exposed to Bortezomib (20nM) at 1 dpi or received no treatment (control group) for two days. Tumor mass growth images were captured at 1dpi and 3 dpi	Tumor mass growth was assessed. Human Ki67 expression (mRNA) was analyzed. Tumor mass increase percentage was graphed. Bortezomib reduced tumor growth in vivo	Tonon et al. [5]
HepG2	Oridonin	n/a	n/a	App. one hundred and fifty fluorescently labelled (DiI dye) HepG2-Luciferase cells were injected to the perivitelline space of 54 hpf fish. The fish then received Oridonin (treatment group), Avastin (positive control group) or no treatment (control group). Fish were then kept	Metastasis was assessed. Heartbeats, body weights in mg and survival times were assessed. Metastasis area was graphed. Oridonin exerted anti-metastatic properties and enhanced survival times	Tian et al. [46]

Table 1 (continued)

Cell line	Tested chemical	Modified gene	Modification	Study design	In vivo assessment and outcome	Reference
HepG2	Polypyridyl Ru (II) complex 1	n/a	n/a	Fluorescently labelled (DiI dye) HepG2 cells, fifty to five hundred in number, were injected to the perivitelline space of 2 dpf Tg (fli1:EGFP) fish. The fish were treated with (1.5, 3 or 6 μM) of Polypyridyl Ru (II) complex 1 for 72 hours. Control and treatment groups were then imaged at 0 h and 72 h for analysis	Proliferation was assessed. Polypyridyl Ru (II) complex exerted anti-tumorigenic activity by suppressing proliferation in vivo	Li et al. [7]
HuH7	4-[4-(4-hydroxyphenoxy)phenoxy]phenol (4-HPPP)	n/a	n/a	App. two hundred fluorescently labelled (DiI dye) HuH7 cells were transplanted to the yolk sac of 2 dpf fish. The fish were then treated with 1 μM of 4-HPPP for 24 and 48 hours	Tumor growth was assessed. Tumor volume percentage was graphed. Anti-tumorigenic effect of 4-HPPP was moderate in vivo. No significant inhibitory effect was detected in vivo	Chang et al. [47]

Current State of the Field

In this review, we provide a detailed table (Table 1) in which we have summarized the existing HCC cell line xenografts performed in zebrafish host via a comprehensive literature search. Accordingly, four independent PubMed searches were made for the "hepatocellular carcinoma zebrafish," "hepatocellular carcinoma zebrafish xenograft," "liver zebrafish xenograft," and "liver zebrafish xenotransplantation" keywords. A through screening of articles for these keywords revealed approximately around 250 studies which were then screened for studies that focused on "gene functionality" or "drug/chemical testing," and hence, we systematically gathered them into Table 1. Overall, the most of HCC/liver cell transplantations, regardless of the biological assessment purpose, were performed on 2 days post fertilization (dpf) zebrafish larvae and frequently injected to the yolk sac. In general, the studies addressed the anti-HCC role for tested drugs, plant extracts, or signaling pathway inhibitors. The remaining part of the xenotransplantation studies were based on deciphering the function of tested/investigated genes in vivo through molecularly modified-cell injections.

Experimental Design and Coverage of Liver Cancer Cell Transplantation Studies in the Zebrafish Literature

With respect to concept and design, the literature on HCC/liver cells xenografted in zebrafish could be broadly parsed into two: (a) studies focusing on gene functionality and (b) those addressing drug and/or chemical testing as summarized in Table 1. The former mainly includes cells modified with small interfering RNAs (siRNAs) [12], short hairpin RNAs (shRNAs) [10, 17], or expression vectors [18–22]. Hence, the effects of protein silencing or overexpression of genes on cells could be easily tested in vivo for different biological assessments including tumor growth, proliferation, and metastasis. The latter (b) approach can also incorporate such molecular modifications, yet the main focus is to test the therapeutic effect of drugs on the xenografts.

