Chapter 4

An inducible oncogenic zebrafish liver cell model to study hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The molecular conservation between zebrafish and human liver tumors makes zebrafish a valuable model to study HCC. We established the first reported inducible oncogenic zebrafish cell model, in which oncogenic human Raf-1 can be post-transcriptionally activated in zebrafish liver cells by administration of 4-hydroxytamoxifen (4HT). The Raf-1 activation promoted zebrafish liver cell growth and proliferation, compensated the absence of growth factors, and inhibited apoptosis.
The anti-apoptotic effect of Raf-1 was also observed *in vivo* in zebrafish embryos treated with 4HT after cell implantation. Gene expression profiling of cells treated by 4HT and a MEK-inhibitor identified Raf/MEK-dependent signature sets. This transcriptome response was compared to zebrafish and human liver cancer transcriptome responses and transcriptional markers of hepatocarcinogenesis were identified, showing that the ZFL-ΔRaf1-ER cell line is a valuable model for HCC.

Keywords: zebrafish, hepatocellular carcinoma, ΔRafER, ZFL, transcriptomics, allo-transplantation

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Introduction

Hepatocellular carcinoma (HCC), the major type of liver cancer, is one of the most common malignancies worldwide, accounting for more than 500 000 new cases annually [1]. Recently, a small molecule inhibitor Sorafenib was approved for advanced HCC treatment [2-4]. Sorafenib is a multi-target inhibitor that inhibits both receptor tyrosine kinases and the serine/theronine kinase Raf-1 [2, 3]. Since the Raf pathway is misregulated in majority of cancer cells including liver tumors, it is an important target for the development of therapeutic drugs. Therefore further study of the function of this pathway in cancer is of great importance.

It is known that Raf-1 and its downstream MEK/ERK cascade play essential roles in cell proliferation, differentiation and apoptosis. Deregulation of the Raf/MEK/ERK pathway contributes to pathogenesis and progression of many types of human cancers [5, 6]. In HCC, the Raf/MEK/ERK pathway can be activated by HBV and HCV infection and Raf-1 overexpression has been found in a high percentage of HCC patient tumors, suggesting the involvement of Raf-1 in hepatocarcinogenesis, which is also supported by the success of Sorafenib treatments [7-10]. However, the underlying mechanisms still remain unclear and Sorafenib inhibits not only Raf-1. In order to improve diagnosis and to develop new specific therapies for HCC, it's crucial to overcome limitation of existing animal models and further understand the molecular events involved in HCC, for example, the role of Raf-1 [4, 9, 11].

The zebrafish, *Danio rerio*, has been recognized as an important model for biomedical research in the last decades. 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. The transparent zebrafish embryos offers great opportunities to monitor cancer, especially the events during early stages of tumor initiation and progression [12, 13]. Importantly, zebrafish develops liver tumor spontaneously or by chemical carcinogenesis [12-15]. Recent studies showed that zebrafish liver tumors are highly similar to human liver tumors at both histology and transcriptome level, highlighting the potential of using zebrafish to model human HCC [14-16].

Deregulation of the Ras/Raf/MEK/ERK pathway in the carcinogen-induced zebrafish liver tumors was revealed by previous transcriptome analysis [15]. It was also reported that HCC was induced in a transgenic zebrafish line with liver specific expression of oncogenic KRas (Gong *et al.*, in preparation). To develop a new model suitable for studying the roles of Raf/MEK signaling in HCC at molecule 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 ortholog. In the obtained ZFL-ΔRaf1-ER cell line, the kinase domain of human Raf-1 (ΔRaf1) can be post-transcriptionally activated by 4-hydroxytamoxifen (4HT), which has been validated using different mammalian models [17, 18].

After addition of 4HT, zebrafish MEK-ERK cascade was activated in the ZFL-ΔRaf1-ER cells and the cells acquired a series of survival and growth advantages. The Raf-1 activation promoted ZFL cell growth and proliferation, compensated the

absence of growth factors, and inhibited apoptosis, which was also observed *in vivo* after cell implantation into zebrafish embryos. Microarray analysis confirmed cellular transformation following the ΔRaf1 activation in the ZFL cells. Transcriptome comparison between the Raf/MEK signatures in ZFL cells and zebrafish liver tumor signatures identified a set of genes transcriptionally regulated by the hyperactive Raf/MEK signaling, which also involved in zebrafish liver tumor progression. A subset of these common genes have been reported in human HCC, suggesting that the *in vitro* zebrafish liver cell model can be used for further studying of the molecular basis of human HCC.

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 [19]. 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 wildtype ZFL cells after 24 hour incubation, which allowed growth of cells in low density during G418 selection procedure. After selection the stable transfected cells were FACS sorted for the YFP-positive cells (Figure 1 and 2A). ΔRaf1-ER expression in the stable cell line remained stable for at least 25 passages (Figure 1).

4HT-dependent ΔRaf1 activation leads to hyper-activation of the zebrafish MEK/ERK cascade

1 μM 4-hydroxytamoxifen (4HT) was applied to the ZFL-ΔRaf1-ER cells to sufficiently activate ΔRaf1 without detectable toxicity effect on the cell growth (Figure 3A). The 4HT administration and ΔRaf1 activation significantly induced phosphorylation of zebrafish MEK1/2 and ERK1/2 in serum-starved ZFL-ΔRaf1-ER cells (Figure 2A). Addition of the MEK inhibitor U0126 to the ZFL-ΔRaf1-ER cells completely abolished induction of the ERK1/2 phosphorylation by 4HT administration, indicating that the ΔRaf1-induced ERK1/2 phosphorylation is dependent on the MEK1/2 activity (Figure 2B). The MEK1/2-dependent ERK1/2 phosphorylation was detected in the ZFL-ΔRaf1-ER cells after short term 4HT administration such as 15 and 30 minutes (Figure 1C). The hyper-activation of ERK1/2 can be sustained for at least 5 days in the presence of 4HT (Figure 2C). 4HT administration didn't induce MEK1/2 or ERK1/2 phosphorylation in wildtype ZFL cells (data not shown).

