Hooked on zebrafish: insights into development and cancer of endocrine tissues

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Abstract

Zebrafish is emerging as a unique model organism for studying cancer genetics and biology. For several decades zebrafish have been used to study vertebrate development, where they have made important contributions to understanding the specification and differentiation programs in many tissues. Recently, zebrafish studies have led to important insights into thyroid development, and have been used to model endocrine cancer. Zebrafish possess a unique set of attributes that make them amenable to forward and reverse genetic approaches. Zebrafish embryos develop rapidly and can be used to study specific cell lineages or the effects of chemicals on pathways or tissue development. In this review, we highlight the structure and function of endocrine organs in zebrafish and outline the major achievements in modeling cancer. Our goal is to familiarize readers with the zebrafish as a genetic model system and propose opportunities for endocrine cancer research in zebrafish.

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Introduction: endocrine tissues in zebrafish

Zebrafish have most organs found in higher vertebrates, including endocrine tissues. Development in zebrafish proceeds rapidly, the embryos are translucent and can be studied using light microscopy, and tissue-specific markers can be used to study the temporal and spatial patterns of gene expression in normal and mutant embryos. The differentiation program of endocrine organs in zebrafish has been the subject of significant study. These studies have led to valuable insights into the genes and pathways that control the induction, specification, and differentiation of endocrine tissues. In many cases, these programs are conserved between zebrafish and higher vertebrates and provide opportunities for making connections between developmental pathways and neoplasia.

Follicular derived thyroid cells

Adult zebrafish possess thyroid follicles, which are distributed in connective tissue along the ventral aorta (Fig. 1). The zebrafish thyroid, like that of other

teleosts, is not encapsulated by connective tissue. The developmental program specifying thyroid follicular cells is conserved in zebrafish (Wendl *et al.* 2002). Zebrafish possess two NK2 homeobox 1 (*NKX2-1*, also known as *TTF1*) homologs due to an ancient gene duplication event (Rohr & Concha 2000). Only one of these, *nkx2.1a* is expressed in precursor and mature thyroid cells. Knockdown of *nkx2.1a* during zebrafish embryogenesis results in failure of thyroid development, similar to the effects observed after targeted disruption of *Nkx2-1* in mouse (Elsalini *et al.* 2003).

Zebrafish embryos develop externally and development of major organs is complete by 5 days post fertilization. Studies from the Rohr laboratory have utilized these attributes to perform a detailed study of the spatial and temporal patterns of key thyroid markers during zebrafish embryogenesis (Alt *et al.* 2006). Expression of *nkx2.1a*, hematopoietically expressed homeobox (*hhx*), and paired box gene 2a (*pax2a*, also called *pax2.1*) can be identified in thyroid primordium at ~24 h post fertilization (hpf). Expression of Tg mRNA begins at ~30 hpf. The first thyroid follicles to bud from pharyngeal endoderm can

Figure 1 Adult zebrafish thyroid follicles. Sagittal section of a 12-month-old adult male zebrafish reveals thyroid follicles near the midline (arrows) in close association with the ventral aorta and gills. 20 $\times$ magnification, scale is indicated (bottom left, 100 μ m). The region of magnification is indicated by the black box in the inset (bottom right). Image available online at the Pennsylvania State University zebrafish atlas website (<http://zfAtlas.psu.edu/view.php?s=279&z=1&c=12486,13767>).

be identified at 55 hpf. Mature zebrafish thyroid follicles express thyroglobulin protein, T4, and show uptake of radioactive iodine (Alt *et al.* 2006, Wendl *et al.* 2007). Zebrafish possess thyrotropes in the adenohypophysis that produce TSH. The developing zebrafish embryo relies on a maternal deposit of thyroid hormone in the yolk for the first 4–6 days of development. Treatment of developing zebrafish with the methimazole or phenylthiourea leads to the absence of T4 immunostaining in zebrafish embryos, and variable effects on other tissues (Elsalini & Rohr 2003).

A genetic analysis of pathways involved in thyroid specification has revealed a conserved developmental program. The Rohr lab has investigated the effects of zebrafish *pax2a* loss of function on thyroid development using a zebrafish mutant *no isthmus (noi)*. Loss of *pax2a* in the *noi* mutants leads to loss of T4-positive follicles and loss of *nkx2.1a* expression (Wendl *et al.* 2002). This is interesting since *Pax2* has no apparent role in thyroid development in mouse, but is part of a paralogous group that includes *Pax2/5/8*, and *Pax8* plays a comparable role in mouse. Using zebrafish mutants with defects in the specification of endoderm it has been possible to establish that nodal signaling is required for thyroid development. Zebrafish mutants with defects in endoderm development such as one-eyed pinhead (*oep*) and cyclops (*cyc*) have significantly reduced or absent thyroid (Elsalini & Rohr 2003). Fate-mapping studies using zebrafish blastula transplants have demonstrated that the zebrafish thyroid follicles are completely derived from endoderm (Alt *et al.* 2006). Studies of the zebrafish mutant hands off have revealed a non-cell autonomous role for the bHLH transcription factor heart and neural crest derivatives expressed 2 (*hand2*) and FGF signaling in

thyroid fate specification (Wendl *et al.* 2007). Since the developmental program, tissue architecture, and function of thyroid are conserved in zebrafish, it is reasonable to assume that functionally relevant models of thyroid cancer can be constructed using the approaches outlined below.

