

## Zebrafish as a Cancer Model

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### Abstract

The zebrafish has developed into an important model organism for biomedical research over the last decades. Although the main focus of zebrafish research has traditionally been on developmental biology, keeping and observing zebrafish in the lab led to the identification of diseases similar to humans, such as cancer, which subsequently became a subject for study. As a result, about 50 articles have been published since 2000 in which zebrafish were used as a cancer model. Strategies used include carcinogenic treatments, transplantation of mammalian cancer cells, forward genetic screens for proliferation or genomic instability, reverse genetic target-selected mutagenesis to inactivate known tumor suppressor genes, and the generation of transgenics to express human oncogenes. Zebrafish have been found to develop almost any tumor type known from human, with similar morphology and, according to gene expression array studies, comparable signaling pathways. However, tumor incidences are relatively low, albeit highly comparable between different mutants, and tumors develop late in life. In addition, tumor spectra are sometimes different when compared with mice and humans. Nevertheless, the zebrafish model has created its own niche in cancer research, complementing existing models with its specific experimental advantages and characteristics. Examples of these are imaging of tumor progression in living fish by fluorescence, treatment with chemical compounds, and screening possibilities not only for chemical modifiers but also for genetic enhancers and suppressors. This review aims to provide a comprehensive overview of the state of the art of zebrafish as a model in cancer research. (Mol Cancer Res 2008;6(5):685–94)

### Cancer Research in Zebrafish

Zebrafish has been used as a laboratory animal for a few decades now. Originally, the main focus was on developmental biology because of the clear advantages of zebrafish such as

large clutch size, transparent embryos, and *ex utero* development of the embryo. While keeping zebrafish in the laboratory environment, however, researchers observed different diseases in adults, including cancer. Studies on the latter revealed that zebrafish spontaneously develop almost any type of tumor (1-4). The most common target tissues for spontaneous neoplasia are the testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland. Less common target tissues include blood vessels, brain, gill, nasal epithelium, and the lymphomyeloid system (2). In the first part of this review, the currently used approaches to induce cancer in zebrafish will be discussed. An overview of these and their main advantages and disadvantages are given in Table 1. Additionally, cancer results of the forward and reverse genetic and transgenic mutant models are summarized in Table 2.

#### *Treatment with Mutagens*

Historically, researchers appreciated the relative ease of treating fish with carcinogens because the chemicals can be dissolved or suspended in water and the animals can be exposed for longer time periods. When exposing zebrafish or guppies to different compounds [e.g., 7,12-dimethylbenz(a)anthracene, *N*-nitrosodimethylamine, and *N*-nitrosodiethylamine], mainly liver and intestinal tumors were observed (5-7). More recently, similar but more extensive studies showed that *N*-nitrosodiethylamine primarily induces liver and pancreas carcinomas (8), and *N*-nitrosodimethylamine induces only liver tumors (9). 7,12-Dimethylbenz[a]anthracene induces the broadest tumor spectrum, including liver neoplasms; epithelial tumors in intestine, pancreas, thyroid, and testis; mesenchymal tumors in cartilage, blood vessels, muscles, and connective and lymphoid tissues; and neural tumors (10). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine can also induce different tumor types, mainly liver and testicular neoplasms, as well as hemangio(sarco)mas and others (11). Some of these carcinogenic treatments have been applied to mutants with a genetic predisposition to cancer but a low spontaneous tumor incidence to show an increased sensitivity of mutants compared with treated wild-type animals (12-14). Not surprisingly, the alkylating mutagen *N*-ethyl-*N*-nitrosourea, which introduces point mutations and is commonly used in forward and reverse genetic screens, has also been found to induce cancer. Following a group of animals from a mutagenesis screen, Beckwith et al. (15) found that all fish developed skin papillomas over time, but not invasive skin cancers.

#### *Transplantation of Mammalian Cancer Cells into Zebrafish*

Different groups have been experimenting with transplantation of mammalian cancer cells into zebrafish embryos. This

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**Table 1. Comparison of Techniques to Induce Cancer in Zebrafish**

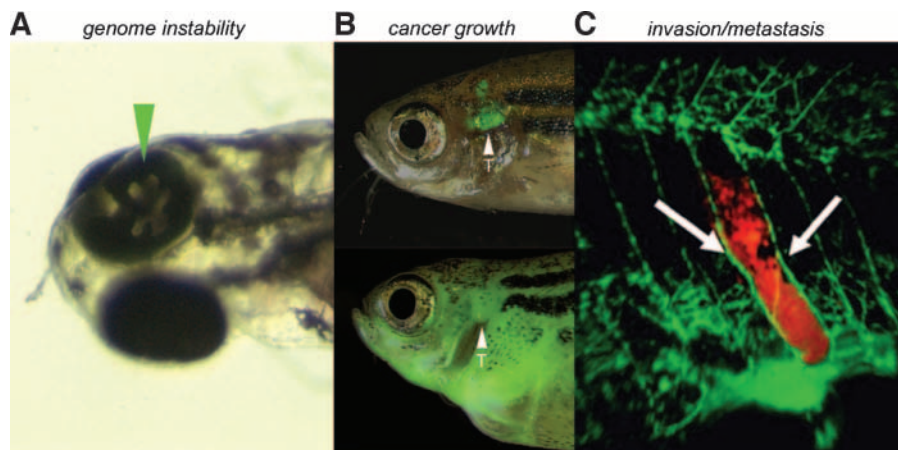
Technique	Advantages	Disadvantages
Chemical treatment	Easy applicable Large numbers of fish Long-term treatment	Unspecific Predominance of liver tumors Potential hazard for researcher
Transplantation of mammalian cells	Rapid onset Study in transparent embryos Use of human cancers Use of fluorescence	Cannot be propagated as a line Low penetrance of tumors
Forward genetic screens	Identification of multiple genes at once Embryonic phenotype as readout Combination with drugs, mutants or transgenics	Laborious Screens for adult phenotypes require space and time Stochasticity of cancer makes it difficult as readout
Reverse genetic knockouts	Inactivation of one specific gene Human-like cancer mutations	Background mutations Late onset and low penetrance Gene duplications
Expression of transgenes	Easy generation by injection Rapid onset Use of human genes Use of fluorescence Conditional and tissue-specific expression	Different tumor spectrum than in humans Laborious if no transgenic line can be established Lack of specific promoters