Tested drugs or compounds are of various origin; herb extracts [23], synthetic antioxidants [24], and kinase inhibitors [13] are among many. For instance, traditional Chinese medicine (TCM) of different kinds has been assessed for their anti-HCC effects in vivo employing zebrafish xenografts. Examples of the tested TCM constituents include compound *Phyllanthus urinaria* L. (a formula composed of different herbs) [21] and arenobufagin (a natural bufadienolide constituent of toad venom) [25] as depicted in Table 1. In addition, pharmaceutical drugs such as kinase inhibitors are often used for testing proliferative capacity of liver cancer cell transplants in vivo. For instance, anti-HCC effects of pan-WNK kinase inhibitors (WNK463) or VEGFR tyrosine kinase inhibitors (PTK787) were tested on liver zebrafish xenograft models [13]. Other kinase inhibitors tested in zebrafish xenograft assessments include JNK-IN-8, a pan-inhibitor of c-JUN N-terminal kinase [26] and SP600125 (JNK inhibitor) [27] as specified in detail in Table 1.

Another category is the use of patient-derived cells injected into the zebrafish host paving the way for precision medicine [3] as also have been the case in mice models [28–30]. Nevertheless, the zebrafish xenograft appli-cations of HCC and/or liver cells [31] remain elusive although development of such models would be essential step towards high-throughput drug screening for liver cancer therapeutics. One such study was performed by Lin and colleagues wherein they have injected patient-derived HCC tissue-derived cells and screened for anti-HCC effects of novel small molecules. In summary, they have studied the proliferative capacity of cells derived from a panel of liver cancer patients in the presence of well-established anti-HCC drug, sorafenib and two other novel multiple tyrosine kinase inhibitors, BPR1J419S1 and BPR1J420S1. Interestingly, they found that BPR1J419S1 and BPR1J420S1 were more effective in terms of their anti-proliferative capacities when compared to sorafenib [31]. This study highlights the potential use of "zebrafish Avatars" for developing personalized therapy towards liver cancer as previously aimed for different types of cancers including colon, gastric, pancreas cancers [32, 33] and melanoma [34].

Conclusions

In summary, we have identified the current state of HCC/liver cell xenotransplantation in zebrafish focusing on the types of cell lines, molecular modifications and drugs used as well as the zebrafish strain and specifications of injection and the biological assessments, which included proliferation activity, tumor growth inhibition, dissemination, intravasation, and metastasis. As illustrated in Table 1, a wide range of HCC and liver cancer cell lines has been used so far in the literature and our review provides an important resource for the selection of the cell line to be injected as well as comparison of the general set-up used for a specific cell line(s). In addition, the articles highlighted throughout Table 1 serve as a platform for researchers to decide on (a) output assessment methods; (b) data analysis methods; and (c) end-point of the undertaken assessments based on indi-

vidual summaries of each study included in the review. Overall, the treatment dosages and/or altered gene dosages in the examined studies have provided often successful anti-cancer, anti-proliferative, or anti-migration responses. These findings using HCC/liver cancer cell lines suggest that zebrafish xenograft models may be first-line screening agents to go further with mammalian models or PDX studies for a given drug, especially for dose determination and toxicity evaluation.

Author Contribution Seniye Targen contributed to conceptualization, performed the literature search, and wrote the manuscript, and Ozlen Konu conceptualized, supervised, wrote, and revised the manuscript.

