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Reeling in the Zebrafish Cancer Models

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Abstract

Zebrafish are rapidly becoming a leading model organism for cancer research. The genetic pathways driving cancer are highly conserved between zebrafish and humans, and the ability to easily manipulate the zebrafish genome to rapidly generate transgenic animals makes zebrafish an excellent model organism. Transgenic zebrafish containing complex, patient-relevant genotypes have been used to model many cancer types. Here we present a comprehensive review of transgenic zebrafish cancer models as a resource to the field and highlight important areas of cancer biology that have yet to be studied in the fish. The ability to image cancer cells and niche biology in an endogenous tumor makes zebrafish an indispensable model organism in which we can further understand the mechanisms that drive tumorigenesis and screen for potential new cancer therapies.

INTRODUCTION

Zebrafish (*Danio rerio*) share 70% of their genome with humans and are commonly used to model human disease (Howe et al. 2013). Rapid external development, high fecundity, and easy, low-cost maintenance make the zebrafish an attractive animal model. Optical transparency has greatly enhanced the ability to visualize internal cell biology using fluorescent reporters and makes the zebrafish uniquely suited to image tumor development, metastasis, and microenvironmental interactions. In addition, zebrafish are excellent model organisms to use in drug screens given the ease with which drugs can be administered to zebrafish embryos in their water. Adult zebrafish can be used for drug screening as well; however, it can be difficult to administer the concentration of the drug necessary exclusively in their water. Techniques such as oral gavage, intraperitoneal injection, and retro-orbital injection have been developed to address this problem (Dang et al. 2016, Kinkel et al. 2010, Pugach et al. 2009). Systems for perturbing candidate genes are readily available in the zebrafish, via either overexpression in a DNA transposon system, knockdown using morpholinos, or knockout with CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9. This can be done with both spatial and temporal control. Fish are known to spontaneously develop cancer, particularly after mutagenesis, and have high conservation of oncogenes (White et al. 2013). One common way to model cancer in zebrafish is through xenotransplantation of human cancer cells (reviewed in White et al. 2013). Although cancers such as head and neck, retinoblastoma, and squamous cell carcinoma have mostly been modeled via a transplant system, this systematic resource review focuses exclusively on genetic models of cancer in zebrafish (**Figure 1**, summarized in **Table 1**).

MELANOMA

Melanoma arises from pigment-producing melanocytes and is the deadliest form of skin cancer (Lo & Fisher 2014). Melanocyte development is conserved between zebrafish and mammals, making zebrafish excellent models of pigmentation and melanoma (Mort et al. 2015). The master regulator of the melanocyte lineage, MITF, is conserved in zebrafish and required for melanocyte development (Lister et al. 1999). Approximately 50% of melanoma cases harbor mutations in *BRAF*, leading to activation of the MAPK pathway (TCGA 2015). Expression of human $BRAF^{V600E}$, under the melanocyte-specific *mitfa* promoter, leads to formation of melanocytic nevi (moles), but

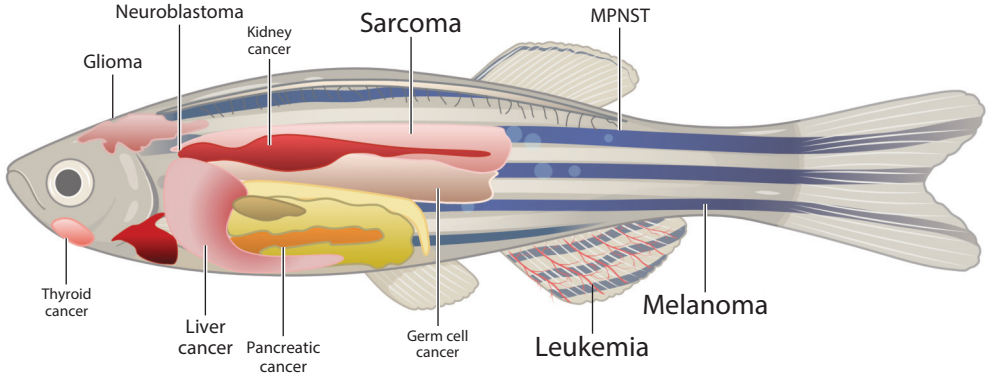

Figure 1

Human cancers that have been genetically modeled in zebrafish. Size of text correlates with how many zebrafish models have been described for that cancer type. Abbreviation: MPNST, malignant peripheral nerve sheath tumor. Figure adapted from an image created with BioRender.com.