creates an *in vivo* system in which the advantages of cultured human cancer cells are combined with those of transparent zebrafish embryos in which development can be followed. Lee et al. (16) transplanted fluorescently labeled human metastatic melanoma cells into zebrafish blastula-stage embryos and showed that these cells survive, migrate, and divide, and are still present in adults but do not cause cancer or metastases. Another study showed that aggressive human melanoma cells are able to induce a secondary axis or an abnormal head when transplanted into 3-hour-old zebrafish embryos, which was shown to be due to Nodal signaling from the tumor cells (17, 18). In contrast to the above studies where no cancer development was observed, similar human melanoma cells as well as a colorectal and a pancreatic cancer cell line were found to induce tumor-like cell masses when transplanted into 2-day-old zebrafish embryos (19).

Transplantation studies can be specifically effective in the study of vasculature remodeling, cancer invasion, and metastasis. Currently, no good *in vivo* model is available in which such dynamic process can be followed in real time. When transplanted into 2-day-old zebrafish embryos, human and murine tumor cell lines expressing fibroblast growth factor or vascular endothelial growth factor induced rapid neovascularization inside the tumor graft, and this could be inhibited by treatment with antiangiogenic chemicals (20). In another elegant study, researchers used i.p. injection of fluorescently labeled human breast cancer cells in 1-month-old zebrafish in combination with three-dimensional modeling to show how the human cells interact with vessels and invade in tissues (Fig. 1C). They showed that expression of vascular endothelial growth factor induces openings in vessel walls that can be used for invasion, which in turn is stimulated by RhoC expression (21).

**Table 2. Cancer Mutants in Zebrafish**

Model	Cancer	Type	Incidence (%)	Onset (mo)	Ref.
Forward genetic mutants					
Ribosomal protein	+	MPNST	35	8-24	(24)
<i>separase</i>	Induced	Liver, intestinal	10	3-12	(13)
<i>bmyb</i>	Induced	Testis, vascular	7	3-12	(14)
Genomic instability	+	MPNST and others	Up to 48	30-36	(25)
Reverse genetic mutants					
<i>tp53</i>	+	MPNST	28	14	(27)
<i>ptenb</i>	+	Ocular	33	7-18	(28)
<i>apc</i>	+	Liver, intestinal	29	15	(12)
<i>mlh1, msh2, msh6</i>	+	MPNST and others	33	17	
Transgenic mutants					
<i>RUNX1-CBF2T1</i>	-				(32)
<i>TEL-AML1</i>	+	Leukemia	3	8-12	(33)
Zebrafish <i>tel-jak2</i>	-				(34)
Mouse <i>c-myc</i>	+	Leukemia	6	1-4	(35)
Mouse <i>c-myc</i> (line)	+	Leukemia	100	2	(36)
Mouse <i>c-myc</i> (conditional)	+	Leukemia	81	4	(37)
Zebrafish <i>bcl2</i>	-				(38)
<i>NOTCH</i>	+	Leukemia	40	5-11	(39)
<i>kRASG12D</i>	+	Rhabdomyosarcoma	47	2	(40)
<i>kRASG12D</i> (conditional)	+	Rhabdomyosarcoma and others	100% of survivors	1	(41)
<i>BRAF-V600E</i>	In <i>tp53</i> <sup>-/-</sup>	Melanoma	6	4	(42)
<i>MYCN</i>	+	Neuroendocrine	2	5	(43)



**FIGURE 1.** The unique possibilities of zebrafish can be used to study different stages of cancer development. **A.** Genome instability will lead to mutation accumulation that can result in cancer. Here, somatic loss of heterozygosity of the *albino* gene is shown in a patch of cells (arrow) in the pigment layer of the eye of an *msh6* mutant zebrafish embryo (H. Feitsma, A. Akay, E. Cuppen, unpublished results). **B.** Cancer growth can be followed in living adult zebrafish. Here, the growth of a green fluorescent protein–labeled T-cell acute lymphoblastic leukemia in stable transgenic fish is shown. Top, *rag2-EGFP* control fish; bottom, *rag2-EGFP-mMyc* fish with massive leukemia; T, thymus. [Reprinted with permission from ref. 36; copyright (2005) National Academy of Sciences.] **C.** Invasion of cancer cells in blood vessels is the first step of metastasis. Here, transplanted dsRED-labeled human cancer cells penetrate green fluorescent protein–labeled blood vessels (arrows) in a zebrafish. [Reprinted with permission from ref. 21; copyright (2007) National Academy of Sciences.]