Calcitonin-producing cells: ultimobranchial body

In humans, parafollicular C-cells are a neural crest-derived lineage that secrete calcitonin. C-cells arise from the ultimobranchial bodies (UBB) that are derived from the fourth and fifth pair of branchial pouches during embryonic development (Biddinger & Ray 1993). These cells migrate into the developing thyroid. In lower vertebrates such as birds and fish, the UBB does not fuse with thyroid and remains a separate organ. In zebrafish, the UBB is a bilateral structure, which expresses calcitonin and is found adjacent to the heart atrium (Alt *et al.* 2006). The developmental differences in C-cells between fish and humans create an opportunity for genetic experiments that target the UBB without interfering with the development or function of follicular thyroid cells. Malignant transformation of C-cells leads to medullary thyroid cancer in humans, so it would be interesting to determine if a similar disease phenotype emerges in a species where C-cells are not colocalized with thyroid epithelial cells. Such studies may shed light on the role of follicular thyroid cells as a niche for C-cells in higher vertebrates.

Parathyroid

Zebrafish express parathyroid hormone and possess the gene for calcium sensing receptor (*casr*), both of which are expressed in gill tissue (Okabe & Graham 2004). Glial cells missing homolog 2 (*Gcm2*), a critical regulator of parathyroid development in mammals, is expressed in pharyngeal endoderm in zebrafish, as in mammals (Hogan *et al.* 2004). Knockdown of *gcm2* in zebrafish embryos leads to defects in formation of internal gill buds. These studies suggest that the gill buds of zebrafish may represent an evolutionary ancestor of the parathyroid gland in terrestrial species.

Parathyroid carcinoma and the hereditary syndrome of hyperparathyroidism–jaw tumor (HPT–JT) are associated with mutations in *CDC73* (*HRPT2*, Parafibromin) (Carpten *et al.* 2002). *CDC73* is part of a highly conserved complex that negatively regulates transcriptional elongation (Rozenblatt-Rosen *et al.* 2005). The tissue specific basis of *CDC73* functioning as a tumor suppressor in human parathyroid is not well understood. Intriguingly, the zebrafish ortholog of *CDC73* was identified in a genetic suppressor screen where it

regulates blood development (Bai *et al.* 2010). The identification of a zebrafish *cdc73* mutant may be a valuable tool for creating a zebrafish model of parathyroid carcinoma.

Adrenal

In zebrafish, steroidogenic and chromaffin cells are found in the anterior portion of the kidney admixed with renal and hematopoietic cells, in a region referred to as the intrarenal or head kidney. Zebrafish produce cortisol in response to stress and production is stimulated by ACTH (Gallo & Civinini 2003). Zebrafish possess a homolog of human SF1, *ff1b* (also known as *nr5a1a*, nuclear receptor subfamily 5) that stimulates the expression of the steroid biosynthesis enzymes *cyp11a* and *3 β -hsb* (Chai *et al.* 2003). Knockdown of *ff1b* leads to impaired differentiation of intrarenal cells and *cyp11a* expression (Hsu *et al.* 2003). Using knockdown of the ACTH receptor (*mc2r*, ACTH receptor), work from the Allolio laboratory has demonstrated that pituitary corticotrophs in zebrafish are responsible for the larval development of the intrarenal organogenesis (To *et al.* 2007). As is the case in mice, early development of the intrarenal organ in fish is independent of pituitary influence, but the animals become dependent on pituitary signals by early larval stages of development. Treatment of zebrafish embryos with dexamethasone leads to suppression of pro-opiomelanocortin in the anterior pituitary and decreased expression of steroidogenic genes in the intrarenal cells, indicating a conserved mechanism of negative feedback.

Adrenocortical carcinoma (ACC) remains a challenging clinical entity with a very poor prognosis for patients with advanced disease (Allolio & Fassnacht 2006). The molecular basis of human ACC remains incompletely understood. Since the basic developmental pathways specifying steroidogenic, and specifically cortisol, producing cells are conserved between mammals and fish, it may be possible to model ACC in zebrafish. As is the case with other lineage-specific transcription factors, an increased dose of the *SF1* (also known as *NR5A1*, nuclear receptor subfamily 5) gene appears correlated with the development of ACC in humans (Doghman *et al.* 2007). Canonical Wnt signaling has been shown to be required for adrenal development, which is interesting since somatic *CTNNB1* (β -catenin) mutations are found in ACC (Kim *et al.* 2008). These observations suggest that the developmental pathways which regulate steroidogenic producing cells may be appropriate starting points for zebrafish models of ACC, and may allow further connections between neoplasia and development to be pursued.

Pancreas

The pancreas is the most intensively studied and best understood endocrine organ in zebrafish (Tiso *et al.* 2009). Anatomically the adult zebrafish pancreas is found as a single islet with exocrine (acinar) cells surrounding a core of endocrine cells. Additional small clusters of endocrine cells can be found adjacent to the intestine. Unlike the mammalian pancreas, the zebrafish pancreas does not develop from an invagination of foregut endoderm, but forms directly from anterior endoderm (Ober *et al.* 2003). Pancreatic progenitor cells can be identified by 15 hpf in the developing zebrafish embryo and are characterized by production of insulin, glucagon, somatostatin, ghrelin, and cells secreting pancreatic polypeptide. The molecular pathways that lead from endoderm induction to specification of pancreas cell identity have been studied in detail during zebrafish embryogenesis (reviewed in Tiso *et al.* (2009)). Knockdown of the zebrafish ortholog of pancreatic and duodenal homeobox 1 gene (*PDX1*), *pdx1*, leads to failure of pancreatic development in zebrafish as in mouse (Yee *et al.* 2001).

The combination of pancreatic specific markers and genetic approaches has led to a detailed understanding of pancreas development in zebrafish. Using a transgenic reporter line of zebrafish that express GFP under the control of the *ela3l* (elastase A) promoter, research from the Lin lab has studied the effects of retinoids on exocrine pancreas development (Jiang *et al.* 2008). Using pancreas-specific promoters it has been possible to create models of both exocrine and endocrine pancreatic tumors (Yang *et al.* 2004, Park *et al.* 2008). It is possible to envision chemical screens using zebrafish embryos to identify compounds capable of promoting β -cell differentiation, or compounds blocking early stages of transformation in tumor models. A detailed understanding of the molecular and genetic pathways that control pancreatic stem cell identity may lead to novel therapies for patients with diabetes, and new drugs and drug targets in tumors arising from the pancreas.