The 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 [20] and it was examined in the ZFL-ΔRaf1-ER cell line, which has the characteristic growth dependence on cell density. Significant cell loss was observed in wildtype ZFL cells within 2 days when cultured in the absence of growth factors with less than 500 cells/mm^2, which cannot be rescued by 4HT administration (Figure 3A). In the ZFL-ΔRaf1-ER cells, 4HT administration significantly delayed and reduced the cell loss (Figure 3A). After 5 days, over 95% of the remained ZFL-ΔRaf1-ER cells in the absence of 4HT were positively stained by trypan blue, indicating cell death, and no colony was observed. In the presence of 4HT, colonies formed by healthy ZFL-ΔRaf1-ER cells remained in the culture which later expanded into confluent cell layers over longer periods, despite of the loss of surrounding cells and the lack of growth factors (Figure 3B). It showed that the 4HT activated ΔRaf1 signaling promoted survival of the ZFL-ΔRaf1-ER cells and rescued the cell arrest and death, suggesting the anti-apoptotic function of ΔRaf1.

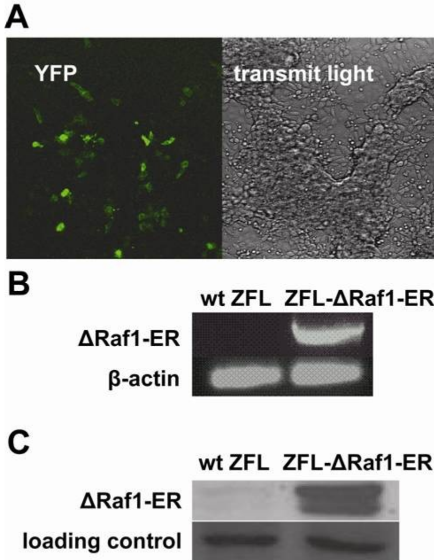

Figure 1. Establishment of the ZFL-ΔRaf1-ER stable cell line.
The ZFL cells were transfected with the ΔRaf1-ER-neo and YFP-CAAX constructs. After G418 selection, the YFP positive cells were sorted by FACS (A). The mRNA expression of ΔRaf1-ER in the ZFL-ΔRaf1-ER stable cell line was detected by One-Step RT-PCR using primers designed against full-length ΔRaf1-ER (B). ΔRaf1-ER protein was detected by western blot using antibody against human ER (Santa Cruz, 1:6000 dilution; C). The wildtype ZFL cells were used as control.

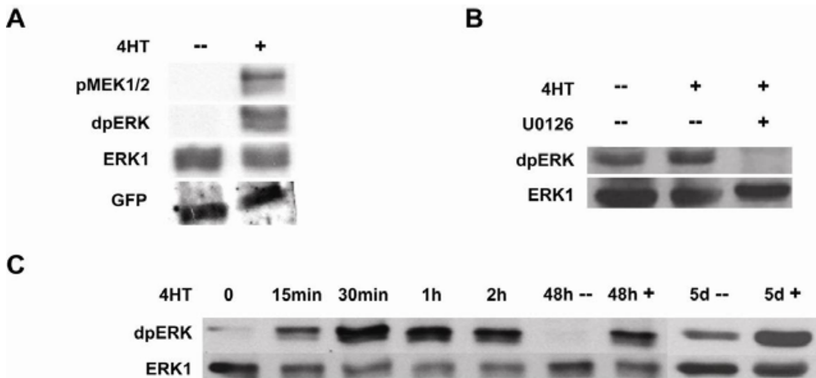

Figure 2. 4HT-dependent ΔRaf1 activation leads to activation of MEK1/2-ERK1/2 cascade.
1 µM 4-hydroxytamoxifen (4HT) was applied to serum-starved ZFL-ΔRaf1-ER cells to activate ΔRaf1. Phosphorylation of MEK1/2 and ERK1/2 was detected by western blot analysis (A) using antibodies against phosphorylated MEK1/2 (Cell Signaling, 1:1000 dilution) or phosphorylated ERK1/2 (Cell Signaling, 1:1000 dilution). The total amount of ERK was visualized using a ERK1 antibody (Santa Cruz, 1:2000 dilution). Expression of YFP-CAAX in the ZFL-ΔRaf1-ER cells was detected using a GFP antibody (Clonetech, 1:1000 dilution). Addition of 30 µM MEK inhibitor U0126 (Cell Signaling) abolished ERK1/2 phosphorylation in presence of 4HT in ZFL-ΔRaf1-ER cells (B). The ERK1/2 hyper-phosphorylation can be detected in the serum-starved ZFL-ΔRaf1-ER cells either after short-term 4HT administration (from15 minutes to 2 hours, C) or after long-term 4HT administration such as 48 hours or 5 days (D).
These results are representive of at least three independent experiments.