Availability of Data and Material Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Code Availability No code was used.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

References

1. Fazio M, Ablain J, Chuan Y, Langenau DM, Zon LI. Zebrafish patient avatars in cancer biology and precision cancer therapy. Nat Rev Cancer. 2020.
2. Drabsch Y, Snaar-Jagalska BE, Ten Dijke P. Fish tales: the use of zebrafish xenograft human cancer cell models. Histol Histopathol. 2017.
3. Targen S, Kaya T, Ender Avci M, Gunes D, Keskus AG, Konu O. ZenoFishDb v1.1: a database for xenotransplantation studies in zebrafish. Zebrafish. 2020;17.
4. Tonon F, Farra R, Zennaro C, Pozzato G, Truong N, Parisi S, Rizzolio F, Grassi M, Scaggiante B, Zanconati F, Bonazza D. Xenograft zebrafish models for the development of novel anti-hepatocellular carcinoma molecules. Pharmaceuticals. 2021;14(8):803. https://doi.org/10.3390/ph14080803.
5. Tonon F, Zennaro C, Dapas B, Carraro M, Mariotti M, Grassi G. Rapid and cost-effective xenograft hepatocellular carcinoma model in zebrafish for drug testing. Int J Pharm. 2016;515.
6. Karkampouna S, van der Helm D, Gray PC, Chen L, Klima I, Grosjean J, et al. CRIPTO promotes an aggressive tumour phenotype and resistance to treatment in hepatocellular carcinoma. J Pathol. 2018.
7. Li Y, Wu Q, Yu G, Li L, Zhao X, Huang X, et al. Polypyridyl ruthenium(II) complex-induced mitochondrial membrane potential dissipation activates DNA damage-mediated apoptosis to inhibit liver cancer. Eur J Med Chem. 2019;164.
8. Bartucci M, Hussein MS, Huselid E, Flaherty K, Patrizii M, Laddha SV, et al. Synthesis and characterization of novel BMI1 inhibitors targeting cellular self-renewal in hepatocellular carcinoma. Target Oncol. 2017;12.
9. Wu L, Chen P, Ying J, Zhang Q, Liu F, Lv B, et al. MAT2B mediates invasion and metastasis by regulating EGFR signaling pathway in hepatocellular carcinoma. Clin Exp Med. 2019;19.
10. Nakayama J, Lu JW, Makinoshima H, Gong Z. A novel zebrafish model of metastasis identifies the HSD11b1 inhibitor adrenosterone as a suppressor of epithelial-mesenchymal transition and metastatic dissemination. Mol Cancer Res. 2020;18.
11. Yang J, Pei H, Luo H, Fu A, Yang H, Hu J, et al. Non-toxic dose of liposomal honokiol suppresses metastasis of hepatocellular carcinoma through destabilizing EGFR and inhibiting the downstream pathways. Oncotarget. 2017;8.
12. Poté N, Cros J, Laouirem S, Raffenne J, Negrão M, Albuquerque M, et al. The histone acetyltransferase hMOF promotes vascular invasion in hepatocellular carcinoma. Liver Int. 2020;40.