Cancer models in zebrafish

Early origins of zebrafish cancer research

The first experimental approaches to creating cancer in zebrafish were reported by Stanton in the mid-1960s (Stanton 1965). In these studies zebrafish treated with the carcinogen diethylnitrosamine (DENa) developed liver tumors. Several decades later, Spitsbergen *et al.*

(2000a,b) conducted detailed studies of the effects of dose, route of administration, and age on zebrafish exposed to the carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 7,12-dimethylbenz[*a*]anthracene (DMBA). Untreated zebrafish have a low rate of spontaneous neoplasia; ~1% of zebrafish over the course of their lifetime will develop a tumor. Zebrafish exposed to either MNNG or DMBA exhibited a range of cancers arising from gill, liver, gastrointestinal tract, pancreas/kidney, and testis. Tumors of epithelial and mesenchymal origin were observed with both agents. Treatment of juvenile zebrafish with DMBA led to the development of cancer in 45–66% of animals at the most potent doses (Spitsbergen *et al.* 2000a). In contrast to MNNG where tumors arise predominantly in epidermal tissues, DMBA treatment is associated with epidermal and mesenchymal tumors. One case of follicular-derived thyroid cancer was observed with DMBA treatment, but none were observed with MNNG treatment. Interestingly, thyroid cancer has been reported at higher levels in other fish species treated with MNNG (Park *et al.* 1993). These and other chemical carcinogenesis studies demonstrate that chemical mutagens retain the ability to cause malignant transformation in zebrafish tissues.

T-cell leukemia

A pivotal study performed in the Look lab led to the creation of a T-cell leukemia model in zebrafish (Table 1; Langenau *et al.* 2003). Microinjection of a plasmid encoding the zebrafish recombination activating gene 2 (*rag2*) promoter, a lymphocyte-specific promoter, driving the expression of murine *c-Myc* led to mosaic transgenic zebrafish with a clonal expansion of T-lymphocytes with hallmarks of leukemia. Fluorescently labeled leukemia cells were serially transplanted and homed to the thymus of immunosuppressed recipient zebrafish. To overcome the early lethality caused by *c-Myc* overexpression in thymocytes, the authors created a system in which microinjection of *Cre* RNA leads to recombination and activation of a floxed *c-Myc* transgene (Langenau *et al.* 2005). In a related study, the authors were able to temporally control the activation of *c-Myc* using a heat shock 70 (*hsp70*) promoter driving *Cre* recombinase (Feng *et al.* 2007). These creative approaches highlight the technical achievements possible in zebrafish and demonstrate that tissue-specific oncogenes from higher vertebrates retain the capacity to cause malignant transformation in zebrafish.

Malignant melanoma

Human melanoma is characterized by a high frequency of mutations in *BRAF* and *NRAS*, similar to papillary thyroid cancer. The most common mutation in human melanoma is *BRAF*^{V600E}, found in ~40–50% of patients with melanoma. A zebrafish model of melanoma created in the Zon lab examined the role of *BRAF*^{V600E} in nevi and melanoma formation *in vivo* (Patton *et al.* 2005). Expression of *BRAF*^{V600E} under the control of the melanocyte-specific promoter, microphthalmia-associated transcription factor (*mitf*), was sufficient to form nevi in zebrafish. Melanocyte-specific expression of *BRAF*^{V600E} on a *p53* mutant background led to animals that developed melanoma with 100% incidence. Zebrafish melanomas were serially transplantable, displayed aneuploidy, and had activation of mitogen-activated protein kinase pathway. A fluorescently labeled *RAS*^{Q61K} driven transgenic model of melanoma also relied on *p53* loss of function for tumor formation (Dovey *et al.* 2009). Gene signature analysis of these tumors indicated that human melanoma tumorigenesis pathways are conserved in the zebrafish.

In the Zon lab, we utilized the Tg (*mitf*:*BRAF*^{V600E/V600E}); *p53*^{-/-} melanoma prone zebrafish to construct a genetic system capable of screening candidate genes for the ability to accelerate or slow melanoma formation (Fig. 2; Ceol *et al.* 2011). Candidate genes were selected from chromosome 1q21.2 based on the identification of a recurrently amplified genomic region in a set of 101 human metastatic melanoma short-term cultures. We filtered amplified genes to select candidates that were also overexpressed based on microarray data. To test candidates we developed a method of rescuing melanocytes in an *mitf*^{-/-} (*nacre*) zebrafish. Zebrafish with a mutation in *mitf* lack melanocytes and are phenotypically albino. Microinjection of embryos from this strain with a vector containing an *mitf* minigene leads to mosaic ‘rescue’ of melanocytes. We built a specialized transposon vector, MiniCoopR, to rescue melanocytes and drive candidate gene expression. When we use MiniCoopR in a triple transgenic/mutant strain of zebrafish: Tg (*mitf*:*BRAF*^{V600E}); *p53*^{-/-}; *mitf*^{-/-} zebrafish, the rescued melanocytes are melanoma prone, because they express oncogenic *BRAF*^{V600E} and carry a *p53* loss-of-function mutation. An advantage of this approach is that we are able to program rescued melanocytes to express any candidate gene product and study the effects on tumor onset. Using the MiniCoopR system we were able to create over 3000 transgenic fish to assay the ability of each candidate gene to

