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ORIGINAL PAPER

A ΔRaf1–ER-inducible oncogenic zebrafish liver cell model identifies hepatocellular carcinoma signatures#

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#The raw array data has been submitted to the Gene Expression Omnibus database (www.ncbinlm.nih.gov/geo).

Abstract

Although the underlying molecular mechanism of hepatocellular carcinoma remains unclear, signalling pathways essential in cell survival and growth are altered, including the Raf–MEK–MAPK pathway. This pathway can be activated by hepatitis B or C virus infections and the ectopic expression of the *Raf-1* oncogene is frequently seen in hepatocellular carcinomas. In addition, the Raf–MEK–MAPK pathway was also shown to be deregulated in zebrafish liver tumours. Based on the genetic conservation between zebrafish and human liver tumours, the zebrafish was used as an animal model to better understand the molecular basis of hepatocellular carcinoma. Here we establish an inducible oncogenic zebrafish cell model, in which oncogenic human Raf-1(ΔRaf1) can be post-transcriptionally activated in zebrafish liver cells by administration of 4-hydroxytamoxifen (4HT). The ΔRaf1 activation resulted in the hyperactivation of the zebrafish MEK–ERK cascade, promoted cell growth and proliferation, and inhibited apoptosis. The mitogenic transformation of the ZFL–ΔRaf1–ER cells was confirmed by *in vivo* allo-transplantation and *in silico* microarray analyses. Gene expression profiling of cells treated with 4HT and a MEK-inhibitor identified a Raf–MEK-dependent signature set. This transcriptome response was compared to zebrafish and human liver cancer transcriptomes. We identified, and validated by quantitative PCR, a set of genes transcriptionally regulated by hyperactive MAPK signalling in ZFL–ΔRaf1–ER cells, zebrafish liver tumours and human liver tumours, suggesting that the *in vitro* zebrafish liver cell model can be used for further study of the molecular basis of human hepatocellular carcinoma. The molecular targeting of the commonly regulated hepatocellular carcinoma genes using the ZFL–ΔRaf1–ER cell model can be applied for high-throughput preclinical target discovery.

Keywords: zebrafish; hepatocellular carcinoma; ΔRaf1–ER; ZFL; transcriptomics; allo-transplantation

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No conflicts of interest were declared.

Introduction

Hepatocellular carcinoma (HCC), the major type of liver cancer, is one of the most common malignancies worldwide [1]. HCC can be induced by risk factors such as hepatitis B or C virus infection, aflatoxin and alcohol, which induce genetic alterations in hepatocytes, causing the transformation into neoplasia [2,3]. Because of its particular heterogeneity, no determinant genetic mutation or molecular pathway of HCC has yet been identified and therapeutic options are limited [3,4]. The major therapeutic strategies for early-stage HCC are mainly hepatic resection or liver transplantation, and effective therapy for late-stage tumours remains lacking [4]. In order to improve diagnosis and to develop new specific therapies for HCC, it is crucial to overcome the limitations of existing animal models and further understand the molecular events involved in HCC [5–7].

A small-molecule inhibitor, sorafenib, which recently gained FDA approval as the first systemic therapy for advanced unresectable HCC, has shown significantly improved survival in clinical trials [2,3,5,8]. Sorafenib inhibits both receptor tyrosine kinases, such as VEGFRs and PDGFR, and the serine/theronine kinase Raf-1 [2,3,8]. It is known that Raf-1 and its downstream MEK–ERK cascade play essential roles in cell proliferation, differentiation and apoptosis. Hyperactivation of the Raf–MEK–ERK pathway contributes to the pathogenesis of many types of human cancers [9,10]. In HCC, the Raf–MEK–ERK pathway can be activated by HBV and HCV infection, and Raf-1 over-expression

has been found in a high percentage of HCC patient tumours, suggesting the involvement of Raf-1 in hepatocarcinogenesis [7,11–14]. Therefore, further study of the function of this pathway in HCC is of great importance.

The zebrafish, *Danio rerio*, has been recently recognized as an animal model for HCC research [15,16]. It is a cost-effective vertebrate model organism sharing the basic molecular cancer biology with human. The *ex utero* development and manipulative genetics of zebrafish allow large-scale genetics or chemical screenings to discover biomarkers for cancer diagnoses, candidate cancer genes for targeted therapies and lead compounds for new cancer treatments [17,18]. The transparent zebrafish embryos offers great opportunities to monitor cancer, especially events during the early stages of tumour initiation and progression [17,18]. Importantly, the zebrafish develops liver tumour spontaneously or by chemical carcinogenesis [15–18]. Lam *et al* established carcinogen-induced zebrafish liver tumours that recapitulated the molecular expression patterns of human liver tumours and possessed features that correlated with progressively higher grades of malignancy [15,16,19].

Transcriptome analysis revealed deregulation of the Ras-Raf–MEK-ERK pathway in the carcinogen-induced zebrafish liver tumours [16]. To develop a new model suitable for studying the roles of Raf–MEK signalling in HCC at the molecular and cellular level, we established a stable zebrafish liver cell line (ZFL) expressing oncogenic human Raf-1, which is 83.6% identical to the zebrafish orthologue. In the ZFL–ΔRaf1–ER cell line obtained, the kinase domain of human Raf-1 (ΔRaf1) can be post-transcriptionally activated by 4-hydroxytamoxifen (4HT) but not by natural oestrogens, as validated using different mammalian models [20,21].

After the addition of 4HT, the zebrafish MEK–ERK cascade was activated and the ZFL–ΔRaf1–ER cells acquired a series of survival and growth advantages. The ΔRaf-1 activation promoted ZFL–ΔRaf1–ER cell growth and proliferation and compensated for the absence of growth factors and inhibited apoptosis. These effects were observed both *in vitro* in cell culture and *in vivo* after cell implantation into zebrafish embryos. Expression microarray analysis confirmed the cellular transformation of ZFL cells upon ΔRaf1 activation and identified a set of genes transcriptionally regulated by the hyperactive Raf–MEK signalling, which was also involved in zebrafish liver tumour progression. A subset of these common genes has been reported in human HCC, suggesting their fundamental importance in hepatocarcinogenesis. Selected genes were validated in zebrafish liver cells, zebrafish and human liver tumours by quantitative PCR (qPCR), demonstrating the translational importance of the zebrafish *in vitro* model for at least some aspects of HCC. The molecular targeting of the common HCC signature genes in the ZFL–ΔRaf1–ER cells could be beneficial for preclinical target discovery.