Table 1 Transgenic zebrafish models of cancer

Cancer type	Genes mutated	Reference(s)
Glioma	*p53*$^{e7/e7}$; *nf1a*$^{+/-}$; *nf1b*$^{-/-}$	Shin et al. 2012
	Tg(ptf1:Gal4; UAS:GFP; UAS:DA-RAC1) *Tg(ptf1:Gal4; UAS:GFP; UAS:DA-AKT1)*	Jung et al. 2013
	Tg(krt5:Gal4VP16; UAS:mCherry-KRAS^{G12V}*)* *Tg(gfap:Gal4VP16; UAS:mCherry-KRAS*^{G12V}*)*	Ju et al. 2015
	Tg(zic4:Gal4TA4; UAS:mCherry; UAS:GFP-HRAS^{G12V}*)* *Tg(zic4:Gal4TA4; UAS:mCherry; UAS:Xmrk)* *Tg(zic4:Gal4TA4; UAS:mCherry; UAS:BRAF*^{V600E}*)* *Tg(zic4:Gal4TA4; UAS:mCherry; UAS:AKT-BFP)* *Tg(zic4:Gal4TA4; UAS:mCherry; UAS:eGFP-EGFRvIII)* *Tg(zic4:Gal4TA4; UAS:mCherry; UAS:YAP*^{S5A}*)*	Mayrhofer et al. 2017
	Tg(sox10:mCherry-NRAS^{wt}*)* +/− *p53*^{M214K} *Tg(sox10:mCherry-NRAS*^{Q61R}*)* +/− *p53*^{M214K} *Tg(sox10:mCherry-NRAS*^{S17N}*)* +/− *p53*^{M214K}	Modzelewska et al. 2016
Neuroblastoma	*Tg(dβh:EGFP; dβh:ALK*^{F1174L}*)* *Tg(dβh:EGFP-MYCN)*	Zhu et al. 2012
	Tg(dβh:EGFP-MYCN); nf1a$^{-/-}$; *nf1b*$^{+/-}$	He et al. 2016
	Tg(dβh:ptpn11^{E69K}*-EGFP; dβh:EGFP-MYCN)* *Tg(dbh:Gab2wt; dbh:EGFP; dβh:EGFP-MYCN)*	Zhang et al. 2017
	Tg(dβh:EGFP-MYCN; dβh:LMO1; dβh:mCherry)	Zhu et al. 2017
	Tg(dβh:MYCN; dβh:EGFP)	Tao et al. 2017
	Tg(dβh:MYC)	Zimmerman et al. 2018
MPNST	*rp*$^{+/-}$ *(rps8a, rps15a, rpl7, rpl35, rpl36, rpl36a, rpl13, rpl23a, rps7, rps18, rps29)*	Amsterdam et al. 2004
	rp$^{+/-}$ or *p53*^{M214K}	Zhang et al. 2013
	brca2$^{Q658X/Q658X}$; *tp53*$^{M214K/+}$	Shive et al. 2014
	Tg(mitfa:atg5^{K130R}*)*; *p53*$^{M214K/+}$	Lee et al. 2016
	Tg(sox10:PDGFRA; sox10:mCherry); nf1a$^{+/-}$; *nf1b*$^{-/-}$; *p53*$^{m/m}$ *Tg(sox10:PDGFRAmut; sox10:mCherry); nf1a*$^{+/-}$; *nf1b*$^{-/-}$; *p53*$^{m/m}$	Ki et al. 2017
	nf1a$^{+/-}$; *nf1b*$^{-/-}$; *p53*$^{m/m}$; *atrx*$^{+/-}$	Oppel et al. 2019
Pancreatic cancer	*z-myod:MYCN* *core-z-myod:MYCN*	Hong et al. 2004
	Tg(ptf1a:eGFP-KRAS^{G12V}*)*	Park et al. 2008
	rpl36$^{+/-}$; *Tg(ptf1a:gal4VP16; UAS:GFP-KRAS*^{G12V}*)*	Provost et al. 2014
	Tg(ptf1a:gal4VP16; UAS:GFP-KRAS^{mut}*)*	Park et al. 2015
	Tg(ubb:Lox-Nuc-mCherry-stop-Lox-GFP::KRAS^{G12D}*)*	Oh & Park 2019
Liver cancer	*Tg(pLF2.8-HCV-core)*	Rekha et al. 2008
	Tg(fabp10:EGFP-KRAS^{G12V}*)* +/− *p53*^{M214K}	Nguyen et al. 2011
	Tg(fabp10:TA; TRE:xmrk)	Li et al. 2012
	Tg(fabp10:TA; TRE:Myc)	Li et al. 2013
	Tg(fabp10:LexPR; LexA:EGFP-KRAS^{G12V}*)*	Nguyen et al. 2012
	Tg(fabp10:LexPR; LexA:Cre; fabp10:loxP-mCherry-loxP-EGFP-KRAS^{G12V}*)*	Nguyen et al. 2016
	Tg(fabp10a:pt-β-cat)	Evason et al. 2015
	apc$^{+/-}$	Haramis et al. 2006
	Tg(fabp10a:tTA; pT2-TRE-gankyrin-HcRed)	Huang et al. 2017
	Tg(fabp10:NRAS^{Q61K}*)*	Wang et al. 2017

(*Continued*)

Table 1 (*Continued*)

Cancer type	Genes mutated	Reference(s)
Thyroid cancer	Tg(*tg:BRAF^{V600E}-TOM*)	Anelli et al. 2017
Rhabdomyosarcoma	*Tg(rag2:KRAS^{G12D}) +/− p53^{-}*	Langenau et al. 2007, Ignatius et al. 2018
	Tg(cdh15:KRAS^{G12D}) *Tg(mylz2:KRAS^{G12D})*	Storer et al. 2013
	Tg(β-actin:LoxP-EGFP-LoxP-KRAS^{G12D})	Le et al. 2007
	Tg(ubi:GFP2A-PAX3FOXO1) *Tg(β-actin:GFP2A-PAX3FOXO1)* *Tg(mitfa:GFP2A-PAX3FOXO1)* *Tg(CMV:GFP2A-PAX3FOXO1)* *Tg(fli1:GFP2A-PAX3FOXO1)*	Kendall et al. 2018
Ewing's sarcoma	*Tg(hsp70:EWS-FLI1) +/− p53^{-}* *Tg(hsp70:EWS-FLI1:IRES-GFP) +/− p53^{-}* *Tg(β-actin:EWS-FLI1:IRES-GFP) +/− p53^{-}*	Leacock et al. 2012
	ewsa$^{-/-}$; p53$^{M214K/M214K}$	Park et al. 2016
Liposarcoma	*Tg(rag2:myr-mAkt2) +/− p53^{M214K}*	Gutierrez et al. 2011b
	Tg(krt4:Has.myrAkt1)^{cy18}	Chu et al. 2012
Melanoma	*Tg(mitfa:BRAF^{V600E}); p53$^{-/-}$*	Patton et al. 2005
	Tg(mitfa:BRAF^{V600E}); p53$^{-/-}$; mitfa$^{-/-}$ + MiniCoopR	Ceol et al. 2011
	Tg(mitfa:EGFP:NRAS^{Q61K}); p53$^{-/-}$	Dovey et al. 2009
	Tg(kita:HRAS^{G12V})	Santoriello et al. 2010
	Tg(mitfa:NRAS^{Q61R})	McConnell et al. 2019
AML	*Tg(spi1:MYST3/NCOA2-EGFP)*	Zhuravleva et al. 2008
	Tg(spi1:FLT3-ITD-2A-EGFP/CG2) +/− spi1:NPM1-Mut-PA/CG2	Lu et al. 2016
	Tg(spi1::loxP-EGFP-loxP::NUP98-HOXA9)	Forrester et al. 2011
	asxl1$^{+/-}$ +/− tet2$^{-/-}$	Gjini et al. 2019
	RUNX1-CBF2T1	Kalev-Zylinska et al. 2002
	Tg(hsp70:AML1-ETO)	Yeh et al. 2008
	Tg(MYCN:HSE:EGFP)	Shen et al. 2013
	Tg(ß-actin-LoxP-EGFP-LoxP-KRAS^{G12D}; hsp70-Cre)	Le et al. 2007
	Tg(fli.1:Gal4FF^{ubs3}; UAS:egfp-HRAS^{G12V})^{io6}	Alghisi et al. 2013
	stat5.1^{N646H} or *stat5.1$^{H298R/N714F}$*	Lewis et al. 2006
	Tg(pu.1:EGFP-CREB)	Tregnago et al. 2016
	irf8$^{-/-}$	Zhao et al. 2018
	idh1$^{-/-}$ *IDH1-R132H*	Shi et al. 2015
CML	*Tg(hsp70:p210$^{BCR/ABL1}$)*	Xu et al. 2020
ALL	*Tg(rag2:cMyc)*	Langenau et al. 2003
	Tg(rag2:EGFP-mMyc)	Langenau et al. 2005
	Tg(rag2:MYC-ER)	Gutierrez et al. 2011a
	TEL-AML1	Sabaawy et al. 2006
	Tg(rag2-ICN1-EGFP)	Chen et al. 2007