Table 1 Zebrafish cancer models

Histology	Oncogene/tumor suppressor	Promoter	Tissue	Notes	Reference
Transgenic zebrafish cancer models					
T-cell ALL	<i>c-Myc</i>	<i>rag2</i>	Lymphocytes	Transplanted tumor cells homed to the thymus of recipient zebrafish	Langenau <i>et al.</i> (2003)
T-cell ALL	<i>NOTCH1</i>	<i>rag2</i>	Lymphocytes	Tumors developed at 5 months	Chen <i>et al.</i> (2007)
B-cell ALL	<i>TEL-AML1</i>	<i>ef1α</i> ^a	B-cells	Leukemia developed with a ubiquitous promoter	Sabaawy <i>et al.</i> (2006)
AML	<i>AML1-ETO</i>	<i>hsp70</i>	Myeloid cells	AML1-ETO promotes progenitor cell fate switch	Yeh <i>et al.</i> (2008)
AML	<i>MOZ-TIF2</i>	<i>spi1</i>	Myeloid cells	Leukemia arose in transgenic zebrafish	Zhuravleva <i>et al.</i> (2008)
Melanoma	<i>BRAF</i>	<i>mitf</i>	Melanocytes	BRAF is sufficient for nevi formation but requires p53 loss of function to form tumors	Patton <i>et al.</i> (2005)
Melanoma	<i>NRAS</i>	<i>mitf</i>	Melanocytes	Melanoma tumorigenesis pathways are conserved in zebrafish	Dovey <i>et al.</i> (2009)
Melanoma	<i>HRAS</i>	<i>kit, mitf</i>	Melanocytes	Gal4-UAS system used to compare <i>mitf</i> versus <i>kit</i> promoters	Santoriello <i>et al.</i> (2010)
Melanoma	<i>HRAS</i>	<i>mitf</i>	Melanocytes	PI3K–AKT signaling required for melanoma progression	Michailidou <i>et al.</i> (2009)
Pancreatic carcinoma	<i>KRAS</i>	<i>ptf1</i>	Exocrine pancreas	Tumors are invasive and demonstrate upregulation of Hedgehog signaling	Park <i>et al.</i> (2008)
Neuroendocrine carcinoma	<i>MYCN</i>	<i>myoD</i>	Islet cells	Tumors arose at 4–6 months and expressed insulin	Yang <i>et al.</i> (2004)
Embryonal rhabdomyosarcoma	<i>KRAS</i>	<i>rag2</i>	Satellite cell	Conserved gene signature of RAS pathway activation in ERMS	Langenau <i>et al.</i> (2007)
Rhabdomyosarcoma, MPD, MPNST	<i>KRAS</i>	<i>β-actin</i>	Muscle	Conditionally induced activation of floxed KRAS using heat shock	Le <i>et al.</i> (2007)
Multiple ^b	<i>smo</i> and myristylated-hAKT	<i>krt4</i>	Glial cells	Activation of Hedgehog and PI3K–AKT pathways cooperated in tumorigenesis	Ju <i>et al.</i> (2009)
Zebrafish mutants with cancer predisposition					
MPNST	<i>tp53</i>		Schwann cell?	28% of mutant zebrafish developed tumors	Berghmans <i>et al.</i> (2005)
Ocular tumor	<i>ptenb</i>		Eye	<i>ptenb</i> has unique function in the zebrafish eye	Faucherre <i>et al.</i> (2008)
Intestinal, pancreatic, and hepatic adenomas	<i>apc</i>		Several	Accumulate <i>B</i> -catenin and express <i>Wnt</i> target genes	Haramis <i>et al.</i> (2006)
Gonadal tumors	<i>brca2</i>		Gonad	Mutants have defects in ovarian and testis development	Shive <i>et al.</i> (2010)
Testicular germ cell tumor	<i>lamc1</i>		Testis	Tumors develop at median age of 7 months and are detectable by micro-ultrasound	Neumann <i>et al.</i> (2009)
MPNST	<i>rp</i>		Schwann cell?	Impaired ability to translate p53 protein	Lai <i>et al.</i> (2009)
Neuroblastoma	<i>fbxw4</i>		Cranial ganglia	Misregulation of neighboring gene, <i>fgf8</i> , associated with tumor development	Amsterdam <i>et al.</i> (2009)
MPNST	<i>emi1</i>		Schwann cell?	<i>emi1</i> homozygotes show defects in hematopoiesis and genome instability	Rhodes <i>et al.</i> (2009)
MPNST, neurofibroma, others	<i>msh2, msh6, mlh1</i>		Various	Mutation in mismatch repair genes lead to cancer in multiple tissues	Feitsma <i>et al.</i> (2008)

ERMS, embryonal rhabdomyosarcoma; MPNST, malignant peripheral nerve sheath tumor.

^a*Xenopus laevis*.^bGlioblastoma, spindle cell sarcoma, rhabdomyosarcoma, ocular melanoma, astrocytoma, myxoma.

Figure 2 Overview of the zebrafish MiniCoopR screen. Candidate genes were identified from a genomic analysis of human patient samples. We focused on genomic regions that were recurrently amplified in metastatic melanoma to identify a region on chromosome 1q21 for further study. Candidate genes were individually cloned into MiniCoopR, a tol2 transposon-based vector. MiniCoopR vectors were microinjected into embryos from the indicated transgenic strain. Embryos with melanocyte rescue were identified at 2 days postinjection. Adult zebrafish with rescued melanocytes were followed for the development of melanoma, which is visible as a raised lesion. Disease-free survival was compared between each candidate gene and a reference control gene (GFP) to identify genes capable of accelerating or slowing the rate of melanoma formation.

accelerate or suppress tumor formation. This approach led to the identification of SET domain bifurcated 1 (*SETDB1*), a histone methyltransferase, as a cooperating oncogene in melanoma. Expression of *SETDB1* accelerates the onset of melanomas, and cooperates with *BRAF* to alter the pigmentation pattern. *SETDB1* overexpressing melanomas were highly invasive, bypassed a senescent arrest, and had genetic signature present in human melanomas. An analysis of human melanomas revealed that ~70% of biopsies

from patients with metastatic melanoma tested ($n=91$) exhibit high levels of *SETDB1* by immunohistochemistry. This study highlights how a genetic model in zebrafish can be adapted to functionally validate driver genes from candidates identified in genomic analyses.