13. Sie ZL, Li RY, Sampurna BP, Hsu PJ, Liu SC, Wang HD, et al. WNK1 kinase stimulates angiogenesis to promote tumor growth and metastasis. Cancers. 2020;12.
14. Avci ME, Keskus AG, Targen S, Isilak ME, Ozturk M, Atalay RC, et al. Development of a novel zebrafish xenograft model in ache mutants using liver cancer cell lines. Sci Rep. Springer US; 2018;8:1570.
15. Khan N, Mahajan NK, Sinha P, Jayandharan GR. An efficient method to generate xenograft tumor models of acute myeloid leukemia and hepatocellular carcinoma in adult zebrafish. Blood Cells Mol Dis. 2019.
16. Kim EA, Lee JH, Heo SJ, Jeon YJ. Saringosterol acetate isolated from Hizikia fusiforme, an edible brown alga, suppressed hepatocellular carcinoma growth and metastasis in a zebrafish xenograft model. Chem Biol Interact. 2021;335.
17. Tu H-C, Hsiao Y-C, Yang W-Y, Tsai S-L, Lin H-K, Liao C-Y, et al. Up-regulation of golgi α-mannosidase IA and down-regulation of golgi α-mannosidase IC activates unfolded protein response during hepatocarcinogenesis. Hepatol Commun. 2017;1.
18. Iscan E, Ekin U, Yildiz G, Oz O, Keles U, Suner A, et al. TAp73β can promote hepatocellular carcinoma dedifferentiation. Cancers. 2021;13.
19. Topel H, Bagirsakci E, Comez D, Bagci G, Cakan-Akdogan G, Atabey N. lncRNA HOTAIR overexpression induced downregulation of c-Met signaling promotes hybrid epithelial/mesenchymal phenotype in hepatocellular carcinoma cells. Cell Communication and Signaling. 2020;18.
20. Akbari S, Kunter I, Azbazdar Y, Ozhan G, Atabey N, Firtina Karagonlar Z, et al. LGR5/R-Spo1/Wnt3a axis promotes stemness and aggressive phenotype in hepatoblast-like hepatocellular carcinoma cell lines. Cell Signal. 2021;82.
21. Huang D, Yang B, Yao Y, Liao M, Zhang Y, Zeng Y, et al. Autophagic inhibition of caveolin-1 by compound Phyllanthus urinaria L. activates ubiquitination and proteasome degradation of β-catenin to suppress metastasis of hepatitis B-associated hepatocellular carcinoma. Front Pharmacol. 2021;12.
22. Chou YT, Chen LY, Tsai SL, Tu HC, Lu JW, Ciou SC, et al. Ribose-5-phosphate isomerase A overexpression promotes liver cancer development in transgenic zebrafish via activation of ERK and β-catenin pathways. Carcinogenesis. 2019;40.
23. Liu JS, Huo CY, Cao HH, Fan CL, Hu JY, Deng LJ, et al. Aloperine induces apoptosis and G2/M cell cycle arrest in hepatocellular carcinoma cells through the PI3K/Akt signaling pathway. Phytomedicine. 2019;61.
24. Wei PL, Huang CY, Chang YJ. Propyl gallate inhibits hepatocellular carcinoma cell growth through the induction of ROS and the activation of autophagy. PLoS ONE. 2019;14.