(*Continued*)

Table 1 (*Continued*)

Cancer type	Genes mutated	Reference(s)
Renal cell	*vhl*$^{-/-}$	Van Rooijen et al. 2009
Germ cell	ENU mutagenesis	Neumann et al. 2009
	Tg(flck:TAg)	Gill et al. 2010
	lrrc50^{Hu255b}	Basten et al. 2013
	dnaaf1^{hu255b}	Litchfield et al. 2016
	ns1402	Shimizu & Matsuda 2019

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; DA, dominant active; MPNST, malignant peripheral nerve sheath tumor; wt, wild type.

not melanoma, in adult zebrafish (Patton et al. 2005). Expression of *mitfa:BRAF*^{V600E}, in conjunction with a homozygous *p53* missense mutation, leads to approximately half of *mitfa:BRAF*^{V600E}*; p53*$^{-/-}$ fish developing melanomas by 4 months old (Patton et al. 2005). This represented the first model of melanoma in zebrafish.

Zebrafish allow for transient overexpression and knockout of melanoma-associated genes. The MiniCoopR system was developed in order to perform tissue-specific overexpression of a candidate gene (Ceol et al. 2011). A *mitfa*$^{-/-}$ mutation in *Tg(mitfa:BRAF*^{V600E}*); p53*$^{-/-}$ fish prevents melanocyte development and melanoma from occurring. Melanocyte development can be rescued in these fish via injection of MiniCoopR, a transposon-based vector that contains a *mitfa* minigene, which sits alongside a candidate oncogene driven by the *mitfa* promoter (Ceol et al. 2011). Modification of the CRISPR/Cas9 system allows for melanocyte-specific knockout of a gene of interest (Ablain & Zon 2016, Ablain et al. 2015). The MAZERATI (modeling approach in zebrafish for rapid tumor initiation) system takes advantage of both MiniCoopR and CRISPR MiniCoopR vectors, allowing for rapid oncogene expression and tumor suppressor inactivation exclusively in the melanocytes of zebrafish (Ablain et al. 2018). Most recently, this system was used to identify SPRED1 as a tumor suppressor in *KIT*-mutant melanomas and *GDF6* as an oncogene in *Tg(mitfa:BRAF*^{V600E}*); p53*$^{-/-}$*; mitfa*$^{-/-}$ melanomas (Ablain et al. 2018, Venkatesan et al. 2018).

Approximately 28% of melanoma cases contain an *NRAS* mutation (TCGA 2015). The *mitfa:NRAS*^{Q61K} mutation causes hyperpigmentation but does not generate melanomas in zebrafish (Dovey et al. 2009). However, as with *BRAF*^{V600E}, *NRAS*^{Q61K} cooperates with *p53* loss to generate melanomas. Melanomas that are *mitfa:EGFP:NRAS*^{Q61K}*; p53*$^{-/-}$ mutants are histologically and transcriptionally similar to human melanomas, as well as transplantable (Dovey et al. 2009). The BRAF and NRAS zebrafish models use *mitfa* to drive oncogene expression and only develop melanomas in the presence of a *p53* mutation. Santoriello et al. (2010) reported a zebrafish melanoma model in which the *kita* promoter drives *HRAS* oncogene expression. This model develops melanomas by 1–3 months of age and does not require an additional tumor suppressor mutation. McConnell et al. (2019) generated a transient *mitfa*:*NRAS*^{Q61R} zebrafish melanoma model that does not require a tumor suppressor mutation. These fish develop large hyperpigmented patches at 2 weeks postfertilization, and by 8 weeks these patches rapidly form tumors. This NRAS model was used to investigate neural crest state activation.

Melanocytes arise from the embryonic neural crest lineage. Zebrafish melanomas in *mitfa:BRAF*^{V600E}*; p53*$^{-/-}$ and *mitfa:NRAS*^{Q61R} fish reactivate a neural crest gene signature, including the zebrafish gene *crestin* (White et al. 2011), which marks the neural crest during development, turns off by 72 hours postfertilization, and turns on again exclusively in zebrafish melanomas. Melanoma initiation can be visualized using the *crestin* reporter. Expression of *crestin*

begins as a single *crestin*-positive cell and progresses to a fully formed, transplantable, *crestin*-positive melanoma (Kaufman et al. 2016). Zon and colleagues utilized a chemical screen with wild-type embryos to identify small-molecule suppressors of the *crestin*-positive neural crest lineage, identifying a DHODH inhibitor, leflunomide (Santoriello et al. 2020, White et al. 2011). Chemical screens have also been conducted using primary zebrafish embryonic cell cultures, identifying caffeic acid phenethyl ester as a suppressor of neural crest development (Ciarlo et al. 2017).

One caveat of the previously described models is the inability to spatially and temporally control melanoma formation. Transgene electroporation in adult zebrafish (TEAZ) was developed to introduce plasmids in zebrafish with both spatial and temporal control (Callahan et al. 2018). Plasmids of interest are injected into the zebrafish flank just below the epithelium and electrodes deliver an electric pulse, creating pores in the cell surface that facilitate uptake of plasmid DNA. TEAZ can be used to generate melanomas in adult *Tg(mitfa:BRAF^{V600E}); p53$^{-/-}$; mitfa$^{-/-}$* fish by electroporation of a MiniCoopR plasmid, *ubb:Cas9*, and *zfU6:sgRNA* against *rb1*. In the future, TEAZ will be a powerful way to serially model oncogene and tumor suppressor mutations and may be used to study metastasis.