The Mione lab has created a model of melanoma in zebrafish using the Gal4-UAS approach (Santoriello *et al.* 2010). With this system, researchers are able to test the effects of an oncogene under the control of

different promoters. By crossing *UAS-HRAS^{G12V}* transgenic fish to a promoter-Gal4 transgenic fish, the expression of an oncogene can be studied under the control of different promoters. This approach was utilized to characterize the differences between the *mitf* versus *kit* (kit receptor a) promoters as they drive the expression of *HRAS^{G12V}*. They found that *kit* driven melanomas were more aggressive and developed earlier than *mitf* driven melanomas. Blastula transplantation was used to demonstrate that *HRAS^{G12V}* transformed cells, driven from the *kit* promoter, proliferate and form melanomas cell autonomously.

The Hurlstone lab used *HRAS^{G12V}* under the zebrafish melanocyte promoter, *mitf*, to dissect the mechanism of RAS pathway activation in melanoma (Michailidou *et al.* 2009). Expression of *HRAS^{G12V}* under the control of the *mitf* promoter resulted in the development of melanoma in 90% of animals by 12 weeks. Coexpression of a dominant negative phosphoinositide 3-kinase (PI3K) regulatory domain mutant, $\Delta p85$, which interferes with interaction with the p110 catalytic domain (PIK3CA), resulted in dramatic suppression of *RAS^{G12V}* induced melanoma in assays of mosaic juveniles at 12 weeks and in stable transgenic lines. Melanomas arising in the *HRAS^{G12V}: $\Delta p85$* animals were less invasive and showed reduced levels of phospho-Akt. These studies demonstrate that PI3K signaling plays a role in *HRAS^{G12V}* induced malignant transformation of melanocytes in the zebrafish model system.

Myeloid leukemia

Acute myeloid leukemia (AML) has several molecularly defined subsets. One subset of AML is defined by the presence of translocation t(8;21) which fuses the runt-related transcription factor 1 (*RUNX1*, also known as *AML*) and *ETO* (also known as *RUNX1T1*, runt-related transcription factor 1; translocated to, 1) genes. Several studies have addressed the genetic basis of t(8;21) leukemia using zebrafish. The Crosier lab performed a detailed analysis of zebrafish *runx1*, which they demonstrated is required for blood formation in fish, as in mouse (Kalev-Zylinska *et al.* 2002). Overexpression of human AML-ETO in zebrafish led to defects in hematopoiesis and vascular development, which mimic the effects observed in a comparable mouse models. Yeh *et al.* (2008) developed a heat-shock inducible *AML-ETO* transgenic line of zebrafish. In this temporally inducible system, expression of AML1-ETO in zebrafish embryos disrupts the differentiation of hematopoietic progenitor cells by promoting a fate switch from erythroid to myeloid lineages.

Treatment of AML1-ETO expressing zebrafish embryos with trichostatin A, an inhibitor of histone deacetylases, reversed the differentiation block (Yeh *et al.* 2009). They performed a chemical screen to identify compounds able to suppress oncogenic effects of AML1-ETO, and a cyclooxygenase 2 (Cox2) inhibitor was identified. In a study by Dayyani *et al.* (2008) the authors use zebrafish embryos to test the ability of *gro3*, a Groucho/transducin-like enhancer of *split* (TLE) homolog, to cooperate with AML-ETO in deregulating hematopoiesis. Groucho/TLE proteins are corepressors involved in multiple aspects of gene regulation and development. The authors perform morpholino knockdown of *gro3* in zebrafish after induction of AML-ETO expression and demonstrate that the combination leads to an absence of circulating blood cells and the presence of blast-like cells that express granulocytic markers. These studies highlight functional insights into the effects of the AML-ETO oncoprotein on hematopoiesis obtained through zebrafish studies.

Other leukemia models

Several other zebrafish models of leukemia have been generated (Zhuravleva *et al.* 2008). *TEL-JAK2* (TEL is also known as ETV6, Ets variant 6) translocation results in constitutive activation of the Janus kinase 2 (JAK2) kinase and is found in lymphoblastic and myeloid leukemia. Mosaic zebrafish embryos expressing the zebrafish *tel-jak2* fusion protein in myeloid cells displayed a block in differentiation of the myeloid and erythroid lineages (Onnebo *et al.* 2005). A stable transgenic zebrafish model was used to explore the cell of origin involved in *TEL-AML1* induced pre-B ALL. Leukemia arose only in the transgenic zebrafish expressing the *TEL-AML1* ubiquitously, but not when specifically expressed in lymphoid progenitors (Sabaawy *et al.* 2006). *NOTCH1* is commonly mutated in T-cell acute lymphoblastic leukemia (T-ALL). In mosaic zebrafish expressing fluorescently labeled activated *NOTCH1* under the lymphocyte *rag2* promoter, malignant disease developed at 5 months (Chen *et al.* 2007). *NOTCH* activation cooperated with B-cell CLL/lymphoma 2 (*bcl2*) overexpression in stable transgenic lines, establishing a genetic interaction in this model of T-ALL.