#### Mutants from Forward Genetic Screens

The largest impetus for zebrafish to become an important animal model was its suitability for forward genetic screens. Since the first mutagenesis experiments in the Nusslein-Volhard lab (22), screens have been carried out for almost any type of phenotype, including phenotypes related to cancer (Table 2). Amsterdam et al. (23) rather coincidentally ran into a set of genes that cause cancer when mutated. Using retroviral insertions, they carried out a screen for embryonic lethality, but while keeping the heterozygous founders they noticed that 12 of their lines displayed an elevated tumor incidence (24). Eleven of those lines carried insertions in ribosomal genes, for which there was no strong relation with cancer at that point. The other line was mutated in one of the two zebrafish homologues of the neurofibromatosis 2 (*nf2*) gene, a known tumor suppressor in humans.

The Zon lab conducted a screen for proliferation defects, taking advantage of the fact that many oncogenes and tumor suppressors are actually essential for development. Homozygous mutant embryos are therefore lethal, and heterozygotes are, due to haploinsufficiency or loss of heterozygosity, predisposed to cancer later in life. Two mutants from this screen have been reported thus far, a loss-of-function mutation in *bmyb* (14) and a loss-of-function mutation in *separase* (13), genes that both had not previously been unequivocally implicated in cancer. Homozygous mutant embryos of both displayed mitotic defects and genomic instability, whereas heterozygous adults showed a marginal increase (2- to 2.5-fold) in cancer incidence when treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

An elegant screen for genomic instability used loss of heterozygosity of the *golden* pigmentation gene as a readout (25). When in *golden* heterozygous embryos, cells of the retinal pigment epithelium of the eye acquire a mutation in the wild-type allele. This results in unpigmented patches that are easily scorable in large batches of embryos (Fig. 1A). The 12 mutant

lines with embryonic genomic instability that were obtained all showed some or more sensitivity to cancer in heterozygous adults, confirming the strong connection between genomic instability and cancer. However, the underlying mutations have not been mapped or cloned yet.

#### Reverse Genetics: Target-Selected Inactivation of Tumor Suppressor Genes

Since knockout technology in zebrafish became available by means of target-selected mutagenesis (26), several mutants for known tumor suppressor genes have been generated (Table 2). For *tp53*, the most frequently mutated gene in human cancers, two zebrafish mutants were isolated: one with a missense mutation in the DNA-binding domain ( $tp53^{M214K}$ ) and one with a missense mutation that affects protein structure in a heat-sensitive manner ( $tp53^{N168K}$ ). Homozygotes of both lines were developmentally normal but showed suppressed apoptosis on irradiation. Twenty-eight percent of  $tp53^{M214K}$  homozygotes developed tumors at an average age of 14 months, which, except for one melanoma, were all diagnosed as malignant peripheral nerve sheath tumors (MPNST; ref. 27).

The second most frequently mutated tumor suppressor in human cancers, *pten*, has undergone a gene duplication in zebrafish. Faucher et al. isolated nonsense mutants for both *ptena* and *ptenb*. Single homozygous mutants of either allele did not display a developmental phenotype, but mutants lacking both *ptena* and *ptenb* died at day 5 postfertilization from pleiotropic defects due to enhanced proliferation and cell survival, indicating redundant functions. However, adult fish lacking only *ptenb* developed ocular tumors with an incidence of 33% at 18 months, whereas no neoplasms were observed in adult *ptena* mutants. Tumors were not further classified, but their appearance and the fact that they occur only intraocular suggest that they are not MPNSTs (28).

Mutations in the adenomatous polyposis coli (*APC*) gene cause the vast majority of human sporadic and inherited

colorectal cancers by constitutively activating the Wnt signaling pathway (29). A nonsense mutation in zebrafish *apc* was found to result in lethality when homozygous (30). Less than 30% of heterozygous fish spontaneously developed liver and intestinal tumors from 15 months of age onward. Treatment of *apc* heterozygotes with 7,12-dimethylbenz[*a*]anthracene enhanced tumorigenesis, resulting in intestinal, hepatic, and pancreatic tumors, with frequencies 3- to 4-fold higher than treated wild-types. The tumors displayed activated Wnt signaling, indicating that the genetic pathway is conserved (12).

The first DNA repair genes that have been mutated in zebrafish are the mismatch repair genes *mlh1*, *msh2*, and *msh6*, which are involved in the repair of small replication errors such as base mismatches and insertion/deletion loops. Homozygous mutants were genomically unstable, as shown by the occurrence of variation in lengths of microsatellite sequences in their DNA. Taking the data for the three mutants together, 33% of homozygotes, on average, developed tumors at an average age of 17 months. Mainly MPNSTs were found at different places in the body, but other tumor types were also observed.<sup>1</sup>

Targeted knockout strategies such as those used for making mouse knockouts are not available in zebrafish. The current strategy makes use of random *N*-ethyl-*N*-nitrosourea mutagenesis combined with targeted selection of mutations in the gene of interest, which means that the researcher is dependent on the random point mutations that are induced. However, the positive aspect of this is that the generated point mutations can be more similar to the type of spontaneous mutations that occur in human cancer patients than the large gene deletions or insertions in mouse knockouts. Indeed, two of the published zebrafish mutants do exactly mimic human cancer mutations: the tp53<sup>M214K</sup> point mutation in zebrafish *tp53* is frequently found in human cancers (27) and the splice site mutation in the zebrafish *msh2* gene results in an in-frame loss of exon 5,<sup>1</sup> which is one of the most frequent familial mutations in hereditary nonpolyposis colorectal cancer, the human syndrome that is caused by defective mismatch repair.