Materials and methods

Plasmids

A neomycin-resistant vector was generated from pECFP-N1 (Clontech) by replacing CFP with a short piece from pEYFP-C1 (Clontech), using *Bam*HI and *Mfe*I. The ΔRaf1–ER fusion was cloned from pLNC–ΔRaf1–ER (kindly provided by Bob van de Water), using forward primer GGTAGCTGACTGTGTGAAGA and reverse primer ACCTACAGGTGGGGTCTTTC. ΔRaf1–ER was cloned into pCR® 4Blunt-TOPO® (Invitrogen), using Phusion (Finnzymes), and subsequently cloned into the neomycin-resistant vector using *Eco*RI.

Cell culture

The zebrafish liver cell line (ZFL; ATCC, CRL2643) was cultured in complete growth medium (CGM; 50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium, 15% Ham's F12 medium, supplemented with 15 mM HEPES, 0.01 mg/ml insulin, 50 ng/ml EGF and 5% fetal bovine serum; all purchased from Invitrogen) at 28 °C without additional CO_2. Cell population doubling time was calculated using the Doubling Time Online Calculator (http://www.doubling-time.com/compute.php?lang=en). For stable transfection, ZFL cells were nucleofected using Amaxa nucleofector according to the manufacturer's instructions and followed by G418 selection (1 mg/ml). Stable transfected cells were maintained with 0.5 mg/ml G418.

Immunoblotting

Cells were lysed in 1× Loading Buffer (Cell Signalling). Western blot and signal detection was performed as described [22]. Antibodies against ERK1, phosphorylated MEK1/2, phosphorylated ERK1/2 [22], GFP (Clontech, 1 : 1000 dilution), human ER (Santa Cruz, 1 : 6000 dilution) and phosphorhistone H3 (Santa Cruz, 1 : 1000 dilution) were applied.

Generation of zebrafish liver tumours

Zebrafish fry, 3 weeks old, were treated with 0.75 ppm 7,12-dimethylbenz(α)anthracene (DMBA) or DMSO (vehicle) for 24 h. The treatment was repeated once at 5 weeks old for another 24 h with 1.25 ppm DMBA or DMSO. Treated fish were rinsed three times in fresh water and transferred into new tanks for maintenance. The fish were samples for 6–10 months after the onset of DMBA exposure. Tumour samples >3 mm in diameter were histopathologically diagnosed before RNA extraction.

RNA preparation and microarray hybridization

For the ZFL–ΔRaf1–ER cells, biological triplications were taken for each condition. Five zebrafish liver tumours and six healthy zebrafish livers were used to

detect gene signatures in zebrafish liver tumours. Total RNA was prepared as described [23]. Hybridization and scanning on the custom-designed Agilent 4 × 44 k zebrafish microarray containing 43 365 oligos (19 122 unigene clusters, build 105) were performed according to standard Agilent protocols. The feature extraction software, version 9.5, protocol ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction, the option 'No background substraction and spatial detrend' was used. The arrays were scanned twice with 10% photomultiplier tube (PMT) and 100% PMT laser power and the XDR function was used to extend the dynamic range by 10-fold. The raw data is submitted to the Gene Expression Omnibus database (www.ncbinlm.nih.gov/geo, accession GSE29307 and GSE29308).

Microarray data analysis

Microarray data were imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modelling. Data analysis was performed at $p \leq 1.00\text{E}-05$ for unigene clusters. The Unigene and Entrez Gene records of the functionally related human homologues of our zebrafish unigene list of gene signatures were automatically retrieved from the NCBI HomoloGene database. General Gene Ontology (GO) analysis at the level of 'biological process' was performed using GeneTools eGOn v 2.0 software [24] and FunNet software [25], using the Entrez Gene codes of human orthologues.

The featured human HCC gene list was obtained from the database of the *Encyclopedia of Hepatocellular Carcinoma Genes Online* (http://ehco.iis.sinica.edu.tw), based on 13 microarray studies and PubMed researches related to HCC [26,27]. The genes regulated by Raf–MEK signalling in the zebrafish liver cells and liver tumours were manually compared with the human HCC gene list, using the Entrez Gene codes of human orthologues. The functionalities of the common genes were manually analysed using the NCBI database, PubMed and the Gene Ontology Consortium (see Supporting information, Table S3).

Quantitative PCR

The 57 common signatures were analysed for known transcripts in the NCBI RefSeq database. Genes without distinct transcript variants were selected for SYBR green-based quantitative PCR (qPCR) analysis. Primers were designed using QuantPrimer (http://www.quantprime.de/). After primer evaluation [28] for both zebrafish and human homologues, four genes were selected for validation in zebrafish liver cells, zebrafish liver tumours and human liver tumours. RNAs of human samples were prepared from −80 °C-stored tissue samples from normal liver as well as hepatocellular carcinomas obtained from the archive of the Department of Pathology, Leiden University Medical Centre. All samples were handled in a coded fashion and handled according to the ethical guidelines of the National Organization of Scientific Societies (FEDERA). The presence of tumour material in the frozen specimens was verified on frozen sections taken immediately before and after the cutting for RNA isolation. qPCR was performed as described [28]. β-*Actin* and *hypoxanthine phosphoribosyltransferase 1* were used as house-keeping genes for zebrafish and human samples, respectively. The primers are listed in Table S4 (see Supporting information).

Cell implantation, zebrafish embryo handling and immunohistochemistry

Cells were labelled with CM-DiI according to the manufacturer's instructions (Invitrogen). The cells remained fluorescent, without any phenotypical changes up to 14 days. The labelled cells were injected into the yolk of 2 day post fertilization (dpf) zebrafish embryos, raised according to the standard procedures [29,30]. After implantation, the embryos were incubated in daily-refreshed egg water supplemented with 5% DMSO and 0.003% 1-phenyl-2-thiourea (PTU), with or without 1 μM 4HT. Immunohistochemistry was performed as described, using the phosphorhistone H3 antibody [31].