Pancreatic cancer

Several labs have utilized zebrafish to model pancreatic cancers and explore early pancreas development. In one study, investigators from the Leach lab studied the effects of *KRAS^{G12V}* expression on pancreas

development (Park *et al.* 2008). By directly observing pancreatic progenitor cells during development, they were able to demonstrate that KRAS^{G12V} expression induces a block in differentiation of exocrine progenitors. In this model, pancreatic cancers arising in zebrafish are invasive, have cytologic characteristics of human adenocarcinoma, and show evidence of upregulation of Hedgehog signaling. In a study from the Look lab, pancreatic neuroendocrine tumors developed in mosaic transgenic zebrafish selectively expressing MYCN (Yang *et al.* 2004). These tumors were locally invasive and normal appearing exocrine cells and ducts were observed in the tumors. These tumors expressed synaptophysin and insulin suggesting islet cells of the pancreas as the cell of origin. An *N*-ethyl-*N*-nitrosourea (ENU) based screen for mutants in exocrine pancreas development identified a mutant in *trpm7*, a cation channel with protein kinase activity. Transient receptor potential cation channel, subfamily m, member 7 (TRPM7) is over-expressed in human exocrine pancreatic cancer, revealing a potential link between developmental regulators and neoplasia (Yee *et al.* 2011).

Rhabdomyosarcoma

Expression of oncogenic KRAS^{G12D} was sufficient to cause embryonal rhabdomyosarcoma (ERMS) in studies from the Zon laboratory (Langenau *et al.* 2007). Surprisingly, these tumors arose in animals where KRAS was driven from *rag2* regulatory sequences. The authors demonstrate that the zebrafish *rag2* regulatory sequences drive expression in satellite cells of muscle in addition to lymphocytes. Injection of *rag2*-KRAS^{G12D} led to highly penetrant and early onset rhabdomyosarcoma tumors in zebrafish. Gene signature analysis of zebrafish and human ERMS indicated a conserved signature of RAS pathway activation in rhabdomyosarcoma. By co-injecting fluorescent reporters driven from different promoters, subpopulations of cells were differentially labeled within zebrafish rhabdomyosarcoma and their self-renewal capabilities were compared in transplantation studies. These studies suggest that the satellite cell may be the cell of origin in rhabdomyosarcoma.

Other transgenic models

Several labs have used promoters with widespread tissue activity to drive the expression of oncogenes in zebrafish. In one approach, heat shock was used to activate Cre recombinase and cause recombination and activation of a β -actin:KRAS^{G12D} allele (Le *et al.* 2007). This led to four types of tumors/hyperplasia

including rhabdomyosarcoma, myeloproliferative disorder (MPD), intestinal hyperplasia, and malignant peripheral nerve sheath tumors (MPNST). Heat shock was also able to induce activation of KRAS *ex vivo* and induce MPD in recipient zebrafish. In studies from the Chen laboratory, a constitutively activated zebrafish smoothened (*smo*) was expressed using a keratin promoter in the Gal4 system with an activated AKT (Ju *et al.* 2009). Multiple tumor types including spindle cell sarcoma, glioblastoma, and astrocytoma resulted from this approach.

Techniques

Clonal zebrafish

A method for generating clonal (isogenic) zebrafish using gynogenetic female homozygous fish as founders has been described (Streisinger *et al.* 1981). In the first step of this method u.v. inactivated sperm are used to fertilize zebrafish eggs. The sperm are capable of activating the embryos but the u.v. damage prevents them from making a genetic contribution, so the resulting organism is haploid. If heat shock or pressure is applied shortly after fertilization the first cleavage division can be prevented, creating a diploid homozygous fish. This fish is raised to adulthood and the procedure is repeated. In the second round, the animals arising from each female are genetic clones of each other. This method has been used to improve upon transplantation studies in zebrafish. Previous transplant experiments required recipient fish to be sublethally irradiated before transplantation to promote immunosuppression and prevent graft rejection (Langenau *et al.* 2003), clonal zebrafish were used to demonstrate that carcinogen-induced hepatobiliary and pancreatic tumors could be serially transplanted into clonal animals without immune suppression (Mizgirev & Revskoy 2006).

To uncover the self-renewal potential in *c-Myc* driven T-cell leukemia, work from the Langenau lab utilized clonal zebrafish (Smith *et al.* 2010). These studies assessed tumor initiating potential of leukemia by performing single-cell transplantations, and developed novel methods of screening adult recipient animals engrafted with fluorescent tumor cells. In a related study, the Revskoy lab serially transplanted T-ALL into syngeneic zebrafish (Mizgirev & Revskoy 2010). These leukemia-bearing animals demonstrated sensitivity to known chemotherapeutics, establishing a model system for screening potential anticancer compounds *in vivo*. These studies have established clonal zebrafish as unique system for transplantation

assays and provide opportunities to study engraftment, drug sensitivity, and to test the cancer stem cell hypothesis.

Reverse genetics and tumor suppressors

In zebrafish, ENU mutagenesis has been the foundation of a reverse genetic approach to generate mutations in specific genes. First, described in zebrafish by Wienholds *et al.* (2002), targeting induced local lesions in genomes (TILLING) uses ENU mutagenized male zebrafish bred with wild-type females. Sperm from the resulting F₁ males is cryopreserved and genomic DNA analyzed for mutation in gene(s) of interest by DNA sequencing. Once a mutation has been identified, the cryopreserved sperm can be thawed and IVF is performed to generate an F₂ generation. Berghmans *et al.* (2005) utilized TILLING to identify a point mutation in the DNA-binding domain of zebrafish *p53*, M214K, which was shown to be a loss-of-function mutation. The *p53*^{M214K} zebrafish fail to undergo apoptosis after exposure to ionizing radiation, and develops MPNST, a type of sarcoma. The *p53*^{M214K} zebrafish has been a valuable resource for creating genetically engineered tumor models and for uncovering suppressors of apoptosis (Patton *et al.* 2005, Sidi *et al.* 2008).