25. Zhao LJ, Zhao HY, Wei XL, Guo FF, Wei JY, Wang HJ, et al. The lipid homeostasis regulation study of arenobufagin in zebrafish HepG2 xenograft model and HepG2 cells using integrated lipidomics-proteomics approach. J Ethnopharmacol. 2020;260.
26. Gao J, Yin Z, Wu Z, Sheng Z, Ma C, Chen R, et al. Probing synergistic targets by natural compounds for hepatocellular carcinoma. Front Cell Dev Biol. 2021;9.
27. Xu J, Yan B, Zhang L, Zhou L, Zhang J, Yu W, et al. Theabrownin induces apoptosis and tumor inhibition of hepatocellular carcinoma HUH7 cells through ask1-jnk-c-jun pathway. OncoTargets and Therapy. 2020;13.
28. Meehan TF, Conte N, Goldstein T, Inghirami G, Murakami MA, Brabetz S, et al. PDX-MI: minimal information for patient-derived tumor xenograft models. Cancer Res. 2017;77.
29. Conte N, Mason JC, Halmagyi C, Neuhauser S, Mosaku A, Yordanova G, et al. PDX Finder: a portal for patient-derived tumor xenograft model discovery. Nucleic Acids Res. 2019;47.
30. He S, Hu B, Li C, Lin P, Tang WG, Sun YF, et al. PDXliver: a database of liver cancer patient derived xenograft mouse models. BMC Cancer. 2018;
31. Lin HS, Huang YL, Wang YR, Hsiao E, Hsu TA, Shiao HY, et al. Identification of novel anti-liver cancer small molecules with better therapeutic index than sorafenib via zebrafish drug screening platform. Cancers. 2019;
32. Usai A, Di Franco G, Colucci P, Pollina LE, Vasile E, Funel N, et al. A model of a zebrafish avatar for co-clinical trials. Cancers. 2020;12.
33. Di Franco G, Usai A, Funel N, Palmeri M, Rosamaria Montesanti IE, Bianchini M, et al. Use of zebrafish embryos as avatar of patients with pancreatic cancer: a new xenotransplantation model towards personalized medicine. World J Gastroenterol. 2020;26.
34. Ikonomopoulou MP, Fernandez-Rojo MA, Pineda SS, Cabezas-Sainz P, Winnen B, Morales RAV, et al. Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. Sci Rep. 2018;8.
35. Yuan Y, Zhang Y, Yao S, Shi H, Huang X, Li Y, et al. The translation initiation factor eIF3i up-regulates vascular endothelial growth factor A, accelerates cell proliferation, and promotes angiogenesis in embryonic development and tumorigenesis. J Biol Chem. 2014;289.
36. Zhang H, Song Y, Li L, Zhang SY, Wu Q, Mei WJ, et al. Phenanthroimidazole derivatives act as potent inducer of autophagy by activating DNA damage pathway. Bioinorg Chem. 2019;88.
37. Dawood M, Hegazy MEF, Elbadawi M, Fleischer E, Klinger A, Bringmann G, et al. Vitamin K3 chloro derivative (VKT-2) inhibits HDAC6, activates autophagy and apoptosis, and inhibits aggresome formation in hepatocellular carcinoma cells. Biochem Pharmacol. 2020;180.
38. Deng LJ, Lei YH, Quan JY, Li BJ, Zhang DM, Tian HY, et al. 1β–OH–arenobufagin induces mitochondrial apoptosis in hepatocellular carcinoma through the suppression of mTOR signaling pathway. J Ethnopharmacol. 2021;266.
39. Huang CY, Chang YJ, Wei PL, Hung CS, Wang W. Methyl gallate, gallic acid-derived compound, inhibit cell proliferation through increasing ROS production and apoptosis in hepatocellular carcinoma cells. PLoS ONE. 2021;16.
40. Chen Y, Yang W, Chen Q, Liu Q, Liu J, Zhang Y, et al. Prediction of hepatocellular carcinoma risk in patients with chronic liver disease from dynamic modular networks. J Transl Med. 2021;19.
41. Zhang C, Li Q, Qin G, Zhang Y, Li C, Han L, et al. Anti-angiogenesis and anti-metastasis effects of polyphyllin VII on hepatocellular carcinoma cells in vitro and in vivo. Chinese Medicine (United Kingdom). 2021;16.
42. Hou Y, Chu M, Du FF, Lei JY, Chen Y, Zhu RY, et al. Recombinant disintegrin domain of ADAM15 inhibits the proliferation and migration of Bel-7402 cells. Biochem Biophys Res Commun. 2013;435.
43. Zhu XY, Guo DW, Lao QC, Xu YQ, Meng ZK, Xia B, et al. Sensitization and synergistic anti-cancer effects of furanodiene identified in zebrafish models. Sci Rep. 2019;9.
44. Gunes A, Bagirsakci E, Iscan E, Cakan-Akdogan G, Aykutlu U, Senturk S, et al. Thioredoxin interacting protein promotes invasion in hepatocellular carcinoma. Oncotarget 2018;9.
45. Tseng CH, Chen YR, Tzeng CC, Liu W, Chou CK, Chiu CC, et al. Discovery of indeno[1,2-b]quinoxaline derivatives as potential anticancer agents. Euro J Medicinal Chem. 2016;108.
46. Tian L, Xie K, Sheng D, Wan X, Zhu G. Antiangiogenic effects of oridonin. BMC Complemen Altern Med. 2017;17.
47. Chang WT, Liu W, Chiu YH, Chen BH, Chuang SC, Chen YC, et al. A 4-phenoxyphenol derivative exerts inhibitory effects on human hepatocellular carcinoma cells through regulating autophagy and apoptosis accompanied by downregulating α-tubulin expression. Molecules 2017;22.