Results

Establishment of the ZFL–ΔRaf1–ER stable cell line

The ZFL cells were transfected with a ΔRaf1-ER-neo construct and a fluorescent membrane marker, YFP–CAAX [32]. Because ZFL cell survival and growth is highly dependent on cell density, probably due to an unknown paracrine mechanism, the transfected cells were maintained in conditioned medium collected from 90% confluent wild-type ZFL cells after 24 h incubation, which allowed growth of cells in low density during the G418 selection procedure. After selection, the stable transfected cells were FACS-sorted [33] for YFP-positive cells (Figures 1, 2A). ΔRaf1–ER expression remained stable for at least 25 passages (Figure 1).

4HT-dependent ΔRaf1 activation leads to hyperactivation of the zebrafish MEK–ERK cascade

1 μM 4-hydroxytamoxifen (4HT) was applied to the ZFL–ΔRaf1–ER cells to sufficiently activate ΔRaf1 without a detectable toxic effect on cell growth (Figure 3A). 4HT administration for 15 or 30 min significantly induced phosphorylation of zebrafish MEK1/2 and ERK1/2 (Figure 2A). Addition of the MEK inhibitor U0126 to the ZFL–ΔRaf1–ER cells completely abolished the induction of ERK1/2 phosphorylation by 4HT administration, indicating that ΔRaf1-induced ERK1/2 phosphorylation is dependent on MEK1/2 activity (Figure 2B). The hyperactivation

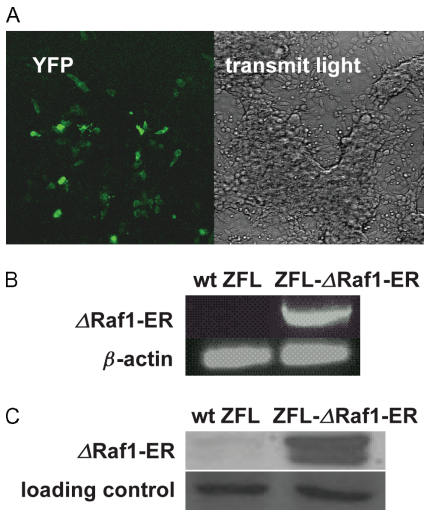

Figure 1. Establishment of the ZFL–ΔRaf1–ER stable cell line. The ZFL cells were transfected with the ΔRaf1–ER–neo and YFP–CAAX constructs. After G418 selection, the YFP-positive cells were sorted by FACS (A). The mRNA expression of *ΔRaf1–ER* in the ZFL–ΔRaf1–ER stable cell line (passage 25) was detected by one-step RT–PCR, using primers designed against full-length ΔRaf1–ER (B). ΔRaf1–ER protein was detected by western blotting, using antibody against human ER (C). Wild-type ZFL cells were used as control.

of ERK1/2 was sustained for at least 5 days in the presence of 4HT (Figure 2C). 4HT administration had no effect on MEK1/2 or ERK1/2 phosphorylation in wild-type ZFL cells (data not shown).

4HT-mediated ΔRaf1 activation promotes cell survival and proliferation *in vitro*

The pro-survival and anti-apoptotic role of Raf-1 has been reported in many cell types [34] and it was examined in the ZFL–ΔRaf1–ER cell line, which grows in a density-dependent manner. Rapid and significant cell loss was observed in wild-type ZFL cells when growth factors were withdrawn from cultures with a density of <500 cells/mm^2 (Figure 3A). In the ZFL–ΔRaf1–ER cells, 4HT administration significantly delayed and reduced cell loss (Figure 3A). After 5 days, >95% of the remaining ZFL–ΔRaf1–ER cells in the absence of 4HT were positively stained with trypan blue, indicating cell death, and no colonies were observed. In the presence of 4HT, colonies were formed by healthy ZFL–ΔRaf1–ER cells, which later expanded into confluent cell layers over longer periods (Figure 3B). This showed that 4HT-activated ΔRaf1 signalling promoted the survival of ZFL–ΔRaf1–ER cells and rescued cell arrest and death, indicating the anti-apoptotic function of ΔRaf1.

Besides the pro-survival function, the Raf–MEK–ERK signalling plays important roles in the cell cycle and proliferation [9], as the proliferation marker phosphorhistone H3 [35] was elevated upon 4HT administration in the ZFL–ΔRaf1–ER cell line (Figure 2B; abolished by U0126). To quantify the effects of ΔRaf1 activation on cell proliferation, the cell population doubling times (T_d) were measured. 4HT administration significantly shortened the doubling time from 104.48 to 62.54 h (Figure 3C). 4HT administration had no significant effect on wild-type ZFL cell growth (T_d = 99.33 and 97.07 h, respectively, in the absence and presence of 4HT; Figure 2A).

When cultured in plain medium, in the absence of serum and growth factors, the ZFL–ΔRaf1–ER cells were forced into a plateau growth phase (T_d = 232.20 h; Figure 3D). With 4HT administration, activated ΔRaf1 signalling promoted the cells to proliferate (T_d = 73.53 h; Figure 3B), which was not observed in wild-type ZFL cells. Thus, 4HT-mediated ΔRaf1 signalling promoted ZFL–ΔRaf1–ER cell proliferation and transformed the cells to growth independence of serum and growth factors.