Besides the pro-survival function, the Raf/MEK/ERK signaling plays important roles in cell cycle and proliferation [5]. To quantify effects of the ΔRaf1 activation on ZFL-ΔRaf1-ER cell proliferation, the cell population doubling time (T_d) were measured. 4HT administration largely promoted ZFL-ΔRaf1-ER cell proliferation and significantly shortened the doubling time, from 104.48 to 62.54 hours (Figure 3C). The same 4HT administration didn't give a significant effect on wildtype ZFL cell growth (T_d = 99.33 and 97.07 hours, respectively, in the absence and presence of 4HT; Figure 2A), indicating that the promoted cell proliferation was specifically induced by the 4HT mediated ΔRaf1 signaling.
When cultured in plain medium, in absence of serum and growth factors, the ZFL-ΔRaf1-ER cells were forced into a plateau growth phase (T_d = 232.20 hours; Figure 3D). With 4HT administration, activated ΔRaf1 signaling promoted the cells to proliferate (T_d= 73.53 hours; Figure 3B), which was not observed in wildtype ZFL cells. Thus, the 4HT mediated ΔRaf1 signaling promoted the ZFL-ΔRaf1-ER cell proliferation and transformed the cells to the growth-independence of serum and growth factors.

The 4HT-mediated ΔRaf1 activation promotes cell survival and inhibits apoptosis *in vivo*

To investigate the role of 4HT inducible ΔRaf1 activation *in vivo* in zebrafish embryos, the ZFL-ΔRaf1-ER cells were labeled with the cell tracker CM-DiI and implanted into the yolk of 2dpf TG(fli1:EGFP) zebrafish embryos, which has endothelial-specific GFP expression (Figure 4) [21, 22]. 5 days post implantation (dpi), over 90% of implanted cells went on apoptosis, indicated by formed apoptotic bodies (Figure 4C). When the embryos were incubated with 1 μM 4HT after implantation, apoptotic bodies were only observed in less than 10% of implanted ZFL-ΔRaf1-ER cells (Figure 4E). The majority of implanted ZFL-ΔRaf1-ER cells remained integrity and showed close association with the host blood vasculatures in 4HT-administrated embryos at 7dpi (Figure 4H). The rescue of cell death by 4HT administration was not observed in embryos implanted with wildtype ZFL cells, where all implanted cells appeared fragmented at 7dpi (Figure 4G). It showed that ΔRaf1 was activated by 4HT in the embryos, which promoted ZFL-ΔRaf1-ER cell survival and inhibited apoptosis *in vivo* after implantation.

Transcriptome analysis of the 4HT-mediated hyperactive Raf/MEK signaling in the zebrafish liver cells

To identify the transcriptional alterations downstream of the hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells, microarray analysis was performed after 12 hours incubation with 4HT, with or without application of the MEK inhibitor U0126. Expression of 1418 zebrafish unigene clusters was specifically up-regulated by the hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells and expression of 913 unigene clusters was down-regulated ($P \leq 1.00E-05$; Figure 5A; Supplementary Table 1). 66% of the Raf/MEK-specific signatures (1541/2331, Figure 6A) have been annotated, including homologs of some known transcriptional targets of the MEK/ERK signaling [23], such as *spred2*, *etv5*, *dusp4*, *b4galt6* and *pycrl* (Supplementary Table 1). GO term analysis of these signatures revealed enrichment of the up-regulated transcripts in biological processes such as

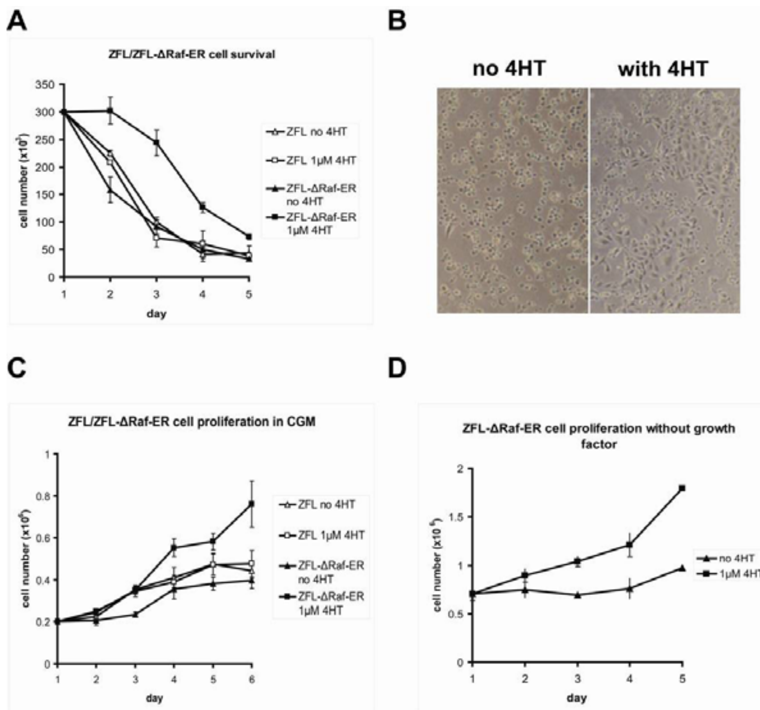

Figure 3. 4HT mediated ΔRaf1 activation promotes cell survival and proliferation *in vitro*.

A: The ZFL-ΔRaf1-ER and wildtype ZFL cells were seeded at a density of 750 cells/mm^2 in CGM to allow attachment. 12 hours 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, whereas not rescuing the wildtype ZFL cells.

B: After 5 day 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 wildtype ZFL cells were cultured in CGM in absence or presence of 1 µM 4HT and counted daily. ZFL-ΔRaf1-ER cell proliferation was promoted by 4HT administration.

D: When cultured in plain medium without serum and growth factors, the ZFL-ΔRaf1-ER cells stopped proliferation in absence of 4HT, whereas cells proliferated in presence of 1 µM 4HT. The effect of 4HT administration was not observed in the wildtype ZFL cells in plain medium (data not shown).

These results are representive of at least three independent experiments.