Melanoma metastasis has previously been studied in the zebrafish using fluorescent zebrafish melanoma cell lines transplanted into irradiated adult or embryonic *casper* recipients. These transparent fish allow for high-resolution imaging of fluorescent metastatic cells and quantification of metastasis (Heilmann et al. 2015). This technique was used by White and colleagues, who found that adipocytes in the melanoma microenvironment increase melanoma lipid content, cell growth, and invasion (Zhang et al. 2018). Due to the difficulty distinguishing metastasis from de novo tumor formation or tumor spreading, the ability to specifically model metastasis in the zebrafish will be an important area of study in coming years.

LEUKEMIA

The pathways regulating hematopoiesis are conserved between mammals and fish, leading to the development of many zebrafish leukemia models (Robertson et al. 2016). Here we review models of hematopoietic malignancies published to date.

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) comprises leukemias of lymphoid origin and is the most common childhood cancer. T cell ALL (T-ALL) is characterized by uncontrolled proliferation of T cell progenitors and accounts for 25% of adult and 15% of pediatric ALL (Durinck et al. 2015). Although most ALL cases are B cell in origin, the vast majority of ALL models in zebrafish recapitulate T-ALL. The first zebrafish T-ALL model was discovered in the Look lab (Langenau et al. 2003). This model expressed mouse *c-Myc* under the lymphoid-specific *rag2* promoter. Approximately 5% of F_0 fish developed tumors about 44 days postfertilization. These leukemias were shown to arise from clonal expansion of transformed T lymphocyte precursors and were transplantable. Langenau and colleagues later went on to create a stable *rag2-EGFP-mMyc* zebrafish line that developed T-ALL but could only be propagated by in vitro fertilization (Feng et al. 2007, Langenau et al. 2005). They later created a Cre/Lox-inducible version that led to the formation of T-ALL (Gutierrez et al. 2011a). Using this model, they found that the PI3K-AKT pathway can cause T-ALL in the absence of MYC induction. Recently, *rag2:Myc* zebrafish were shown to develop B cell ALL (B-ALL) in a fraction of fish (Borga et al. 2019, Garcia et al. 2018).

B-ALL is characterized by uncontrolled proliferation of immature B cell precursors and is the most common childhood leukemia. Despite the fact that B-ALL comprises 75% of human ALL

cases, it is challenging to model B-ALL in zebrafish because the *rag2* promoter has a T cell bias. Sabaawy et al. (2006) generated transgenic zebrafish that express TEL-AML1 (ETV6-RUNX1), a fusion common in B-ALL. Ubiquitous TEL-AML1 expression led to progenitor cell expansion and transplantable B-ALL in 3% of fish.

Since the development of these models, several studies have been conducted to investigate the mechanism of leukemia formation. Rudner et al. (2011) compared 17 T-ALLs from 4 zebrafish T-ALL models to human T-ALL and found that they were comparable at a genomic level. T-ALL clones in a Myc-induced T-ALL fish model continuously evolve to drive leukemia progression, in particular via Akt pathway activation (Blackburn et al. 2014). In Myc-induced T-ALL fish, proapoptotic Bim is downregulated by Myc and Akt in treatment-resistant T-ALL (Reynolds et al. 2014). Bcl2 was found to accelerate T-lymphoblastic lymphoma, but inhibited progression to T-ALL in Myc-induced fish models due to an inability to intravasate the vasculature (Feng et al. 2010). In addition, Myc-induced zebrafish T-ALL was not affected by *tp53* mutations (Gutierrez et al. 2014a). This is consistent with zebrafish having no functional ortholog for CDKN2A (ARF), which links Myc-induced stress to *tp53* tumor suppression. As a result, the Myc-induced zebrafish T-ALL model is ideal for studying the role of CDKN2A-independent pathways in T-ALL.

Over 60% of T-ALL patients have activating mutations in *NOTCH1*. A zebrafish model of NOTCH1-induced T cell leukemia has been developed (*rag2-ICN1-EGFP*) (Chen et al. 2007). Notch signaling led to an expansion of T cell clones, but they were not fully transformed and did not initiate leukemia when transplanted. Zebrafish expressing both Notch and Myc have more aggressive T-ALL, and *TOX* was identified as an oncogene that synergized with *MYC* and *NOTCH1* to accelerate T-ALL by expanding transformed clones and increasing genomic instability (Blackburn et al. 2012, Lobbardi et al. 2017). Synergy between the Notch pathway and *bcl2* was also observed in these fish (Chen et al. 2007).

Several groups have used T-ALL zebrafish models to conduct screens for leukemia drugs and genetic modifiers. One group used T cell reporter fish to conduct a small-molecule screen for compounds that act against immature T cells, leading to the identification of Lenaldekar (Ridges et al. 2012). This group then used the Myc-induced T-ALL model to determine that Lenaldekar induces long-term remission in these fish. Similarly, perphenazine was identified to be toxic to Myc-overexpressing thymocytes in the fish and was shown to induce apoptosis of T-ALL in a Myc-induced fish model (Gutierrez et al. 2014b). Most recently the Look laboratory identified a class of small-molecule activators of protein phosphatase 2A that are effective against T-ALL cells from *Tg(rag2:Myc; rag2:EGFP)* zebrafish (Morita et al. 2020).

Chronic Myeloid Leukemia

Recently, a heat shock–inducible BCR/ABL1 zebrafish model was reported that develops chronic myeloid leukemia in 76% of adult fish aged 6 months to 1 year (Xu et al. 2020). Chronic myeloid leukemia in this fish line was similar to that in humans and these fish were used in a small-drug screen.