4HT-mediated ΔRaf1 activation promotes cell survival and proliferation *in vivo*

To investigate the role of 4HT-inducible ΔRaf1 activation *in vivo* in zebrafish embryos, ZFL–ΔRaf1–ER cells were labelled with the cell tracker CM-DiI,

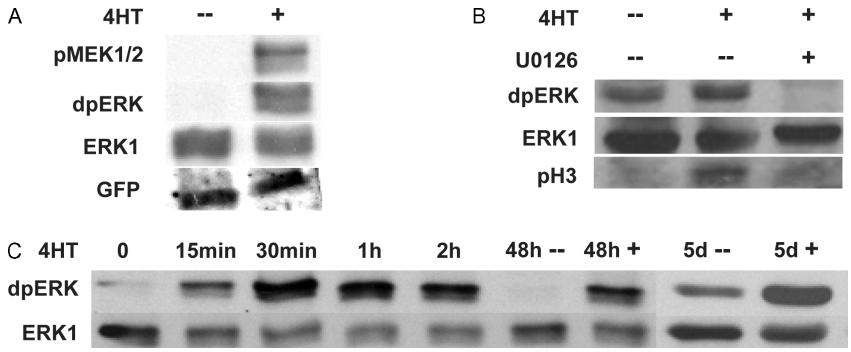

Figure 2. 4HT-dependent ΔRaf1 activation leads to activation of the MEK1/2–ERK1/2 cascade. 1 μM 4-hydroxytamoxifen (4HT) was applied to serum-starved ZFL–ΔRaf1–ER cells to activate ΔRaf1. Phosphorylation of MEK1/2 and ERK1/2 was detected by western blot analysis (A). The total amount of ERK and expression of YFP–CAAX were visualized using an ERK1 antibody and a GFP antibody, respectively. The addition of 30 μM MEK inhibitor U0126 (Cell Signalling) abolished ERK1/2 and histone H3 phosphorylation in the presence of 4HT in ZFL–ΔRaf1–ER cells (B). ERK1/2 hyperphosphorylation can be detected in the serum-starved ZFL–ΔRaf1–ER cells either after short-term (15 min–2 h) 4HT administration (C) or after long-term (48 h or 5 days) 4HT administration (D). These results are representive of more than three independent experiments.

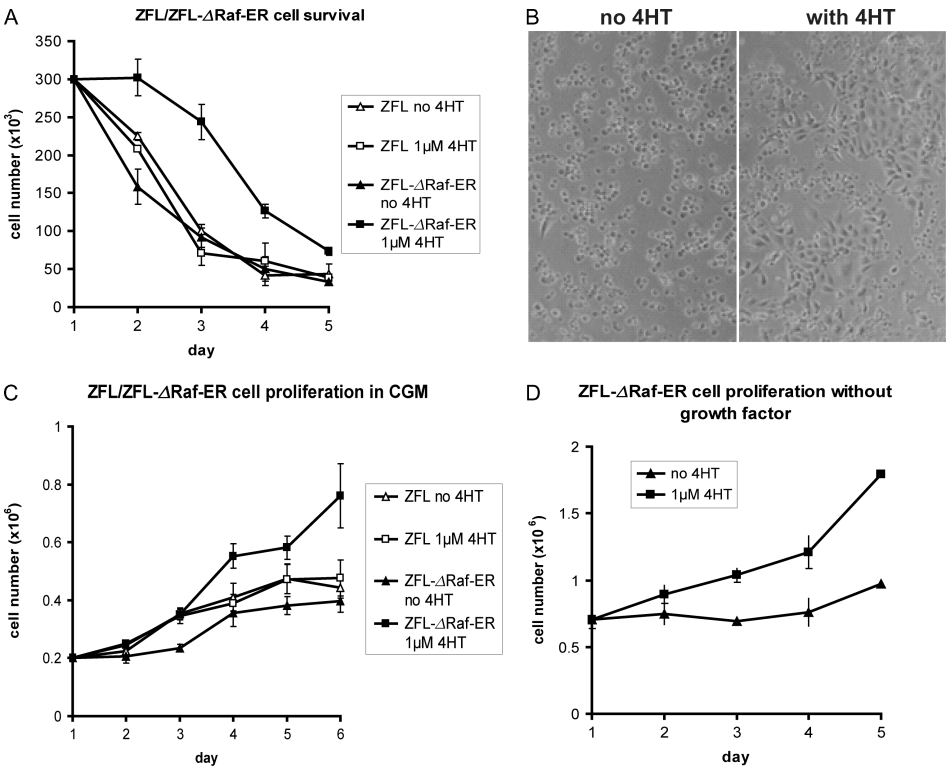

Figure 3. 4HT-mediated ΔRaf1 activation promotes cell survival and proliferation *in vitro*. (A) The ZFL–ΔRaf1–ER and wild-type ZFL cells were seeded at a density of 750 cells/mm^2 in CGM to allow attachment. 12 h after seeding, CGM was replaced by plain medium with or without 1 μM 4HT. The cells were counted daily to record the cell loss. 4HT partially rescued cell loss in the ZFL–ΔRaf1–ER cells, while not rescuing the wild-type ZFL cells. (B) After 5 days in the lethal condition, healthy cell colonies were only observed in the ZFL–ΔRaf1–ER cells with 4HT administration, which later expanded into confluent cell culture despite the absence of growth factors (data not shown). (C) The ZFL–ΔRaf1–ER and wild-type ZFL cells were cultured in complete growth medium (CGM) in the absence or presence of 1 μM 4HT and counted daily. ZFL–ΔRaf1–ER cell proliferation was promoted by 4HT administration. Note that the 4HT had no significant impact on ZFL cells in (A, C). (D) When cultured in plain medium without serum and growth factors, the ZFL–ΔRaf1–ER cells stopped proliferation in the absence of 4HT, whereas cells proliferated in the presence of 1 μM 4HT. The effect of 4HT administration was not observed in the wild-type ZFL cells in plain medium (data not shown). These results are representive of more than three independent experiments.

which is fluorescent and photostable when incorporated into membranes, including the plasma membrane and cytoplasmic vesicles. Subsequently, labelled cells were implanted into the yolk of 2dpf TG(fli1:EGFP) zebrafish embryos expressing vascular-specific GFP (Figure 4) [29,36]. 1 day post implantation (dpi), the proliferation of implanted cells was elevated in embryos incubated with 1 μM 4HT, as detected by phosphorhistone H3 antibody (Figure 4C). two dpi, implanted cells retained proliferation in 4HT-treated embryos, but not in non-treated embryos (Figure 4C), indicating that 4HT-mediated ΔRaf1 activation promoted ZFL–ΔRaf1–ER cell proliferation *in vivo*. At 5 dpi, >90% of implanted cells formed apoptotic bodies in wild-type embryos (Figure 4D, E). In contrast, <10% of implanted ZFL–ΔRaf1–ER cells showed apoptotic bodies when the embryos were treated with 4HT (Figure 4F, G). In these embryos, the majority of implanted ZFL–ΔRaf1–ER cells showed close association with the host blood vasculatures. This demonstrated that ΔRaf1 was activated by 4HT in the embryos, which promoted ZFL–ΔRaf1–ER cell survival and proliferation *in vivo* after implantation. 4HT administration had no effect on embryos implanted with wild-type ZFL cells (data not shown).