One of the disadvantages of the use of random mutagenesis in the knockout procedure is that each fish that is retrieved will contain, besides the mutation of interest, several background mutations. Those additional mutations are heterozygous and will probably not have a large effect on developmental phenotypes, but they may of course be of influence on the process of mutation accumulation that is necessary for tumor development. Therefore, *N*-ethyl-*N*-nitrosourea-induced mutants should be crossed out for several generations to exclude confounding effects of unknown background mutations.

#### Transgenic Zebrafish Expressing Mammalian Oncogenes

The largest number of studies on cancer development in zebrafish thus far come from transgenic zebrafish expressing mammalian oncogenes (Table 2). This approach makes use of another advantage of zebrafish as a laboratory animal: the convenience of introducing foreign DNA into zebrafish cells and getting it expressed by injection into one-cell embryos.

Many of the models concern lymphomas and leukemias, cancers that rarely occur spontaneously in zebrafish but for which the transgenic model may be of great aid in searching for new treatments. This is especially important for this class of diseases when considering that human patients usually need therapies severely affecting quality of life.

Frequently, hematologic malignancies arise because of genetic rearrangements resulting in misexpression of certain oncogenes (e.g., by fusion to lymphoid-expressed genes). Although many of the genes and even chromosome regions known to be involved have been conserved in zebrafish (31), researchers have mostly limited their experiments to expressing human fusion constructs. For example, injection of a human RUNX1-CBF2T1 fusion, which is frequent in acute myeloid leukemia, caused circulation defects, hemorrhages, abnormal vascular development, and defective hematopoiesis, but not leukemia, in zebrafish embryos (32). The human TEL-AML1 fusion is responsible for 25% of childhood pre-B acute lymphoblastic leukemia cases. When expressing a TEL-AML1 fusion construct in zebrafish, acute lymphoblastic leukemia developed in 3% of fish that expressed the transgene ubiquitously, but not when expression was restricted to lymphoid cells using the *rag2* promoter (33). In an attempt to model a molecular rearrangement associated with human lymphoblastic and myeloid leukemias, Onnebo et al. (34) injected embryos with a zebrafish tel-jak2 fusion under control of the myeloid *spi1* promoter, which caused not only severe perturbation of hematopoiesis but also high mortality, making it impossible to study adults.

Langenau et al. have put an enormous effort into generating optimal zebrafish models for leukemia. They developed zebrafish expressing mouse *c-myc* under the zebrafish *rag2* promoter to restrict the expression to lymphoid cells. MYC is known to play an important role in human and mouse lymphoid malignancies. Six percent of injected fish developed infiltrative T-cell leukemia (Fig. 1B) with a latency of 30 to 131 days (35). A germ line-transmitting transgenic line of these fish displayed 100% cancer incidence with a mean latency of 80 days (36), but because disease onset mostly precedes reproductive age, the line can only be propagated via *in vitro* fertilization, which makes experiments with this model labor intensive. However, by creating a conditional transgene in which a *dsRED* gene flanked by loxP sites was put in between the *rag2* promoter and the mouse *c-myc* (*mMyc*) gene (*rag2-loxP-dsRED-loxP-EGFP-mMyc*), and using this transgenic line in combination with CRE injection, leukemia development was made inducible. Unfortunately, only partial recombination was obtained, resulting in leukemia in only 12 of 186 CRE-injected animals (36). A second improvement was made by crossing the conditional line to a transgenic line expressing CRE under a heat shock promoter (37). On heat shock, double transgenic fish developed T-lymphoblastic lymphoma that rapidly progressed to T-cell acute lymphoblastic leukemia. The best heat shock treatment gave an induction of cancer in 81% of fish, with a mean latency of 120 days, but in the next generations of the transgenic line the cancer latency was higher (37). The same lab also developed a transgenic line overexpressing the zebrafish B-cell leukemia 2 (*bcl2*) gene under the *rag2* promoter, in which lymphoid apoptosis is blocked (38). Irradiation treatment of fish

<sup>1</sup> H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman, E. Cuppen, submitted for publication.

that were transplanted with leukemic cells from *mMyc* transgenic fish (36) ablated cancer in wild-type fish but not in the *bcl2*-overexpressing fish (38). In another leukemia model, the knowledge was used that activating mutations in NOTCH1 are found in ~60% of T-cell acute lymphoblastic leukemia. Zebrafish expressing constitutively active human NOTCH under the *rag2* promoter developed T-cell leukemia at 5 to 11 months in ~40% of the cases (39). When crossing these fish with the *bcl2*-overexpressing line (38), disease onset was accelerated and the leukemias were not radiation sensitive anymore, as apoptosis was inhibited.