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a clonal hematopoietic cancer of the bone marrow (extensively reviewed in Ferrara & Schiffer 2013). The first zebrafish model of a human AML fusion was created by fusing histone acetyl-transferases MYST3 and NCOA2 under the control of the early myeloid specific promoter *spi1* (Zhuravleva et al. 2008). One percent of adult fish went on to develop AML characterized by invasion of myeloid blast cells in the kidney. A variety of

zebrafish models utilize *spi1*-driven oncogene expression. Zebrafish expressing *spi1:FLT3-ITD-2A-EGFP/CG2* present myeloid hyperplasia in kidney marrow at 6 months, followed by the leukemia phase at 9 months postfertilization (Lu et al. 2016). Double *spi1:FLT3-ITD-2A-EGFP/CG2* and *spi1:NPM1-Mut-PA/CG2* transgenic fish developed leukemia by 6 months, indicating synergy between the two mutations. Several fusion oncogenes have been reported in AML (Forrester et al. 2011, Lutterbach & Hiebert 2000). A *cre/lox*-inducible *Tg*(*spi1::loxP-EGFP-loxP::NUP98-HOXA9*) fusion was generated in zebrafish, resulting in expansion of the myeloid lineage and suppression of the erythroid lineage (Forrester et al. 2011). In all, 23% of these adult fish developed myeloproliferation, similar to human myeloproliferative neoplasm, and epigenetic therapies restored normal hematopoiesis (Deveau et al. 2015). RUNX1-CBF2T1 (AML1-ETO) zebrafish embryos exhibit disrupted hematopoiesis, abnormal circulation, internal hemorrhages, and cellular dysplasia, consistent with phenotypes seen in Runx1-Cbf2t1 mice, therefore validating the zebrafish as a leukemia model (Kalev-Zylinska et al. 2002). A heat shock–inducible zebrafish AML1-ETO model was generated as well, and these embryos exhibited reprogramed hematopoietic cell fate and cytological and transcriptional similarities to human AML (Yeh et al. 2008). As timing is critical when modeling AML, several other inducible models have been generated.

Amplification of MYCN is a frequent event with poor prognosis in human AML. *Tg(MYCN:HSE:EGFP)* zebrafish were created by introducing murine Mycn upstream of heat shock–driven Egfp (Shen et al. 2013). Overexpression of Mycn led to increased myeloid and precursor cells, while decreasing erythrocytes. Mycn reprograms hematopoietic cell fate by upregulating expression of hematopoietic transcription factors *scl*, *lmo2*, *pu.1*, and *mpo* and downregulating *gata1*.

In total, 25–50% of myeloid leukemias have mutations activating RAS family members. The Zon lab has developed a heat shock–inducible Cre/Lox-mediated approach to activating human KRAS (Le et al. 2007). *Tg(ß-actin-LoxP-EGFP-LoxP-KRAS^{G12D}; hsp70-Cre)* fish developed four different tumor types: rhabdomyosarcoma, intestinal hyperplasia, malignant peripheral nerve sheath tumor (MPNST), and myeloproliferative disorder. Isolated kidney marrow cells were heat shocked ex vivo and transplanted into irradiated recipient fish, generating a zebrafish model of myeloproliferative disorder. Like *KRAS*, *HRAS* is frequently mutated in myeloproliferative disorders. Zebrafish with *HRAS* expression driven by the endothelial cell promoter *fli1* have abnormal hematopoiesis including increased myelo-erythroid progenitor cells in the caudal hematopoietic tissue, which requires Notch signaling repression (Alghisi et al. 2013).

In addition to Notch, other signaling pathways are involved in AML and have been used to model this disease in zebrafish. Constitutively active *stat5.1* zebrafish mutants present increased numbers of early and late myeloid cells, erythrocytes, and B cells (Lewis et al. 2006). In addition, CREB is frequently overexpressed in AML. CREB overexpression in the myeloid lineage results in 79% of adult zebrafish with a block in myeloid differentiation mediated by C/EBPδ (Tregnago et al. 2016). These fish developed AML with clonal mature monocytic blasts, loss of myeloid precursors, and a transcriptional signature reminiscent of human AML. Zebrafish with an *irf8* mutation developed a myeloproliferative neoplasm characterized by the expansion of myeloid precursors (Zhao et al. 2018). Similarly, zebrafish *idh1* knockdown blocked myeloid differentiation and overexpression of mutant human IDH1-R132H resulted in myeloid expansion (Shi et al. 2015). Mutations in genes encoding other epigenetic regulators have also been explored in these disease models. In total, 50% of *asxl1*$^{+/-}$ fish also develop myeloproliferation by 5 months, and the concomitant homozygous deletion of *tet2* leads to AML in some fish (Gjini et al. 2019). The aforementioned zebrafish models recapitulate some, but not all, aspects of human AML, with mostly myeloproliferative and embryonic phenotypes. There will be a need in the coming years to simultaneously and tissue-specifically manipulate several driver genes.

SARCOMA

Rhabdomyosarcoma

Rhabdomyosarcoma is a rare cancer that forms in soft tissue, particularly skeletal muscle, in children and young adults. Zebrafish models of sarcomas, including rhabdomyosarcoma, chordoma, hemangiosarcoma, and liposarcoma, have been extensively reviewed, so here we focus on common transgenic models in zebrafish (Hayes & Langenau 2017). The majority of embryonal rhabdomyosarcomas have ectopic activation of the RAS pathway; this was replicated in zebrafish in 2007 by overexpressing oncogenic *KRAS*^{G12D} under the control of the *rag2* promoter (Langenau et al. 2007). These fish develop tumors at 10 days postfertilization that transcriptionally and histologically resemble the human disease. Loss of *tp53* in this model system increased tumor incidence, as well as invasion and metastasis (Ignatius et al. 2018, Langenau et al. 2007). Imaging of developing tumors using fluorescent reporters gives zebrafish a distinct advantage over other species and, in the embryonal rhabdomyosarcoma model, enabled the visualization of tumor and blood vessel formation (Ignatius et al. 2012).

In addition to the *rag2* promoter, *KRAS*^{G12D} was also driven by the early muscle progenitor promoter *cdh15* and the differentiated myoblast promoter *mylz2* (Storer et al. 2013). Interestingly, RAS activation in progenitor cells has been shown to lead to decreased survival and the formation of less differentiated tumors. To create an inducible model system, Le et al. (2007) inserted a floxed stop cassette preceding *KRAS*^{G12D} that could be removed by a heat shock–inducible Cre. This resulted in rhabdomyosarcoma formation but also yielded intestinal hyperplasia, myeloproliferative disorder, and MPNSTs. Recently, another rhabdomyosarcoma model was developed using the *PAX3/7-FOXO1* fusion oncogene, which was placed under the control of various promoters, including *ß-actin*, *ubi*, *CMV*, *mitfa*, and *fli1*, which resulted in the formation of rhabdomyosarcoma, undifferentiated sarcoma, and primitive neuroectodermal tumors (Kendall et al. 2018).