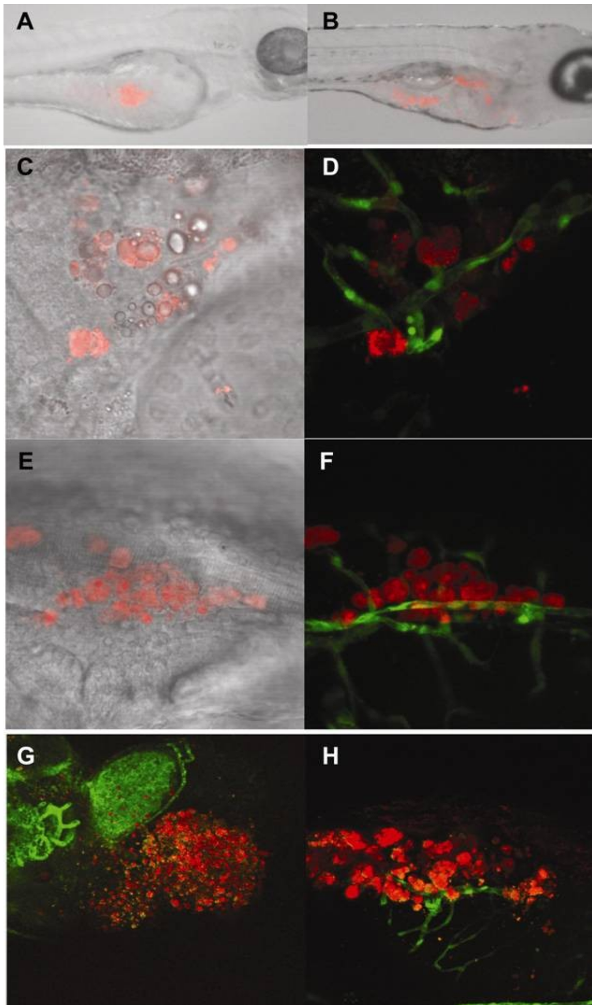

Figure 4. 4HT mediated ΔRaf1 activation promotes cell survival and inhibited apoptosis *in vivo*.

A-B: The ZFL-ΔRaf1-ER cells were labeled with CM-DiI and injected into yolk of 2 dpf zebrafish Fli:GFP embryos. Representive embryos 1 day and 5 days post implantation are shown in A and B, respectively (images taken by Leica fluorescent stereo microscope).

C-F: 5 days post implantation, apoptotic bodies were observed in over 90% of the remained implanted cells in embryos without 4HT administration (C, D), whereas only detected in approximately 5% of remained implanted cell in the embryos administrated with 1 μM 4HT (E, F).

G-H: 7 days post implantation, in 4HT administrated embryos, all the implanted wildtype ZFL cells were fragmented (G), whereas the implanted ZFL-ΔRaf1-ER cells remained integrity and showed close association with host vasculature in the Fli:GFP embryos (H).

Images in C-H were taken by Leica confocal microscope.

These results are representive of two independent experiments with at least 5 embryos in each condition.

signal transduction, transcription, protein synthesis, cell migration, angiogenesis and cell differentiation. A high percentage of the down-regulated transcripts was involved in the 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 signaling pathway, which is frequently associated with human HCC [24, 25]. For example, the expression of *wnt4a*, *wnt4b*, *wnt8a*, *wnt10b*, *fzd3*, *fzd6*, *fzd8c* and *fzd10* was significantly altered (Supplementary Table 1). Taken together, the results indicated that the ZFL cells were transcriptionally reprogrammed towards a malignant phenotype after the ΔRaf1 activation. The microarray analysis also identified a set of novel genes which were transcriptionally regulated by the hyperactive Raf/MEK signaling, potentially as transcriptional targets of the Raf/MEK/ERK signaling.
It is known that activation of Raf-1 can deliver signal to downstream molecules other than MEK [5, 6]. The use of MEK inhibitor showed that 311 unigene clusters were transcriptionally regulated in a MEK-independent manner (Figure 5A). It was also found that the phosphorylation of histone H3 induced by the Raf-1 activation was not completely abolished by addition of U0126, suggesting that the cell proliferation enhanced by the ΔRaf1 activation is partially due to MEK-independent signaling.

Genes regulated by the hyperactive Raf/MEK signaling in the zebrafish liver cells are associated with zebrafish hepatocarcinogenesis

It is known that the ZFL cell line showed characteristics common to hepatocytes [26], which make up 70-80% of the cytoplasmic mass of the liver. Gene expression profiling indicated that 97% (41987/43365) of the oligonucleotides present on the microarray were commonly expressed in the ZFL cells and livers isolated from healthy adult zebrafish, suggesting the ZFL cells can be used to model signaling in zebrafish liver *in vitro*.
To further dissect the involvement of Raf/MEK signaling in zebrafish hepatocarcinogenesis, the gene signatures regulated by the hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells were compared with the transcriptome of zebrafish liver tumors (Figure 6A) [14, 15]. This comparison identified 556 unigene clusters commonly regulated in both the zebrafish liver tumors and by the hyperactive Raf/MEK signaling in ZFL cells (513 annotated zebrafish genes and 43 novel transcripts). Significance of the intersections was confirmed by hypergeometric test (P=0.00E+00), indicating that these genes regulated by the hyperactive Raf/MEK signaling in ZFL cells were associated with zebrafish hepatocarcinogenesis. The proteins coded by these common gene signatures were involved in biological processes associated with tumor initiation and progression, including cell cycle and proliferation, cell death and apoptosis, cell adhesion and motility, angiogenesis and inflammation responses (Figure 6B), as well as transcription, RNA processing and protein synthesis, transport, metabolism and signal transduction (Supplementary Table 2). The molecular association of the ΔRaf1-reprogrammed ZFL cells and the zebrafish liver tumors validates the ZFL-ΔRaf1-ER cell line as an *in vitro* model to study hepatocarcinogenesis using zebrafish.