Several transgenic models for solid tumors have also been generated. Langenau et al. observed that the zebrafish *rag2* promoter that they frequently used showed ectopic expression in undifferentiated cells of the muscle, making it possible to express human activated RAS (kRASG12D), a common mutation in human oncogenesis, in these cells, resulting in the development of rhabdomyosarcoma in 47% of the cases by 80 days of age and in only one lymphoid hyperplasia (40). Tumorigenesis was accelerated when these experiments were done on the *tp53* mutant genetic background. A conditional version of the same activated human RAS under a zebrafish  $\beta$ -actin promoter with a floxed *EGFP* gene inserted in between was combined with the previously mentioned heat shock-inducible CRE. This resulted in overall reduced viability in heat-shocked as well as non-heat-shocked animals. The latter is most likely due to leakiness of the heat shock promoter of the CRE line. Surviving fish were found to develop four types of tumors at ~35 days: rhabdomyosarcomas, myeloproliferative disorders, intestinal epithelial hyperplasmas, and two cases of MPNST (41).

To test the role of activated BRAF in melanoma development, transgenic zebrafish expressing activated human BRAF-V600E under the zebrafish melanocyte *mitfa* promoter were generated. These fish developed pigmented nevi in 10% of the cases, but not melanoma (42). Although *tp53* mutations are rare in human melanoma, in *tp53* mutant background 4 of 66 transgenic fish developed highly invasive and transplantable melanoma.

Finally, human *MYCN* under the zebrafish *myod* promoter is expressed in neural tissue and pancreas and, in combination with a human core enhancer, also in muscles. Only 5 of 250 fish injected with this construct developed tumors at ~5 months of age, one cranial and four abdominal, but all neuroendocrine (43).

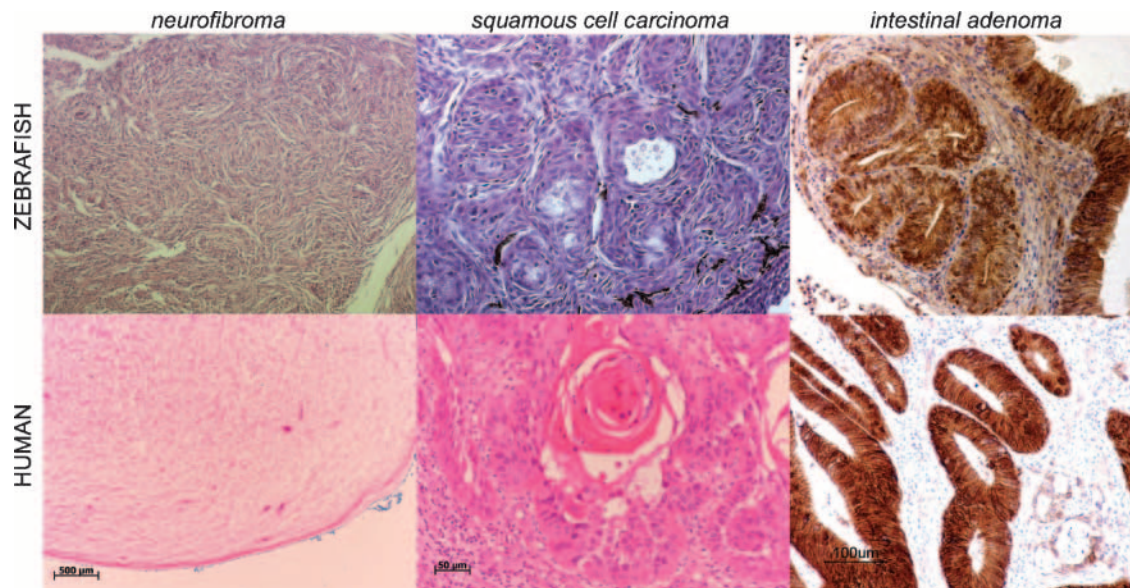
## Zebrafish Cancers Compared with Mammalian Cancers

As previously mentioned, zebrafish can develop almost any type of cancer (2). Moreover, a tremendous asset of zebrafish as a cancer model is that many tumors histologically resemble human tumors (Fig. 2; ref. 44). In addition, more general cancer characteristics such as genomic instability, invasiveness, transplantability, and the existence of cancer stem cells (40) apply to zebrafish tumors as well (Fig. 1), and many tumor suppressor genes and oncogenes have been conserved. Taken together, these studies validate zebrafish as a bona fide cancer model. However, many details are still unknown and some important differences with regard to human tumorigenesis have also become clear, which will be discussed below.

## Cancer Incidence and Onset

The tumor suppressor genes that have been mutated in zebrafish have thus far been limited to the few well-studied classic examples, for which mouse knockouts have been generated as well, and human hereditary diseases are known. Mouse *apc*( $\Delta$ 716) knockouts are embryonic lethal in the homozygous state, similar to what was found in zebrafish. However, all heterozygous mice develop intestinal polyps by 7 weeks of age (45). *Pten* homozygous mutant mice are also early embryonic lethal, and heterozygotes die before 3.5 months of age from malignant tumors (46). Likewise, 74% of *p53* homozygous knockout mice develop cancer before 6 months of age (47). The *msh2* and *mlh1* mouse knockouts were found to have a 50% cancer incidence at 6 months. For the *msh6* knockout this was less severe because *msh6* is partially redundant with *msh3* (48). The conditions caused by mutations in the above genes in humans are Li-Fraumeni syndrome for *TP53* (49), familial adenomatous polyposis for *APC* (50), Cowden disease and Bannayan-Riley-Ruvalcaba syndrome for *PTEN* (51), and hereditary nonpolyposis colorectal cancer for mismatch repair genes (52). These human diseases are considered early-onset syndromes, becoming clinically relevant around the age of 40 to 45 years. Tumor incidences are probably high but mostly unknown because unaffected carriers of disease mutations are usually not noticed.