Inhibitors of MEK and mTOR signaling, as well as activation of the Wnt signaling pathway, decreased tumor formation in *rag2:KRAS*^{G12D} zebrafish (Chen et al. 2014, Le et al. 2013). Since the development of these models, the Langenau lab has performed several elegant studies that highlight the importance of developmental pathways in rhabdomyosarcoma formation. They found that the muscle-specific transcription factors *MYOD* and *MYF5* promote tumor growth by binding to muscle development and cell cycle genes (Tenente et al. 2017). They also found that *NOTCH1* activation increases tumorigenesis via *SNAIL1* activation and *MEF2C* suppression (Ignatius et al. 2017). Furthermore, they discovered that the noncanonical Wnt pathway member VANGL2 is active in highly proliferative tumor propagating rhabdomyosarcoma cells (Hayes et al. 2018). These studies underscore the prominent role that developmental signaling pathways play in tumorigenesis.

Ewing's Sarcoma

Ewing's sarcoma is a rare childhood cancer of the bone that occurs through an *EWS* gene fusion. When one of the most common fusions, *EWS-FLI1*, is injected under the control of either a ubiquitous promoter or a heat shock–inducible promoter alongside p53 loss, a small subset of fish form tumors that histologically and molecularly resemble Ewing's sarcoma (Leacock et al. 2012). The *EWS-FLI1*-fusion, *p53*-mutant zebrafish also developed MPNSTs and leukemia-like cancer, likely due to the lack of promoter specificity. Ewing's sarcoma and MPNST tumor induction was also found in *ewsa*$^{-/-}$; *p53*$^{M214K/M214K}$ zebrafish containing no fusion protein (Park et al. 2016). Interestingly, these fish formed tumors at a much higher rate than the *EWS-FLI1*-fusion, *p53*-mutant zebrafish (~60–70% versus ~25%, respectively). Ewing's sarcoma is the second most common type of bone cancer behind osteosarcoma. To our knowledge, no transgenic osteosarcoma zebrafish models exist, making this a promising area of research in the future.

Liposarcoma

Liposarcoma is a cancer of the adipose tissue and is one of the most common types of sarcoma. To model liposarcoma in the fish, constitutively active *Akt2* was expressed under the control of the zebrafish *rag2* promoter in the context of p53 loss (Gutierrez et al. 2011b). Well differentiated liposarcomas formed in 30% of the p53-homozygous-mutant fish at 1–3 months of age. They also found that about one-third of human liposarcomas have aberrant AKT signaling. Interestingly, overexpressing constitutively active *Akt2* in the skin using the *krt4* promoter induces lipoma formation via hyperplastic growth of adipocytes in adult fish (Chu et al. 2012). These studies point to a major role for AKT signaling in adipocyte and liposarcoma development.

NEUROLOGICAL CANCERS

Glioma

Gliomas are brain tumors that are thought to arise from neuroglial stem or progenitor cells (Molinaro et al. 2019). The first transgenic zebrafish model of glioma was generated in the Look lab, who showed that the $p53^{e7/e7}$; $nf1a^{+/-}$; $nf1b^{-/-}$ mutation results in high-grade glioma and MPNST formation beginning at around 30 weeks of age (Shin et al. 2012). In 2013, two additional transgenic glioma models were generated by expressing either dominant active (DA) AKT1 or RAC1 under a central nervous system promoter, *ptf1* (Jung et al. 2013). Both models result in gliomas with heterogenous grades, with DA-RAC1 tumors inducing more aggressive high-grade tumors. Given that the AKT-PI3K pathway is hyperactive in the majority of glioblastoma tumors, this clinically relevant model could be used to identify novel therapeutic targets. Another model was developed by driving $KRAS^{G12V}$ under the *krt5* or *gfap* promoter (Ju et al. 2015). The *krt5:*$KRAS^{G12V}$ mutation induced brain tumors at low frequency, but the majority of tumors that formed were MPNST-like malignancies. However, when driven under the glial cell–specific *gfap* promoter, $KRAS^{G12V}$ brain tumors in both ventricular zones and parenchyma were formed at a high frequency (Ju et al. 2015).

Additional transgenic zebrafish glioma models were developed by the Mione lab, who drove a variety of MAPK pathway members including Xmrk (oncogenic EGFR), EGFRvIII, $KRAS^{G12V}$, $BRAF^{V600E}$, AKT1, and $HRAS^{G12V}$ under the *zic4* promoter, which is specific to the proliferating zones of the central nervous system (CNS), resulting in a wide range of benign and malignant brain tumors (Mayrhofer et al. 2017). The most aggressive brain tumors were formed when driving $HRAS^{G12V}$ alongside DA-YAP, which was not seen when driving $HRAS^{G12V}$ alone. These studies emphasize the role for developmental signaling pathways in cancer formation and provide a new model for aggressive human mesenchymal glioblastoma subtypes.

In 2016, a model for primitive neuroectodermal tumors of the CNS (a glioblastoma variant) was developed by driving oncogenic *NRAS* in *sox10* oligoneural precursor cells alongside $p53^{M214K/M214K}$ (Modzelewska et al. 2016). The wide variety of oncogenic or tumor suppressor drivers used in these transgenic zebrafish glioma models is representative of the heterogeneity found in human brain tumors. Although these models are excellent systems to study glioma biology, more work needs to be done to elucidate the mechanisms of brain tumor initiation, progression, and metastasis.

Neuroblastoma

Neuroblastoma is a tumor of the peripheral sympathetic nervous system that arises from neural crest progenitor cells in children. The development of transgenic zebrafish neuroblastoma models has been spearheaded by the Look laboratory. Use of the zebrafish dopamine-δ-hydroxylase

(*dβh*) promoter to drive human *MYCN* fused to EGFP specifically in the sympathetic nervous system results in the formation of tumor masses that histopathologically, immunohistochemically, and ultrastructurally mimic human neuroblastoma (Zhu et al. 2012). They also showed that overexpression of both *MYCN* and ALK^{F1174L} in *dβh*-expressing cells rapidly induces neuroblastoma formation starting at 5–7 weeks of age. The Look lab also discovered that *MYCN* overexpression in combination with the loss of zebrafish *nf1* results in neuroblastoma formation with high penetrance at 4 weeks of age (He et al. 2016). Interestingly, neuroblastoma formation was dependent on loss of the GAP-related domain of NF1 and could be abrogated using MEK inhibitors and retinoids. Induction of the MAPK pathway by overexpressing mutant *ptpn11* or *Gab2* induces neuroblastoma with a high penetrance (Zhang et al. 2017). As with the *nf1*-mutant model discussed above, treatment of *MYCN; Gab2wt* (wild-type *Gab2*) transgenic fish with the MEK inhibitor trametinib inhibits neuroblastoma formation. A fourth gene that acts in concert with *MYCN* to induce neuroblastoma is *LMO1* (Zhu et al. 2017). The most striking thing about this model is that it induces widespread metastasis, which is rarely seen in other transgenic zebrafish models of cancer.