Zebrafish harbor two orthologs of the tumor suppressor phosphatase and tensin homolog (*PTEN*), *ptena* and *ptenb* (Croushore *et al.* 2005). TILLING was used to identify *ptena* and *ptenb* mutant zebrafish lines, which have redundant roles in embryonic development, but distinct tumorigenic functions (Faucherre *et al.* 2008). Intriguingly, *ptenb* mutant zebrafish developed ocular tumors in the presence of normal *ptena* function. Gene duplications are relatively common in the zebrafish genome and often lead to tissue-specific functions. Studies from the laboratory of den Hertog demonstrated that PI3K inhibition with a small molecule, LY294002, suppresses the hyperplastic–dysplastic changes in eyes, heart, and brain observed in *ptena*^{−/−}; *ptenb*^{−/−} embryos. These studies suggest that one could perform a chemical screen for chemical suppressors of the *ptena*^{−/−}; *ptenb*^{−/−} phenotype which could lead to the discovery of novel PI3K inhibitors, such compounds would potentially have therapeutic utility in *PTEN* mutant tumors.

TILLING has been used to identify a mutation in the zebrafish homolog of adenomatous polyposis coli (*APC*; Hurlstone *et al.* 2003). Characterization of *apc*^{MCR} mutant zebrafish led to the characterization of *apc* as a tumor suppressor in zebrafish (Haramis

et al. 2006). Heterozygous *apc*^{MCR} zebrafish developed tumors of the digestive tract spontaneously, primarily in liver and intestine. Tumor incidence was accelerated with the addition of DMBA. These tumors demonstrated upregulation of the *Wnt/β-catenin* signaling pathway. Further studies of *apc* mutant zebrafish have demonstrated a novel role for *Wnt/β-catenin* signaling in liver regeneration after hepatectomy (Goessling *et al.* 2008).

Hereditary mutations in breast cancer 2 (*BRCA2*, early onset) are associated with increased risk of ovarian and breast cancer. TILLING has been used to isolate a missense mutation in the zebrafish *brca2* ortholog (Shive *et al.* 2010). *Brca2*^{Q658X} homozygous mutant zebrafish fail to undergo ovarian development and exhibit abnormal spermatogenesis. Examination of *brca2*^{Q658X}; *p53*^{−/−} adults revealed an increased incidence of testicular neoplasia. These studies reveal a role for *brca2* in ovarian development and tumor formation in zebrafish. TILLING has also been used to generate several mutations in mismatch repair genes and study the effects on tumor development (Feitsma *et al.* 2008).

Zinc finger nucleases

Zinc finger nucleases (ZFN) are chimeric fusions between C2H2 zinc fingers and the Fok I endonuclease that are selected for high-affinity binding to specific genomic loci. Dimerization of two zinc fingers at a locus leads to endonuclease dimerization and the generation of a double-stranded DNA break (Porteus & Carroll 2005). Because DNA strand breaks are imperfectly repaired by non-homologous end joining, small insertions or deletions are formed. ZFN have been successfully used to generate loss-of-function alleles in plants, invertebrate organisms, cell lines, and zebrafish (Doyon *et al.* 2008). The Wolfe and Lawson labs have demonstrated the efficacy of using ZFN in zebrafish to selectively induce mutations at specific zebrafish genomic loci (Meng *et al.* 2008). Using the target locus *kdr* (also known as *kdrl*, kinase insert domain receptor like), a receptor tyrosine kinase involved in vascular development, they created mutations with high efficiency, specificity, and germline transmission. Founder heterozygous zebrafish were morphologically normal and mutations were identified by PCR and complementation studies with a known mutant. Oligomerized pool engineering (OPEN) is an alternative method of creating ZFN with streamlined library screening (Maeder *et al.* 2008). This method has been used to efficiently design ZFN to target specific zebrafish genomic loci

(Foley *et al.* 2009). Using *in silico* analysis, the authors estimate that 86% of transcripts in the zebrafish genome can be targeted using OPEN. These studies suggest that ZFNs are becoming a powerful method for performing site-selected mutagenesis in zebrafish.

Forward genetic screens

Forward genetic screens in zebrafish have had a major impact on developmental biology (Driever *et al.* 1996, Haffter & Nusslein-Volhard 1996, Haffter *et al.* 1996). Several labs have performed screens in zebrafish to uncover cancer-related genes. Work from the Zon lab utilized phosphorylated histone H3 staining, a marker of proliferation, in a cell cycle screen to identify cancer prone mutants (Shepard *et al.* 2005, 2007, Neumann *et al.* 2009). These studies identified a zebrafish mutant of separase (also known as *espl1*, extra spindle poles like 1), an anaphase promoting cysteine protease (Shepard *et al.* 2007). Heterozygous *espl1* mutants were more susceptible to epithelial tumors after exposure to the chemical carcinogen MNNG. A loss-of-function mutant of *mybl2*, the zebrafish *MYBL2* (*B-MYB*) ortholog, was also identified (Shepard *et al.* 2005). These mutant zebrafish exhibited genome instability and increased cancer susceptibility. The cell cycle screen also identified a zebrafish model of germ cell tumors (Neumann *et al.* 2009). Zebrafish harboring a mutation in laminin, γ 1 (*lamc1*) exhibited, both spontaneous and carcinogen-induced, germ cell tumors. Micro-ultrasound, a useful tool for imaging zebrafish (Goessling *et al.* 2007), was utilized to detect subclinical germ cell tumors in *lamc1*^{cz61} mutants. These studies demonstrate the ability of forward genetic screens in zebrafish to uncover novel genes involved in cancer.