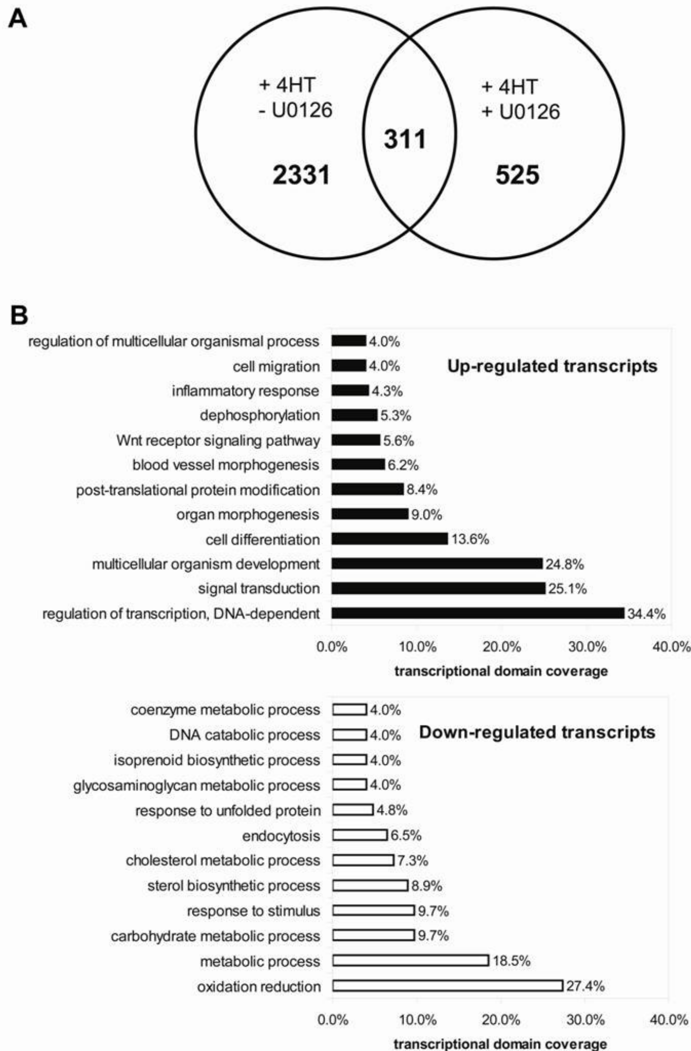

Figure 5. Transcriptome analysis of hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells.

A: After 12 hours 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≤1.00E-05). The MEK inhibitor U0126 was used to specify the gene signatures downstream of MEK-independent signaling. After subtraction of MEK-independent transcripts, a set of gene transcripts specifically downstream of the Raf/MEK signaling was identified and highlighted.

B: GO functional analysis of the 2331 genes regulated by the Raf/MEK signaling. Genes were categorized based on GO term analysis at the level of 'biological process'. The functional categories with highest enrichment of regulated transcripts are listed.