In 2017, the Look lab uncoupled the EGFP and MYCN fusion and injected each separately under the control of the *dβh* promoter, which resulted in higher levels of MYCN and increased penetrance (Tao et al. 2017). Using this model, they studied the role for DEF, a protein involved in preribosomal RNA processing, and found that DEF overexpression significantly accelerates neuroblastoma onset, while DEF loss-of-function mutations prevent neuroblastoma formation. This study highlights the importance of transcription in tumor development and identifies a potential pathway that could be therapeutically targeted. In addition to *MYCN* amplification, about 11% of neuroblastomas have high levels of c-MYC without amplification of the gene itself. Expression of *dβh:MYC* results in rapid tumor formation at 4–8 weeks with almost complete penetrance in zebrafish (Zimmerman et al. 2018). Further analysis using whole-genome sequencing of human neuroblastomas revealed that *MYC* overexpression is caused by amplification of enhancers distal to *MYC* or translocations that place highly active enhancers physically close to the *MYC* coding sequence. These findings highlight the importance of understanding gene regulation and its role in cancer development.

Malignant Peripheral Nerve Sheath Tumors

MPNSTs are a rare type of cancer that forms in the connective tissue surrounding peripheral nerves. A zebrafish model of MPNSTs was first developed in 2004 when 11 zebrafish lines containing mutations in ribosomal protein genes were found to have elevated tumor incidence (Amsterdam et al. 2004). In total, 80% of these tumors were MPNSTs, which were highly aneuploid, with amplifications and deletions in oncogenes and tumor suppressors including *cyclinD2*, *cdk6*, and *fgf6a* (Zhang et al. 2010). In searching for copy number alterations in *rp*- and *tp53*-mutant zebrafish MPNSTs, 34 genes were found to be amplified in both zebrafish and human MPNSTs (Zhang et al. 2013). Although this MPNST model is slow to develop (11–20 months), it is representative of the heterogeneity seen in patient tumors and can be used to study natural disease progression.

Since then, several transgenic MPNST models have been generated in zebrafish. $Brca2^{Q658X/Q658X}$; $tp53^{M214K/+}$ zebrafish have an average MPNST onset age of 15 months (Shive et al. 2014). In addition to the histologic similarity to human MPNSTs, these tumors highly express S100 and CD57, indicating that they are from a neural crest origin, as in humans (White et al. 2017). Another MPNST model, $Tg(mitfa{:}atg5^{K130R})$; $p53^{M214K/+}$, was created when a dominant-negative autophagy protein was expressed under the control of the *mitfa* promoter in the context

of p53 loss (Lee et al. 2016). Interestingly, the mutation status of p53 altered the tumor type, with fish heterozygous for p53 forming predominantly MPNSTs, whereas the p53-homozygous fish developed a variety of tumors, including neuroendocrine and small cell. This study also confirmed the neural crest origin of MPNSTs, suggesting that Schwann cell precursors are the cell of origin of MPNSTs.

A more targeted model of MPNSTs was generated when *PDGFRA* was driven under the *sox10* neural crest–specific promoter in *nf1a*$^{+/-}$; *nf1b*$^{-/-}$; *p53*$^{m/m}$ zebrafish, which formed MPNSTs at around 80% penetrance by 30 weeks of age (Ki et al. 2017). Recently the Look lab inactivated the SWI/SNF chromatin remodeling factor ATRX using CRISPR/Cas9 to generate *p53*$^{-/-}$; *nf1b*$^{-/-}$; *nf1a*$^{+/-}$; *atrx*$^{+/-}$ zebrafish (Oppel et al. 2019). They found that ATRX loss does not promote pathogenesis and instead widens the number of tumor types formed, as compared to 100% MPNSTs formed in the control *p53*$^{-/-}$; *nf1b*$^{-/-}$; *nf1a*$^{+/-}$ zebrafish. The zebrafish MPNST models highlighted here are ideal platforms for identifying novel therapies for this rare cancer.

GASTROINTESTINAL CANCERS

Pancreatic Cancer

The two main types of pancreatic cancer are pancreatic adenocarcinoma, which arises in exocrine cells and comprises almost 95% of cases, and pancreatic neuroendocrine tumors, which arise in the endocrine cells of the pancreas. The first zebrafish model of pancreatic cancer was developed in 2004 when human *MYCN* was driven under control of the zebrafish *myoD* promoter, resulting in the formation of pancreatic neuroendocrine tumors (Hong et al. 2004). Since then, the Leach lab has paved the way for models of pancreatic adenocarcinoma. In 2008 they discovered that when oncogenic *KRAS*^{G12D} is expressed under the pancreas progenitor promoter *ptf1a*, it blocks normal differentiation and causes an expansion in progenitor cells, leading to pancreatic cancer formation (Park et al. 2008).

Interestingly, deletion of one copy of the ribosomal protein *rpl36* in addition to *KRAS*^{G12D} activation resulted in accelerated tumor progression, with 100% of fish developing tumors by 7.5 months (Provost et al. 2014). Leach and colleagues went on to comprehensively screen 12 different *KRAS* mutations and found a significant increase in pancreatic tumor formation in fish injected with one of the *ptf1a*-driven *KRAS* mutations found in human pancreatic cancer, as compared to those not associated with pancreatic cancer (Park et al. 2015). However, when expressing *KRAS*^{G12D} in *elastase3l* pancreatic cells, pancreatic neuroendocrine tumors are formed, highlighting the need to test additional promoters to better understand pancreatic cancer development (Oh & Park 2019).