Transcriptome analysis of 4HT-mediated hyperactive Raf–MEK signalling in zebrafish liver cells

To identify transcriptional alterations downstream of hyperactive Raf–MEK signalling in ZFL–ΔRaf1–ER cells, microarray analysis was performed after 12 h incubation with 4HT, with or without the MEK inhibitor U0126. The MEK-dependent transcriptional signatures were extracted and analysed (Figure 5A). Expression of 1418 zebrafish unigene clusters was specifically up-regulated by hyperactive Raf–MEK signalling in ZFL–ΔRaf1–ER cells and the expression of 913 unigene clusters was down-regulated ($p \leq 1.00E-05$; Figure 5A; see also Supporting information, Table S1). 66% of the Raf–MEK-specific signatures (1541/2331, Figure 6A) have been annotated, including homologues of some known transcriptional targets of MEK–ERK signalling [37], such as *spred2, etv5, dusp4, b4galt6* and *pycrl* (see Supporting information, Table S1). GO term analysis of these signatures revealed enrichment of the up-regulated transcripts in biological processes such as signal transduction, transcription, protein synthesis, cell migration, angiogenesis and cell differentiation. A high percentage of the down-regulated transcripts were involved in

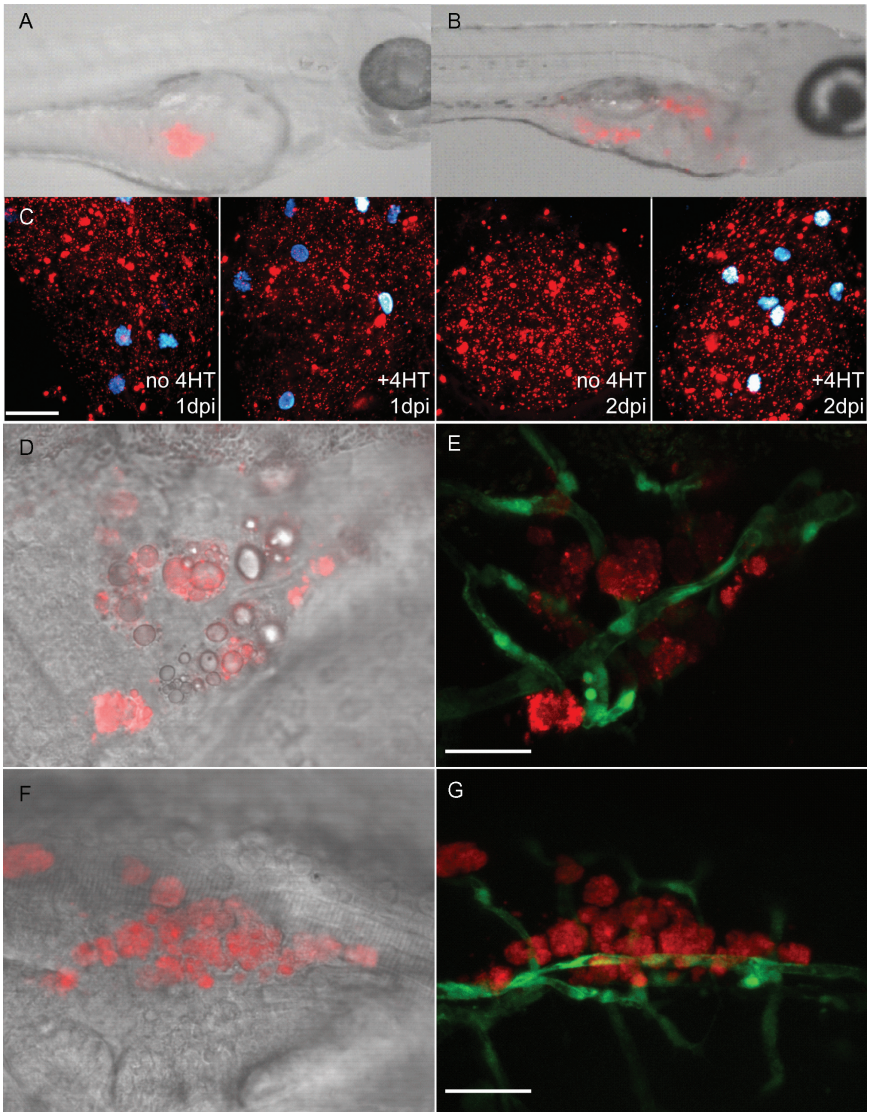

Figure 4. 4HT-mediated ΔRaf1 activation promotes cell proliferation and inhibited apoptosis *in vivo*. (A, B) The ZFL–ΔRaf1–ER cells were labelled with CM-DiI and injected into the yolks of 2dpf Fli : GFP zebrafish embryos. Representative embryos at 1 and 5 days post implantation (dpi) are shown in (A) and (B), respectively (images taken with a Leica fluorescent stereo-microscope). (C) Implanted ZFL–ΔRaf1–ER cells (shown in red, CM-DiI mainly localized in cytoplasmic vesicles due to endocytosis) were stained with the proliferation marker phosphorhistone H3 (shown in 'cyan to white'). (D–G) Five days post implantation, apoptotic bodies were observed in >90% of the remained implanted cells in embryos without 4HT administration (D, E), whereas they were only detected in approximately 5% of the remaining implanted cells in embryos administered with 1 μM 4HT (F, G). Implanted cells are shown in red (CM-DiI; mainly localized on the plasma membrane at this stage) and the host vasculature is shown in green (Fli : GFP). These results are representive of two independent experiments with more than five embryos in each condition, with images taken using a Leica confocal microscope. Scale bar = 25 μm.