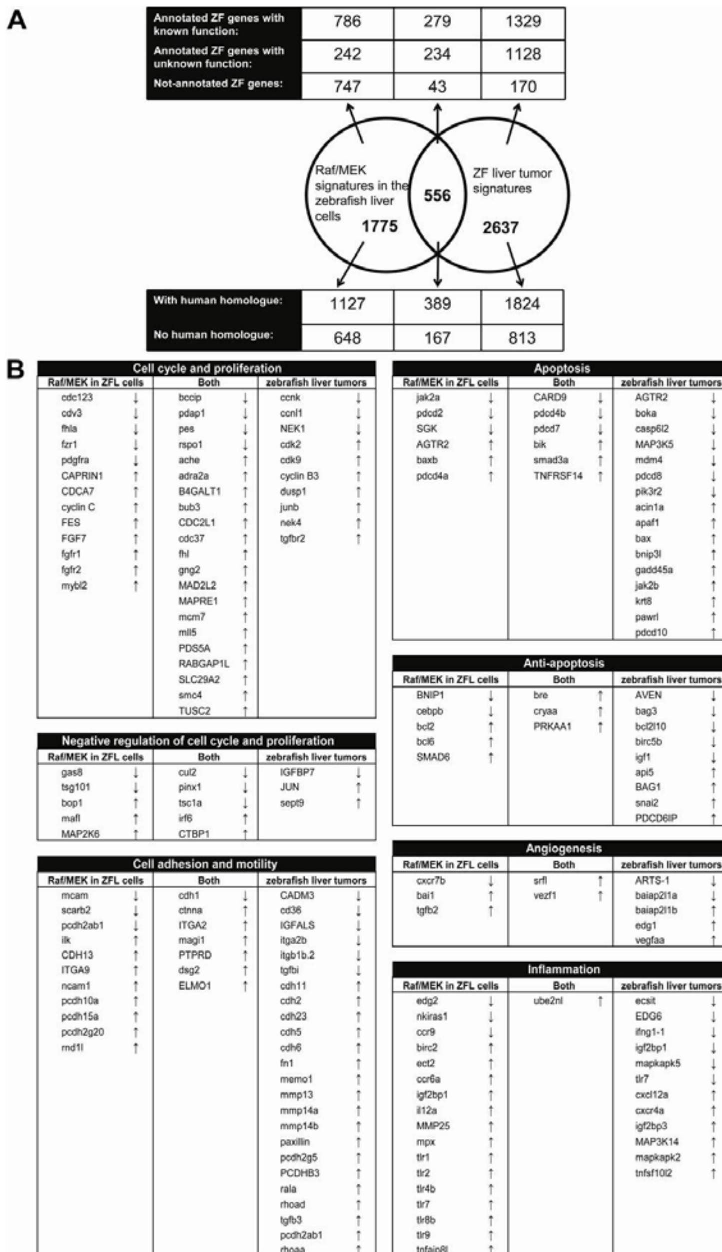

Figure 6. Genes regulated by Raf/MEK signaling in the ZFL cells are associated with zebrafish hepatocarcinogenesis. Comparison of the transcriptome of zebrafish liver tumors to the Raf/MEK signaling induced gene signatures in the ZFL-ΔRaf1-ER cells revealed a pool of 556 common transcripts (A), suggesting their association with zebrafish hepatocarcinogenesis. The signatures were analyzed for gene functionalities involved in tumor progression. Examples of genes are listed (B). The genes in capital letters were analyzed based on functionality of their human homologs.

Common genes regulated by the hyperactive Raf/MEK signaling in the zebrafish liver cells and liver tumors are associated with human HCC

Previous comparative microarray analysis has revealed the molecular conservation between the zebrafish and human hepatocarcinogenesis, suggesting the usefulness of zebrafish to model human HCC [15]. To investigate whether the ZFL-ΔRaf1-ER cells can be used to model human HCC, the 556 common gene signatures regulated by the hyperactive Raf/MEK signaling in ZFL cells and zebrafish liver tumors were analyzed for their human homologs. 389 zebrafish genes were identified with human homologs in the NCBI HomoloGene database. Their human homologs were in turn compared to a number of selected human HCC feature genes, which have been collected in the database of Encyclopedia of Hepatocellular Carcinoma genes Online [27, 28]. The comparison resulted in a subset of 57 genes commonly regulated by the hyperactive Raf/MEK signaling in ZFL cells and in zebrafish liver tumors, which were also correlated to known genetic features of human HCC, comprising 36 up-regulated genes and 21 down-regulated genes (Table 1, Supplementary Table 3). 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 been associated with known functionality yet. The common regulation of these genes in zebrafish liver cells, zebrafish liver tumors and human liver tumors suggested the strong association and fundamental importance of these genes in hepatocarcinogenesis across species, which can be further studied in the ZFL-ΔRaf1-ER cell line model.

Table 1. Genes commonly regulated by the Raf/MEK signaling in the ZFL cells, in the zebrafish liver tumors and in human HCC.

A, up-regulation

Unigene	Gene symbol	Gene name	GO
Dr.116619	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	cell adhesion
Dr.114415	FUS	fusion (involved in t(12;16) in malignant liposarcoma)	nuclear mRNA splicing, via spliceosome
Dr.114603	UBE2N	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	positive regulation of NF-kappaB transcription factor activity
Dr.119248	RNF5	ring finger protein 5	modification-dependent protein catabolic process
Dr.119918	WHSC1	Wolf-Hirschhorn syndrome candidate 1	transcription
Dr.12263	SDPR	serum deprivation response (phosphatidylserine binding protein)	unknown
Dr.132819	CALM2	calmodulin 2 (phosphorylase kinase, delta)	unknown
Dr.24686	RPS16	ribosomal protein S16	translational elongation
Dr.31674	STX5	syntaxin 5	intracellular protein transport
Dr.31830	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	cell motion, regulation of action polymerization
Dr.33356	RRM1	ribonucleotide reductase M1	DNA replication, deoxyribonucleotide biosynthetic process
Dr.35479	RUVBL2	RuvB-like 2 (E. coli)	regulation of transcription, DNA-dependent
Dr.361	C11orf31	chromosome 11 open reading frame 31	cell redox homeostasis
Dr.138569	AGRN	agrin	signal transduction
Dr.47436	MCM7	minichromosome maintenance complex component 7	cell proliferation, transcription
Dr.114210	POLD1	polymerase (DNA directed), delta 1, catalytic subunit 125kDa	DNA synthesis during DNA repair, S phase of mitotic cell cycle
Dr.51273	DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	morphogenesis of an epithelial sheet, protein complex assembly
Dr.76816	SCNM1	sodium channel modifier 1	mRNA processing
Dr.76826	AKAP8L	A kinase (PRKA) anchor protein 8-like	unknown
Dr.77687	UBAP2L	ubiquitin associated protein 2-like	unknown
Dr.77769	SMC4	structural maintenance of chromosomes 4	cell cycle
Dr.78038	ELMO1	engulfment and cell motility 1	apoptosis, cell motion, Rac protein signal transduction
Dr.78272	C20orf149	chromosome 20 open reading frame 149	cell differentiation
Dr.97335	VPS28	vacuolar protein sorting 28 homolog (S. cerevisiae)	protein transport
Dr.78291	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	cell adhesion and motility
Dr.78635	BUB3	budding uninhibited by benzimidazoles 3 homolog (yeast)	cell proliferation, mitotic cell cycle checkpoint
Dr.79080	TNFRSF14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	apoptosis, cell surface receptor linked signal transduction
Dr.79208	WDR68	WD repeat domain 68	unknown
Dr.80005	NDRG3	NDRG family member 3	negative regulation of cell growth
Dr.80218	CDC37	cell division cycle 37 homolog (S. cerevisiae)	regulation of cyclin-dependent protein kinase activity
Dr.80917	CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	cell adhesion
Dr.8177	PAX8	paired box 8	positive regulation of transcription, ,DNA-dependent
Dr.82226	CTTN	cortactin	cell adhesion and motility
Dr.83913	MAPRE1	microtubule-associated protein, RP/EB family, member 1	cell cycle, cell proliferation
Dr.89520	TBC1D13	TBC1 domain family, member 13	regulation of Rab GTPase activity
Dr.89609	URM1	ubiquitin related modifier 1 homolog (S. cerevisiae)	modification-dependent protein catabolic process