Overall, the tumor incidences in zebrafish are generally lower and the onset is later as compared with the orthologous mouse models. Although animal numbers of the initial studies are limited, it turns out that the cancer incidence is quite similar for all zebrafish mutants, close to 30% (12, 27, 28).<sup>1</sup> In addition, the average tumor frequency in the ribosomal gene mutant lines of the retroviral insertion screen was also ~35% (24). This high similarity in frequencies is remarkable, and suggests that this is the maximum frequency of cancer that spontaneously develops in genetically predisposed zebrafish. Furthermore, zebrafish develop tumors generally in the second year of life (12, 27, 28),<sup>1</sup> whereas the comparable mouse mutants develop cancer within the first 6 months of life. Because the overall life span is similar for mice and fish, the difference could be due to the lower number of cells in fish, which simply decreases the chance that one body cell will acquire all oncogene activations and tumor suppressor truncations necessary for tumor development. However, considering the zebrafish body weight is ~100-fold lower than that of mouse (0.3 versus 30 g) and the number of cells will be more or less accordingly, a time to disease onset that is four times larger (2 years versus 6 months) and a disease incidence that is three times smaller (30% versus 100%) are actually relatively small differences. In addition, the lower zebrafish disease incidence as compared with mice is experimentally not a major problem because generating and keeping a 3-fold larger number of fish is practically easy. In any case, the frequency of cancer in mutant fish is much higher than in wild types, which spontaneously develop tumors in the order of 0% (27) to 11% (24) of the population. Another long-term study of spontaneous neoplasms determined that wild-type fish have 1% cancer incidence in the first year, which increases in the second year (10). However, most groups agree that more data need to be generated on tumor incidence in wild-type fish to improve the model.



**FIGURE 2.** Zebrafish tumors resemble various types of human tumors at the histologic level. Comparison of histology of neurofibroma (*left*; stained with H&E), squamous cell carcinoma (*middle*; stained with H&E), and intestinal adenoma (*right*; immunostained for  $\beta$ -catenin) in zebrafish (*top*) and human (*bottom*).

Carcinogen treatments on zebrafish give more robust induction of cancer and are considerably easier to perform as compared with the mouse system. Although early studies of transgenic fish expressing oncogenes yielded highly variable tumor incidences, it is exciting and promising to see that technical improvements boosted cancer incidences to 80% to 100% (36).

A recurrent problem in zebrafish is the presence of duplicated genes resulting from a recent partial or complete genome duplication in teleosts (53), which may influence the role of oncogenes and tumor suppressors in carcinogenesis. An example is the presence of two forms of *pten* in the zebrafish genome, which were found to be functionally redundant in development but not in oncogenesis: no loss of *ptena* was observed in tumors of *ptenb* homozygous mutants (28). In addition, although loss of heterozygosity is expected to be the frequent mechanism in humans to explain the cancer predisposition in individuals with heterozygous tumor suppressor mutations (54), it was hardly ever observed in the comparable zebrafish mutants: not in *apc* (12), not in all mutant lines of the retroviral insertion screen (24), only once in the whole set of genomic instability mutants (25), twice in the *separase* mutant (interestingly, these particular mutants did not show a polyploidy phenotype; ref. 13), and never in the *bmyb* mutant (14). An interesting study in this respect is the *N*-nitrosodimethylamine induction of liver tumors in triploid and diploid zebrafish (9). When assuming that loss of function of tumor suppressor genes is an important step in cancer development, one would expect that in case of a triploid fish, three alleles need to be hit to lose the function of the gene, consequently taking longer for tumors to develop. Indeed, triploid fish had a slightly later onset of cancer, but concomitantly these fish showed a higher incidence of tumors, which indicates that activation of oncogenes is equally important.

#### Tumor Spectrum

Most tumor classifications in zebrafish are still rather broad, lacking the more sophisticated identification marker stainings available for mammals. Unfortunately, many commonly used assays for human and mouse do not work on zebrafish material, despite repeated efforts from probably almost all zebrafish tumorigenesis labs. The Cheng lab is working towards a systematic zebrafish tumor histology database,<sup>2</sup> which is a very valuable initiative to standardize tumor classification. Nevertheless, the need for zebrafish-specific antibodies is evident for this and many other research areas.