oxidation–reduction and cellular metabolic processes, especially carbohydrate, lipid and coenzyme metabolic processes (Figure 5B). Such transcriptional alterations are consistent with the molecular characteristics of HCC. GO analysis also showed the deregulation of the Wnt receptor signalling pathway, which is frequently associated with human HCC [4,38]. For example, the expression of *wnt4a, wnt4b, wnt8a, wnt10b, fzd3, fzd6, fzd8c* and *fzd10* was significantly altered (see Supporting information, Table S1). Taken together, the results indicated that the ZFL cells were transcriptionally reprogrammed towards a malignant phenotype after ΔRaf1 activation. The microarray analysis also identified a set of novel genes which were transcriptionally regulated by hyperactive Raf–MEK signalling, potentially as transcriptional targets of Raf–MEK–ERK signalling.

Genes regulated by the hyperactive Raf–MEK signalling in zebrafish liver cells are associated with zebrafish hepatocarcinogenesis

It is known that the ZFL cell line shows morphological and functional characteristics common to hepatocytes [39], which make up 70–80% of the cytoplasmic mass of the liver. Gene expression profiling indicated that 97% (41 987/43 365) of the oligonucleotides present on the microarray were commonly expressed in ZFL cells and livers isolated from healthy adult zebrafish, suggesting that ZFL cells can be used to model signalling in zebrafish liver *in vitro*.

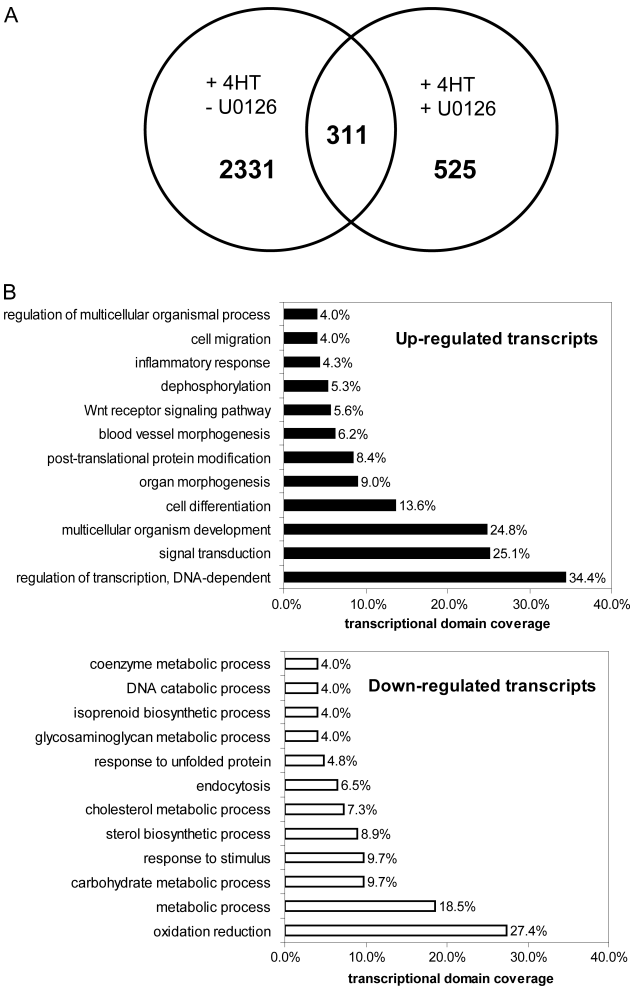

Figure 5. Transcriptome analysis of hyperactive Raf–MEK signalling in the ZFL–ΔRaf1–ER cells. (A) After 12 h administration of 1 μM 4HT, expression of 1563 unigene clusters was up-regulated in the ZFL–ΔRaf1–ER cells, whereas expression of 1079 unigene clusters was down-regulated ($p \leq 1.00E-05$). The MEK inhibitor U0126 was used to specify the gene signatures downstream of MEK-independent signalling. After subtraction of MEK-independent transcripts, a set of gene transcripts specifically downstream of the Raf–MEK signalling was identified and highlighted. (B) GO functional analysis of the 2331 genes regulated by Raf–MEK signalling. The genes were categorized based on GO term analysis at the level of 'biological process'. The functional categories with the highest enrichment of regulated transcripts are listed.

To further dissect the involvement of Raf–MEK signalling in zebrafish hepatocarcinogenesis, the gene signatures regulated by hyperactive Raf–MEK signalling in ZFL–ΔRaf1–ER cells were compared with the transcriptome of zebrafish liver tumours (Figure 6A) [15,16]. This comparison identified 556 unigene clusters commonly regulated in zebrafish liver tumours and by hyperactive Raf–MEK signalling in ZFL cells (513 annotated genes and 43 novel transcripts). Significance of the intersections was confirmed by the hypergeometric test ($p = 0.00E+00$), indicating that these genes regulated by hyperactive Raf–MEK signalling in ZFL cells were associated with zebrafish hepatocarcinogenesis. The proteins coded by these common gene signatures were involved in