B, down-regulation

Unigene	Gene symbol	Gene name	GO
Dr.105268	SUCLG2	succinate-CoA ligase, GDP-forming, beta subunit	metabolic process
Dr.11467	PANK1	pantothenate kinase 1	coenzyme A biosynthetic process
Dr.115953	CDH1	cadherin 1, type 1, E-cadherin (epithelial)	cell adhesion
Dr.117252	DHTKD1	dehydrogenase E1 and transketolase domain containing 1	glycolysis, oxidation reduction
Dr.139822	PHYH	phytanoyl-CoA 2-hydroxylase	lipid metabolic process
Dr.14625	COPB2	coatomer protein complex, subunit beta 2 (beta prime)	intracellular protein transport
Dr.28434	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	cellular alcohol metabolic process
Dr.32174	DHRS1	dehydrogenase/reductase (SDR family) member 1	oxidation reduction
Dr.3514	COX4NB	COX4 neighbor	unknown
Dr.3583	SULT6B1	sulfotransferase family, cytosolic, 6B, member 1	unknown
Dr.40063	ACO1	aconitase 1, soluble	metabolic process
Dr.124584	FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	metabolic process
Dr.74875	OAZ1	ornithine decarboxylase antizyme 1	polyamine biosynthetic process
Dr.75635	PINX1	PIN2-interacting protein 1	negative regulation of cell cycle
Dr.76347	C19orf63	chromosome 19 open reading frame 63	unknown
Dr.76704	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	cellular alcohol metabolic process
Dr.78089	COX5A	cytochrome c oxidase subunit Va	unknown
Dr.82487	CCDC94	coiled-coil domain containing 94	unknown
Dr.83978	UPP1	uridine phosphorylase 1	pyrimidine nucleotide metabolic process
Dr.86220	ETFA	electron-transfer-flavoprotein, alpha polypeptide	transport
Dr.116730	C21orf59	chromosome 21 open reading frame 59	unknown

Discussion

Cancer genetics have been studied in human and rodent cell cultures for many years. Validation of the information acquired from these *in vitro* studies into different animal models has largely enhanced our knowledge about cancer. The zebrafish has entered the field of cancer research recently, as a cost-effective model organism sharing the basic molecular cancer biology with human [12, 13]. A few approaches, such as chemical carcinogenesis, forward and reverse genetics, transgenesis and tumor transplantation, have already been used in the zebrafish to model specific cancer types. Comparative oncogenomic studies have validated the carcinogen-induced zebrafish liver cancer as a model to study HCC [14, 15]. In addition, transgenic fish lines have been generated that the KRas oncogene induced liver tumorgenesis and HCC (Gong *et al*., in preparation). However, an *in vitro* inducible tumor system is not available yet in zebrafish.

In this study, 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 [26]. 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 signaling was activated by 4HT administration, which validated the usage of the 4HT/ER system to control oncoprotein expression and function for the first time in zebrafish cells. After 4HT administration, hyperactivation of the Raf/MEK signaling in the ZFL-ΔRaf1-ER cells resulted in survival and proliferation advantages, acquired self-sufficiency with respect of growth factors, and rescued cell death. The unconstrained proliferation and impaired apoptosis are both key hallmarks of cancer, suggesting that the zebrafish liver cells were (partially) transformed by the hyperactive Raf/MEK signaling.

The 4HT treatment also promoted cell survival and inhibited apoptosis of the ZFL-ΔRaf1-ER cells after implantation into zebrafish embryos, which validated the usage of the 4HT/ER system *in vivo*. The growth advantage resulted from the hyperactive Raf/MEK signaling allowed studying of longer term events such as cell proliferation and primary tumor 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 signaling (Figure 6B), suggests the usefulness of the implantation approach to study cellular interaction with the host vasculature and angiogenesis in context of oncoprotein activation.

It has been suggested that cancer is the net outcome of series of incorporated alterations in complex signaling networks. Accelerated cell division and proliferation promoted by the Raf/MEK activation increases susceptibility to the step-wise acquisition of additional genetic mutations necessary for malignant transformation [24]. To further understand the involvement of Raf-1 in the zebrafish liver cell transformation, microarray analysis was performed to systematically identify signaling networks and target genes downstream of the hyperactive Raf/MEK signaling. 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 analyzed the cellular transcriptome after 12 hours of ΔRaf1 activation. The transcriptional alterations resulted from the hyperactive Raf/MEK signaling were involved in many biological processes related to cellular transformation of the ZFL cells. Up-regulation of genes with human homologues known as transcriptional targets by the Raf/MEK signaling 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 signaling (Figure 6). Longer term of 4HT administration, such as 24 hours, resulted in similar gene signatures.

After identifying a pool of transcripts downstream of Raf/MEK signaling in ZFL-ΔRaf1-ER cells, we performed comparative microarray analysis to systematically validate these gene signatures to zebrafish liver tumors. We discovered that many genes involved in cell cycle, proliferation and apoptosis were commonly regulated by hyperactive Raf/MEK signaling in ZFL cells and in zebrafish liver tumors, indicating the essential role of cell survival and hyper-proliferation in hepatocarcinogenesis. Common genes involved in angiogenesis, cell adhesion and motility were also identified, suggesting their roles in zebrafish liver tumor progression. Strikingly, many genes involved in inflammation, especially the Toll-like receptors, were transcriptionally regulated by hyperactive Raf/MEK signaling in ZFL cells but not in zebrafish liver tumors. It might reflect the dynamic association of inflammation and cancer progression, but the mechanism is currently not understood yet.