Liver Cancer

Liver cancer, particularly hepatocellular carcinoma, is the leading cause of cancer-related death worldwide (ACS 2020). The role for zebrafish in hepatocellular carcinoma research has been extensively reviewed by Goessling and colleagues; here, we summarize the major findings in the field (Wrighton et al. 2019). The first transgenic zebrafish model of hepatocellular carcinoma was developed in 2008 by overexpressing the hepatitis C core protein in the context of hepatotoxin treatment (Rekha et al. 2008).

A more targeted transgenic model was generated when *KRAS*^{G12V} was driven under the liver-specific *fabp10* promoter and resulted in hepatocellular carcinoma formation penetrance between 2 and 12 weeks, which was accelerated with p53 loss (Nguyen et al. 2011). Since then, the Gong lab has made several transgenic hepatocellular carcinoma models, including LexPR, Cre-LoxP

and Tet-inducible systems for *KRAS*^{G12V}, *xmrk*, and *Myc* that are molecularly similar to human hepatocellular carcinoma (Li et al. 2012, 2013; Nguyen et al. 2012, 2016; Zheng et al. 2014). In studying these models, the Gong lab discovered a major role for the immune system, particularly neutrophils and macrophages, differential signaling pathway activation, and sex hormone–induced differences in tumor onset, as observed in humans (Li et al. 2019; Yan et al. 2015, 2017; Yang et al. 2018; Zhao et al. 2016). The difference between sexes was independently confirmed in a DMBA-induced liver cancer model, which showed that treatment with 17β-estradiol accelerated tumor formation largely in the male zebrafish (Chaturantabut et al. 2019).

An additional hepatocellular carcinoma model was developed by overexpressing activated β-catenin under the *fabp10* promoter, leading to tumor formation at 78% penetrance by 6 months of age (Evason et al. 2015). As in human hepatocellular carcinoma, these tumors had hyperactivation of the JNK pathway and inhibition of this pathway resulted in a decrease in tumor growth. Another group found that by truncating the tumor suppressor adenomatous polyposis coli, or APC, zebrafish form spontaneous adenomas in the liver and small intestine and, when combining the APC mutation with DMBA treatment, also induced pancreas and bile duct tumors (Haramis et al. 2006).

Another type of liver cancer, intrahepatic cholangiocarcinoma, has also been modeled in zebrafish by overexpressing the oncoprotein Gankyrin in *fabp10*-expressing liver cells using a Tet-Off system (Huang et al. 2017). An additional intrahepatic cholangiocarcinoma model was generated by using the *fabp10* promoter to overexpress *NRAS*^{Q61K}, which resulted in 81.5% of fish developing tumors at one year postfertilization (Wang et al. 2017). Despite these advances in modeling pancreatic and liver cancers, there are many gastrointestinal tumors that have yet to be modeled using a transgenic zebrafish system. Additional combinations of gastrointestinal-specific reporters driving oncogenes with and without tumor suppressor loss warrant further investigation.

THYROID CANCER

The endocrine and neuroendocrine system is made up of clusters of cells in several organs and glands, including the pituitary, thyroid, parathyroid, adrenal, and pineal glands. A common tumor type in this system is thyroid cancer. In 2017, the Houvras lab developed a model of papillary thyroid cancer by overexpressing *BRAF*^{V600E} under the zebrafish thyroglobulin promoter (Anelli et al. 2017). These fish exhibited developmental abnormalities in their thyroid follicle structure, which was reversed by BRAF and MEK inhibitors, and formed thyroid cancer at 12 months postfertilization. In the future, researchers should explore the mechanisms driving thyroid cancer formation.

GENITOURINARY

Renal Cell Carcinoma

The zebrafish kidney represents a useful model of kidney development and disease (Drummond & Davidson 2010). The van Eeden lab generated two zebrafish lines with inactivated *vhl*, a very common mutation in clear cell renal cell carcinoma (Maher & Kaelin 1997; Santhakumar et al. 2012; TCGA 2013; Van Rooijen et al. 2009, 2010). Erythropoiesis, angiogenesis, abnormal cardiac contractility, and early lethality were ameliorated by HIF2α inhibition in these fish, and the kidney of the *vhl*$^{-/-}$ larvae recapitulated clear cell histology, indicating that these fish may be used as a model of early-stage renal cell carcinoma (Martins Metelo et al. 2015, Noonan et al. 2016). Generating tissue-specific and inducible models and introducing secondary mutations will be important in coming years.

Germ Cell Tumors

Germ cell tumors (GCTs) arise from primordial germ cells and occur in infants, children, and young adults in testis, ovary, or extragonadal sites (Sanchez & Amatruda 2016). Zebrafish germline development is reminiscent of that of mouse and human (reviewed in Sanchez & Amatruda 2016). The Amatruda lab developed a zebrafish model of testicular GCT, characterized by undifferentiated germ cells, that is highly penetrant and heritable (Neumann et al. 2009). Fish develop testicular tumors that are sensitive to radiation, by approximately 7 months postfertilization. These fish were later found to have an inactivating mutation in *alk6b*, a bone morphogenetic protein receptor (Neumann et al. 2011, Sanchez et al. 2019). Other groups have shown that expression of Simian virus 40 large T-antigen, *slc*, the ciliary gene *lrcc50*, and *ns1402* generates testicular GCTs (Basten et al. 2013, Gill et al. 2010, Litchfield et al. 2016, Shimizu & Matsuda 2019). While progress has been made to identify testicular GCT zebrafish models, zebrafish models of ovarian GCTs have yet to be described.

CONCLUSION

The zebrafish models outlined here have enhanced our understanding of the mechanisms underlying the initiation, progression, and metastasis of human cancer. Additionally, high-throughput drug screens in zebrafish have identified promising new therapies for cancer and other diseases. Despite these instrumental advances, there is still much more to learn using this powerful model system. The future of zebrafish cancer modeling is moving towards more tissue-specific and inducible models using CRISPR/Cas9, Cre/Lox, and Gal4/UAS (upstream activation sequence) technologies. Zebrafish are also an ideal model in which to test serial oncogene activation or tumor suppressor loss using novel techniques like electroporation. The ability to rapidly generate transgenic animals and the ease of imaging make the zebrafish an ideal model in which to study the tumor microenvironment, a largely underexplored area in this field. Studying interactions among tumor cells, the immune system, vasculature, and supporting niche cells is likely to reveal novel insights into tumorigenesis and could lead to the development of new therapeutics. Transgenic modeling of cancer in the zebrafish is a powerful tool to investigate basic biology and identify drugs that will stop cancer development.

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