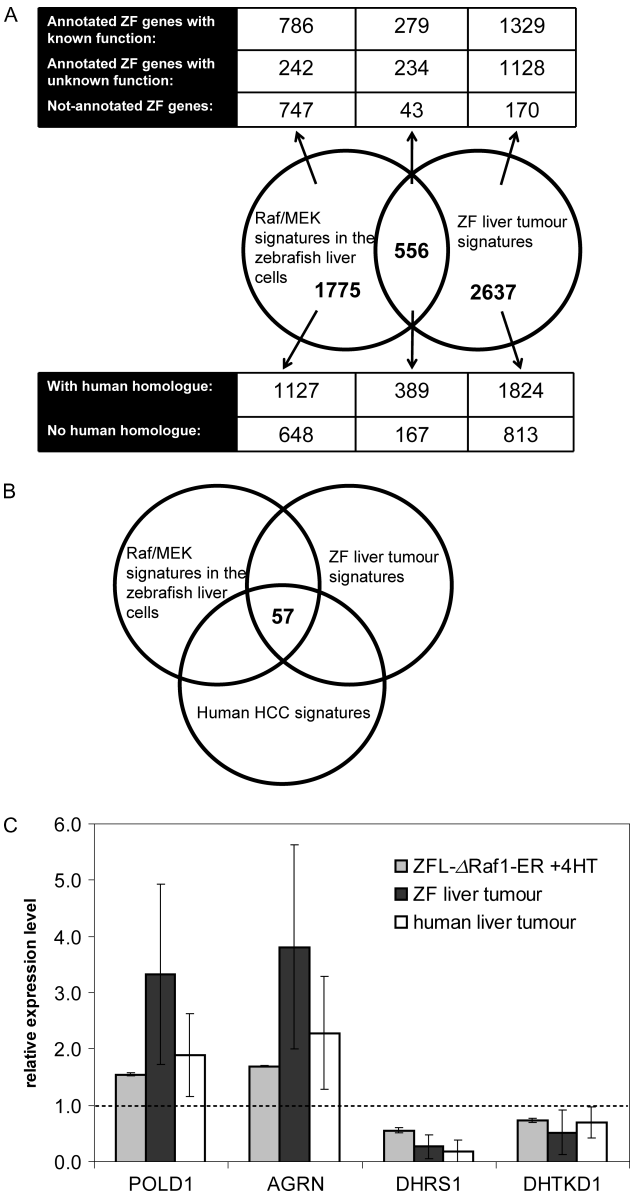

Figure 6. Genes regulated by Raf–MEK signalling in ZFL cells are associated with zebrafish and human hepatocarcinogenesis. Comparison of the transcriptome of zebrafish liver tumours to the Raf–MEK signalling-induced gene signatures in the ZFL–ΔRaf1–ER cells revealed a pool of 556 common transcripts (A), suggesting their association with zebrafish hepatocarcinogenesis. These common signatures were compared with human HCC signature genes selected by the EHCO II HCC database and homologues of 57 genes have been associated with human HCC (B). From the 57 genes, the expressions of four genes were examined by SYBR-green based qPCR in two cultures of ZFL–ΔRaf1–ER cells, four independent zebrafish liver tumours and six human HCC tissue samples. Relative expression levels normalized against non-treated cells or healthy liver tissues are shown in (C). Despite the genetic heterogeneity of liver tumour samples, which causes the variation in expression, POLD1 (DNA-directed DNA polymerase-δ1) and AGRN (Agrin) were significantly up-regulated (relative expression >1, $p > 0.05$), whereas DHRS1 (dehydrogenase/reductase (SDR family member 1) and DHTKD1 (dehydrogenase E1 and transketolase domain-containing 1) were significantly down-regulated (relative expression <1, $p > 0.05$) by 4HT in the ZFL–ΔRaf1–ER cells, in zebrafish and human liver tumours.

biological processes associated with tumour initiation and progression, including cell cycle and proliferation, cell death and apoptosis, cell adhesion and motility, angiogenesis and inflammation responses (see Supporting information, Figure S1), as well as transcription, RNA processing and protein synthesis, transport, metabolism and signal transduction (see Supporting information, Table S2). The molecular association of ΔRaf1-reprogrammed ZFL cells and zebrafish liver tumours validates the ZFL–ΔRaf1–ER cell line as an *in vitro* model to study hepatocarcinogenesis using zebrafish.

Common genes regulated by hyperactive Raf–MEK signalling in zebrafish liver cells and liver tumours are associated with human HCC

Previous comparative microarray analysis has revealed molecular conservation between zebrafish and human hepatocarcinogenesis, suggesting the usefulness of zebrafish to model human HCC [16]. To investigate whether ZFL–ΔRaf1–ER cells can be used to model human HCC, 556 common gene signatures regulated by hyperactive Raf–MEK signalling in ZFL cells and zebrafish liver tumours were analysed for their human homologues; 389 zebrafish genes were identified with human homologues in the NCBI HomoloGene database. Their human homologues were in turn compared to a number of selected human HCC feature genes, which have been collected in the database of the *Encyclopedia of Hepatocellular Carcinoma Genes Online* [26,27]. The comparison resulted in a subset of 57 genes commonly regulated by hyperactive Raf–MEK signalling in ZFL cells and in zebrafish liver tumours, which were also correlated to known genetic features of human HCC, comprising 36 up-regulated genes and 21 down-regulated genes (Figure 6B, see Supporting information, Table S3). The up-regulated common genes are involved in biological processes such as cell cycle and proliferation (*MCM7, POLD1, SMC4, BUB3, NDRG3, MAPRE1*), cell adhesion and motility (*ITGA2, ACTR3, ELMO1, B4GALT1, CTNNA1, CTTN*), transcription (*UBE2N, WHSC1, RUVBL2, PAX8*), RNA processing and translation (*FUS, RPS16, SCNM1*) and signal transduction (*AGRN, TNFRSF14, CDC37, TBC1D13*). Most down-regulated common genes are involved in oxidation–reduction (*DHTKD1, DHRS1*) and cellular metabolic processes (*SUCLG2, PANK1, PHYH, ALDH2, ACO1, FAH, OAZ1, UPP1*). Some of the common genes are novel and have not yet been associated with known functions. To validate the cross-species comparison, *POLD1, AGRN, DHRS1* and *DHTKD1* were selected for SYBR green-based qPCR and their common regulation patterns were confirmed in zebrafish liver cells, zebrafish liver tumours and human liver tumours (Figure 6C). These results suggested their fundamental importance in hepatocarcinogenesis across species, which can be further studied in the ZFL–ΔRaf1–ER cell line model.