Our study has shown that the zebrafish cell lines can be used to bridge cancer approaches at different levels. For example, the anti-apoptotic role of ΔRaf1 activation in the ZFL-ΔRaf1-ER cells was demonstrated *in vitro* by cell growth in culture (Figure 3), *in vivo* by survival of implanted cells in zebrafish embryos (Figure 4), and *in silico* by microarray analysis which showed alteration of apoptosis related gene expression (Figure 6B). It not only adds value to the zebrafish as model organism, but also provides a novel platform to expand basic cancer research at the molecular and cellular level towards the tissue, organ and the entire organism level. In addition, we found hundreds of novel transcripts that were specifically regulated by hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells (Figure 6A). Many of these transcripts were not previously annotated. Most of them have been linked to human homologs, but for many of these the function remains unknown. The ZFL-ΔRaf1-ER cell line can be used for the future functional characterization of these genes, which will bring new insight to understand Raf/MEK signaling and the molecular mechanism of HCC. We believe that modeling HCC using the zebrafish and ZFL cells will provide more knowledge about the molecular and preclinical phases of HCC, and help us to identify a subset of key regulatory genes, which may be subsequently chosen for evaluation as candidate targets for HCC therapies.

Material and Methods

Plasmids

A neomycin resistant vector was generated from the pECFP-N1 (Clontech) by replacing CFP with a short piece from the pEYFP-C1 (Clontech) using BamHI and MfeI. To generate the ΔRaf1-ER-neo construct, primers were designed against the ΔRaf1-ER fragment in pLNC-ΔRaf1-ER (kindly provided by Bob van de Water): forward primer, GGTAGCTGACTGTGTGAAGA; reverse primer, ACCTACAGGTGGGGTCTTTC. ΔRaf1-ER was cloned into pCR®4Blunt-TOPO® (Invitrogen) following PCR with Phusion (Finnzymes) and subsequently cloned into the neomycin resistant vector using EcoRI.

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 (T_d) was calculated using Doubling Time Online Calculator (http://www.doubling-time.com/compute.php?lang=en). For stable transfection, ZFL cells were nucleofected using Amaxa nucleofetor under the manufacturer's instruction (Amaxa, program T27 and buffer V) and followed by G418 selection (1 mg/ml). Stable transfected cells are maintained in CGM with 0.5 mg/ml G418.

Immunoblotting

Cells were lysed in 1x Loading Buffer (Cell Signaling). Proteins were electrophoresed in SDS-polyacrylamid gel and transferred onto nitrocellulose membranes (Schleicher & Schuell). The membranes were subsequently blocked with 5% skim milk in TBS-Tween 20 (TBST) and incubated with appropriate antibodies in TBST with 3% bovine serum albumin (Sigma). Signal was detected using a 1:5000 dilution of horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence methods (Amersham). Western Re-probe kit (Omnilabo) was used to stripe the blot.

Generation of zebrafish liver tumors

Three-week-old zebrafish fry were treated with 0.75 ppm 7,12-dimethylbenz(α)anthracene (DMBA) or dimethyl sulfoxide (DMSO, vehicle) for 24 h and the treatment was repeated once at 5 weeks old for another 24 hours with 1.25 ppm DMBA or DMSO. Treated fish were rinsed three times in fresh water and transferred into new tanks for maintenance. Fish were sampled during 6-10 months after the onset of DMBA exposure. Tumor samples used for the microarray study were all bigger than 3 mm in diameter. Partial liver tumors were sampled for RNA exaction and the rest of liver tissues were examined by histopathological diagnosis.

RNA preparation and microarray hybridization

For the ZFL-ΔRaf1-ER cells, biological triplications were taken for each condition. 5 zebrafish liver tumors and 6 healthy zebrafish livers were used to detect gene signatures in zebrafish liver tumors. Total RNA was isolated and prepared as described [29]. Hybridization and scanning on the custom-designed Agilent 4x44k zebrafish microarray containing 43365 oligos (19122 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% PMT and 100% PMT laser power and the XDR function was used to extend the dynamic range with 10-fold. The raw data will be submitted to the Gene Expression Omnibus database (www.ncbinlm.nih.gov/geo).

Microarray data analysis

Microarray data was imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modeling. Data analysis was performed at P≤1.00E-05 for unigene clusters. The Unigene and Entrez Gene records of the functionally related human homologs 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 the GeneTools eGOn v2.0 software (www.genetools.microarray.ntnu.no) [30] and the FunNet software (www.funnet.info) [31] using the Entrez Gene codes of human orthologs.

The featured human HCC gene list was obtained from the database of Encyclopedia of Hepatocellular Carcinoma genes Online (http://ehco.iis.sinica.edu.tw), based on 13 microarray studies and PubMed researches related to HCC [27, 28]. The genes regulated by Raf/MEK signaling in the zebrafish liver cells and liver tumors were manually compared with the human HCC gene list using the Entrez Gene codes of human orthologs. The functionalities of the resulted common genes were manually analyzed by searching the NCBI database, PubMed and the Gene Ontology Consortium (www.geneontology.org; Supplementary Table 3).

Cell implantation and zebrafish embryo handling

Cells were labeled with CM-DiI according to the manufacturer's instruction (Invitrogen). After labeling, the cells remained fluorescent up to 14 days, without detected deficiency in survival and proliferation. The labeled cells were injected into the yolk of 2 dpf zebrafish embryos [21]. Zebrafish embryos were raised according to the standard procedures [32]. After implantation, embryos were incubated in daily-refreshed egg water supplemented with 5% DMSO and 0.003% PTU, with or without 1 μM 4HT.

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