The most common target tissues for spontaneous tumors in wild-type fish are testis and liver (2). In contrast, genetic mutant lines most commonly develop MPNSTs, as observed in the *p53*, mismatch repair, ribosomal protein, and genomic instability mutants (24, 25, 27).<sup>1</sup> Likewise, the transgenic line overexpressing human *MYCN* develops MPNSTs (43). MPNSTs form a large group of tumors that includes, for example, neurofibromas, the tumor type that occurs in human neurofibromatosis conditions (Fig. 2). In the *nf2* mutant fish line from the retroviral screen, this type of tumors can therefore certainly be expected (24). As already mentioned, defective mismatch repair in humans is linked to hereditary nonpolyposis colorectal cancer, which is a colorectal cancer. However, this concerns patients that are heterozygous for the mismatch repair gene mutation. Interestingly, the rare patients with biallelic inactivation of mismatch repair genes have been reported to develop a more neurofibromatosis type 1–like syndrome with skin neurofibromas and brain tumors. Mouse mismatch repair knockouts mainly develop lymphomas (48), indicating that the zebrafish models important aspects of the human disease that are not

<sup>2</sup> K. Cheng, personal communication.

observed in mice (Table 3). *p53* mutants in human and mouse do not develop MPNSTs but rather osteosarcomas, rhabdomyosarcomas, breast cancer, brain tumors, and leukemias in human (49), and lymphomas and sarcomas in mouse (Table 3; ref. 47).

The intestinal and liver tumors in zebrafish *apc* mutants (12) are comparable to those in mouse mutants and human patients, also with regard to constitutive activation of the Wnt pathway in tumors (Table 3; Fig. 2; refs. 45, 50). Because the zebrafish gut is much smaller than that of mouse and human, the absolute chances for developing gut cancer are probably proportionally smaller, possibly explaining the observed difference in penetrance. Surprisingly, in zebrafish *ptenb* mutants, only one type of tumors inside the eye was found (28), whereas mouse and human *pten* mutants show a broad spectrum of cancers (Table 3; refs. 46, 51).

To get a more detailed view on similarities of fish and human cancers, gene expression profiles of zebrafish and human tumors have been compared. For the *bmyb* mutant, gene expression profiles of homozygous embryos showed significant correlation with human cancers (14), which is noteworthy, as a developmental zebrafish phenotype was compared with a human cancer. A more extensive expression array study was done on 7,12-dimethylbenz[*a*]anthracene- and 1,4-diphenyl-1,3-butadiene-induced liver tumors in zebrafish, showing significant similarities with human liver tumors, but not with other human tumor types (55-57). The two human types of rhabdomyosarcoma, embryonic rhabdomyosarcoma and alveolar rhabdomyosarcoma, have a distinct molecular signature. Microarray analysis on rhabdomyosarcomas of activated RAS transgenic fish showed that these have a signature similar to human embryonic rhabdomyosarcoma but not to alveolar rhabdomyosarcoma. The profile also showed similarities to other RAS-induced tumors such as human pancreatic adenocarcinoma and mouse lung adenocarcinoma, in addition to a more specific embryonic rhabdomyosarcoma signature of muscle genes (40). These studies collectively indicate that the genetic pathways involved in cancer development are conserved between fish and mammals.

### Specific Advantages of Zebrafish

The differences of zebrafish cancer from mammalian cancers do not compromise the organism as a cancer model, but should rather help focusing on the specific strengths of zebrafish to unravel mechanisms in carcinogenesis, complementary to other models.

#### Screens

The suitability of zebrafish for genetic screens has long been recognized also in cancer research. However, although very elegant strategies were chosen for the first screens for cancer

genes that were done in zebrafish, the results were promising but not completely convincing. Two new genes for which a relation to cancer was unknown or unclear were identified in the proliferation screen, but the cancer predisposition in heterozygous mutants is marginal and only visible when carcinogen treatment was used (13, 14). In relation to this, no mutations in *separase*, one of the identified genes, were found in 82 human tumor cell lines. In addition, although the ribosomal protein mutants from the retroviral insertion screen were clearly cancer-prone, a clear connection of this important and large group of genes to cancer had never been observed in other organisms (24). Certainly, further research is necessary to qualify these genes as real "cancer genes." The mutants from the genomic instability were all clearly predisposed to cancer (25), but because the embryonic phenotype is a matter of chance and not fully penetrant, mapping the mutants is labor intensive and time-consuming. Nevertheless, there are many options for advanced novel screens by performing mutagenesis in a cancer-prone background or crossing mutants to a cancer-prone line to identify genetic modifiers of specific cancers. Additionally, making use of transgenic lines with fluorescent reporters and/or chemical compound libraries (see below) will certainly result in new steps in cancer research.

#### Imaging

The lauded advantage of zebrafish embryos being transparent does not, except for some transplantation studies, apply to most cancer studies in zebrafish that involve adult animals. However, a relatively transparent adult zebrafish line that lacks all types of pigment has been generated by Znomics (58). Additionally, Goessling et al. (59) have successfully applied high-resolution microscopic ultrasound to follow tumor development and regression by treatment in living adult fish. Other existing imaging techniques that should, in principle, be possible in zebrafish are tomography and magnetic resonance imaging (58).

Most effective, however, and specific for zebrafish is the use of fluorescence, as even adult zebrafish are small enough to be able to visualize fluorescent organs or tumors inside the living body. Tumor development and cancer spreading can therefore be followed over time in a living fish. Indeed, transplantation studies described above often used fluorescently labeled mammalian cells, facilitating monitoring of behavior inside the host (16, 17, 19). Furthermore, by combining transgenic zebrafish lines expressing fluorophores in the vasculature with transplantation of differentially labeled mammalian cancer cells, the dynamics of vascular remodeling within the tumor can be visualized in a detailed fashion over time in a living model (20, 21), which is a unique opportunity for this research field (Fig. 1C).