Discussion

The majority of HCCs arise in the setting of underlying chronic hepatitis or cirrhosis [3,8]. Due to the complex aetiology of this malignancy, the molecular pathogenesis of HCC has not yet been clarified. However, several carcinogenic pathways have been indentified in HCC, including VEGFR, EGFR, FGFR, IGFR, PDGFR pathways [3]. These studies also highlighted the importance of the Ras-Raf-MEK-ERK and PI3K-Akt-mTOR pathways, which are often activated downstream of the tyrosine receptor kinases and underlie growth advantages during the early stages of carcinogenesis, such as cell proliferation and anti-apoptosis [3]. Further study of these pathways in animal models will help to understand their involvement in HCC and contribute to target and drug discovery.

The zebrafish has recently been validated as a model for studying HCC [15,16]. The tractable nature of the zebrafish for large-scale forward and reverse genetic screens makes it ideal for the discovery of novel gene functions in disease processes at a high-throughput level that can not be matched by rodent models. Here we used the ZFL cell line to establish an *in vitro* HCC model. The ZFL cell line is an epithelial cell line derived from adult zebrafish liver [39]. We showed that it largely represents normal zebrafish liver tissues at the transcriptome level. In the ZFL–ΔRaf1–ER cell line, ΔRaf1 and its downstream signalling was activated by 4HT administration, which validated the use of the 4HT–ER system to control oncoprotein expression and function for the first time in zebrafish cells. After 4HT administration, hyperactivation of Raf–MEK signalling in ZFL–ΔRaf1–ER cells resulted in survival and proliferation advantages, acquired self-sufficiency with respect to growth factors, and rescued cell death. Unconstrained proliferation and impaired apoptosis are both key hallmarks of cancer, suggesting that zebrafish liver cells were partially transformed by hyperactive Raf–MEK signalling.

4HT treatment also promoted cell survival and inhibited apoptosis of ZFL–ΔRaf1–ER cells after implantation into zebrafish embryos, which validated the use of the 4HT/ER system *in vivo*. The growth advantage resulting from hyperactive Raf–MEK signalling allowed the study of longer-term events, such as cell proliferation and primary tumour formation, following the implantation approach. In addition, the close association of implanted cells with the host vasculature (Figure 4), together with the transcriptional regulation of angiogenesis-related genes by hyperactive Raf–MEK signalling (see Supporting information, Table S3), suggests the usefulness of the implantation approach for studying cellular interaction with the host vasculature and angiogenesis in the context of oncoprotein activation.

Accelerated cell division and proliferation promoted by Raf–MEK activation increases susceptibility to the step-wise acquisition of additional genetic mutations

necessary for malignant transformation [38]. To further understand the involvement of Raf-1 in the zebrafish liver cell transformation, microarray analysis was performed to systematically identify signalling networks and target genes downstream of hyperactive Raf–MEK signalling. The specificity of the transcriptome analysis was determined by application of the MEK inhibitor U0126.

Because cell transformation is usually due to long-term exposure to cellular alterations caused by the genetic changes, we analysed the cellular transcriptome after 12 h of ΔRaf1 activation. The transcriptional alterations resulting from hyperactive Raf–MEK signalling were involved in many biological processes related to the cellular transformation of ZFL cells. Up-regulation of genes with human homologues known as transcriptional targets by Raf–MEK signalling indicated the molecular conservation between zebrafish and human cell cultures. A subset of novel zebrafish genes was discovered, as potential transcriptional targets of the Raf–MEK signalling (Figure 6). A longer term of 4HT administration, eg 24 h, resulted in similar gene signatures.

After identifying a pool of transcripts downstream of Raf–MEK signalling in ZFL–ΔRaf1–ER cells, we performed comparative microarray analysis to systematically validate these gene signatures to zebrafish liver tumours. We discovered that many genes involved in the cell cycle, proliferation and apoptosis were commonly regulated by hyperactive Raf–MEK signalling in ZFL cells and in zebrafish liver tumours, indicating the essential role of cell survival and hyperproliferation in hepatocarcinogenesis. Common genes involved in angiogenesis, cell adhesion and motility were also identified, suggesting their roles in zebrafish liver tumour progression. Strikingly, many genes involved in inflammation were transcriptionally regulated by hyperactive Raf–MEK signalling in ZFL cells but not in zebrafish liver tumours. This might reflect the dynamic association of inflammation and liver carcinogenesis, but the mechanism is currently not understood.

Our study has shown that zebrafish cell lines can be used to bridge cancer approaches at different levels, including *in vitro* in cell cultures, *in vivo* after implantation and *in silico* by microarray analysis. It provides a novel platform to expand basic cancer research at the molecular and cellular levels towards the tissue, organ and the entire organism levels. In addition, we found hundreds of novel transcripts that were specifically regulated by hyperactive Raf–MEK signalling in ZFL–ΔRaf1–ER cells (Figure 6). Many of these transcripts have not previously been annotated. Most of them have been linked to human homologues, but for many of these the function remains unknown. Comparison with the human HCC transcriptome data revealed 57 genes commonly regulated by ΔRaf1 activation in ZFL–ΔRaf1–ER cells, in zebrafish liver tumours and in human HCC. The common regulation patterns of *POLD1, AGRN, DHRS1* and *DHTKD1* were confirmed by quantitative PCR. The ZFL–ΔRaf1–ER cell model can be used for the future functional characterization of these genes, which will bring new insight to the understanding of Raf–MEK signalling and the molecular mechanism of HCC.

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Author contributions

SH conducted all experiments and wrote the paper; SFGK provided experimental help; HZ and ZG generated zebrafish liver tumour material; PCWH provided human liver tumour material and clinical relevant impact; and SH, PCWH, HPS and BES were involved in writing the paper. All authors had final approval of the submitted and published versions.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Figure S1. Genes regulated by Raf–MEK signalling in the ZFL cells are associated with zebrafish hepatocarcinogenesis.

Table S1. Genes up-regulated by 12 h ΔRaf1 activation in zebrafish liver cells, dependant on MEK.

Table S2. Genes down-regulated by Raf–MEK signalling in zebrafish liver cells and down-regulated in zebrafish liver tumours.

Table S3. Genes associated with human HHC, which were down-regulated by Raf–MEK signalling in zebrafish liver cells and down-regulated in zebrafish liver tumours.

Table S4. Primers used in this study.