**Table 3. Cancer Types in Tumor Suppressor Mutants in Zebrafish, Mouse, and Human**

Gene	Zebrafish	Mouse	Human
<i>TP53</i>	MPNSTs	Lymphomas and sarcomas	Sarcomas, breast cancers, brain tumors, leukemias (Li-Fraumeni)
<i>PTEN</i>	Intraocular tumors	Broad spectrum	Broad spectrum
<i>APC</i>	Liver and intestinal tumors	Intestinal polyps	Colorectal cancer (FAP)
<i>MMR</i>	MPNSTs and others	Lymphomas and gastrointestinal tumors	Heterozygous: colorectal cancer (HNPCC) Homozygous: brain tumors and neurofibromas, lymphomas and leukemias

Abbreviations: FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colorectal cancer.

Another example of the versatility of fluorescence in zebrafish is the generation of transgenic zebrafish lines expressing mammalian oncogenes, where the expression cassettes are usually provided with fluorescence markers to visualize the carcinogenesis process (Fig. 1B; refs. 33, 35, 39, 41, 43). For example, Langenau et al. (38) used fluorescence of labeled leukemic cells to show that radiation treatment of wild-type fish transplanted with leukemia cells resulted in disappearance of the cancer, whereas in *bcl2*-overexpressing, apoptosis-deficient fish the cancer remained present. Fluorescence was also very elegantly used for tracking recombination events in *cre/lox*-regulated systems in whole fish. A loxed *dsRED* gene in between the *rag2* promoter and *EGFP-mMYC* transgene resulted in dsRED expression in thymocytes, but on recombination in the presence of CRE, resulted in a switch to enhanced green fluorescent protein (EGFP) expression in the same cells (36, 37). In the rhabdomyosarcoma model mentioned above, different fluorescent markers were successfully used for the fluorescence-activated cell sorting of four distinguishable cell types to identify cancer stem cells that have the capacity to induce new cancers in transplanted fish (40).

The above examples are pioneering studies, but they already show the enormous possibilities of using fluorescence in zebrafish cancer research, being indicative of a proportional number of opportunities for new studies. For example, some of the transgenic lines will be very well suited for genetic or chemical screens to identify modifiers of carcinogenesis, potentially in automated high-content screening setups.

#### Chemical Treatments in Search for Drugs

As already mentioned, chemical treatment of both embryos and adult animals is relatively easy for zebrafish. In this respect, zebrafish can be a versatile model in the search for cancer therapeutics. Some proof of principle comes from the effective use of known angiogenesis inhibitors in transplantation-induced vascular remodeling. The neovascularization inside mammalian tumor grafts in zebrafish embryos could be inhibited by treatment with antiangiogenic drugs, whereas development of the normal vasculature in these embryos was not influenced (20). One of those compounds was also shown to block the angiogenic response to human tumor cell-secreted vascular endothelial growth factor in zebrafish embryos (21). Another type of search for therapeutics concerns that of radioprotective agents, which are of great clinical relevance considering the importance of radiotherapy in human cancer treatment. As an example, the nanoparticle DF-1 was shown to reduce ionizing radiation damage in zebrafish embryos (60).

The state of the art would be to screen chemical compound libraries to identify novel drugs that inhibit certain aspects of cancer development. For this purpose, mutants that are embryonic lethal in the homozygous state and cancer-prone in the heterozygous state, such as those from the retroviral insertion and proliferation screens or the *apc* mutant (12-14, 24, 30), can be very useful because the early embryonic phenotype can be used as a readout whereas the obtained compound may very well be applicable to the adult cancer phenotype. In a nice and successful example of this, the *bmyb* mutant was used for a small-molecule screen in which the novel

compound persynthamide was found to rescue its embryonic phenotype (61, 62). Unfortunately, the effectiveness of this compound in adults has not yet been reported. A similarly nice small-molecule screen identified the compound 4-bromo-3-nitropropionophenone as a radiation sensitizer specifically for cancer cells. In zebrafish embryos transplanted with human cancer cells and treated with this compound, tumor growth was inhibited by irradiation while there was no effect on embryonic development (63).

#### Outlook

Although the area of cancer research in zebrafish is still relatively young, from this overview, it becomes clear that enormous progress has been made since the year 2000. Versatile tools and infrastructure for studying various aspects of carcinogenesis have become available and have been validated to various degrees. However, new possibilities may be contained in technologies that have successfully been used in cancer research in other models but are not yet established for zebrafish. For example in transgenesis, elegant systems for spatial and temporal control of gene expression, such as those driven by tetracycline or using GAL4 upstream activating sequences, are available in fruitfly and mouse (64, 65). Tetracycline-responsive systems (Tet-On and Tet-Off) allow for on and off switching of gene expression, which, when combined with oncogenes or tumor suppressors, would give the opportunity to follow tumor progression and regression in zebrafish. The GAL4 upstream activating sequence system makes use of endogenous enhancers to target expression. Because the currently available tissue-specific promoters in zebrafish are limited and sometimes unspecific, generating a collection of specific enhancer-trap lines, such as successfully done by the Becker lab (66), would be valuable in strategies to target cancer gene expression. Nevertheless, whereas more universal standards are being developed, many opportunities are now on hand for exciting cancer-related studies in this small but multi-purpose vertebrate model. As a result, the zebrafish can be expected to contribute to novel insights in tumor biology and cancer drug development in the near future.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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