Insertional mutagenesis

The Hopkins lab performed a large-scale insertional mutagenesis screen to identify genes essential for early embryonic development (Amsterdam *et al.* 1999). Blastula stage zebrafish embryos were injected with a retrovirus to produce loss-of-function alleles. The F3 progeny were screened for recessive mutants and 500 unique zebrafish lines representing insertions in ~370 genes were identified. This study provides an unparalleled collection of mutants in genes required for early vertebrate development. Many developmental genes have important links to cancer and endocrine disorders. For example, they isolated a mutant, hi548, which disrupts expression of *vhnl1* (also known as *hnf1ba*, HNF1 homeobox Ba), a transcription factor linked to kidney abnormalities and maturity onset diabetes in

humans (Golling *et al.* 2002). A study of the hi2648 mutant, which disrupts expression of *emi1* (also known as *fbxo5*, F-box protein 5), leads to hematopoietic defects and genome instability, and accelerated tumor formation on a p53 mutant background (Rhodes *et al.* 2009). Intriguingly, the Hopkins group noticed that 17/28 zebrafish lines with mutations in ribosomal proteins have a tendency to develop highly aneuploid MPNST sarcomas. This suggests that alterations in protein translation may lead to a predisposition to the develop cancer and that zebrafish may be used to model genome instability in cancer (Lai *et al.* 2009, Zhang *et al.* 2010). A detailed study of tumor susceptibility in this collection revealed a neuroblastoma-like tumor in four dominant F-box and WD domain protein 4 (*fbxw4*) mutant alleles, all of which appeared to upregulate the neighboring *fgf8* (also known as *fgf8a*, fibroblast growth factor 8a) gene (Amsterdam *et al.* 2009).

Xenotransplantation

Several groups have demonstrated that human melanoma cells can be transplanted into zebrafish larvae where they proliferate and migrate (Lee *et al.* 2005, Haldi *et al.* 2006). Human tumor xenografts induce angiogenesis in zebrafish embryos that can be visualized using transgenic fish that express GFP in blood vessels (Nicoli & Presta 2007, Nicoli *et al.* 2007). Studies from the Klemke lab have utilized confocal microscopy to quantify extravasation of metastatic cells and explore the genetics of vascular remodeling (Stoletov *et al.* 2007, 2010).

Studies from the Hendrix lab have proposed using the zebrafish embryo as a biosensor for the micro-environment of tumor cells (Lee *et al.* 2005). They have demonstrated that human melanoma cells survive after transplantation into blastula stage zebrafish embryos. Transplanted melanoma cells maintain a dedifferentiated state and co-express mesenchymal and epithelial cell markers. They identified a melanoma cell line capable of inducing aberrant axis development in the zebrafish embryo after transplantation, similar to the effects observed when nodal, a developmental morphogen, is ectopically expressed (Topczewska *et al.* 2006). Inhibition of nodal blocks the ability of the melanoma cells to induce an ectopic axis and causes the melanoma cells to become pigmented, suggesting a more differentiated state. By using the phenotype induced in a zebrafish embryo the authors are able to uncover a signaling pathway active in the tumor cells.

Metastatic models

Several groups have developed models of metastasis using xenotransplantation in zebrafish embryos. The Cao lab has developed a novel method of inducing hypoxia in zebrafish (Cao *et al.* 2010, Rouhi *et al.* 2010). Hypoxia induces dissemination of transplanted mouse fibrosarcoma cells in zebrafish embryos and neo-angiogenesis in the tumor. Increasing the level of VEGF in the transplanted tumor cells also promotes invasion and dissemination of tumor cells in the zebrafish assay (Lee *et al.* 2009). The authors show that treatment of zebrafish embryos with sunitinib, a tyrosine kinase inhibitor of VEGFR2 (also known as KDR, kinase insert domain receptor), or morpholino knockdown of *vegfr2*, results in decreased tumor invasion and dissemination. In a report from the Bagowski lab, both human cancer cell lines and primary tumors were shown to enter the circulation and colonize distant sites after transplantation into zebrafish embryos (Marques *et al.* 2009). By mating together two pigment mutants (*roy* and *nacre*) a transparent zebrafish, *casper*, was created and used to study transplantation (White *et al.* 2008). Using *casper* it is possible to visualize GFP-labeled hematopoietic stem cells (HSC) homing to the kidney marrow in adult recipients, and follow the migration of melanoma cells after transplantation. These emerging models may lead to novel chemical and genetic screens to identify suppressors of angiogenesis and metastasis.

Chemical genetic screens

Zebrafish embryos can be used in chemical screens in which hundreds or thousands of compounds are tested for the ability to induce a specific phenotype. Such phenotypes can include expression of a particular gene or suppression of a genetic defect. Phenotype-based screens have the advantage that the chemical hits can occur on any pathway that leads to the desired outcome. In a screen from the MacRae lab, 100 drugs in clinical use were tested for the ability to induce bradycardia in zebrafish embryos (Milan *et al.* 2003). Remarkably, 22/23 drugs known to cause QTc prolongation and bradycardia in humans, also caused bradycardia in zebrafish. The assay in zebrafish embryos was also capable of identifying drug–drug interactions that result from metabolism, a unique advantage of screening in the whole organism.

Zebrafish mutants may have complex phenotypes in specific tissues or organs. A mutation in the hairy/enhancer-of-split related with YRPW motif 2 (*hey2*)

gene, a transcriptional repressor, leads to aortic coarctation (Weinstein *et al.* 1995). The Peterson lab performed a chemical suppressor screen to identify compounds capable of reversing this phenotype (Peterson *et al.* 2004). They identified two compounds that reversed the cardiovascular effects observed in the *hey2* mutant zebrafish. One compound, GS4012, induces vegf, which is effective in reversing this phenotype as well. These experiments demonstrate that a chemical screen in embryos can result in the discovery of drugs that suppress complex phenotypes. This approach to discovery is unbiased and it is independent of understanding the precise molecular mechanism of the mutant.

In zebrafish, the lateral line is a mechanosensory organ, which consists of hair cells organized into clusters known as neuromasts (Dambly-Chaudiere *et al.* 2003). The sensory hair cells in the inner ear of humans have functional similarities to the zebrafish lateral line hair cells. Hair cell injury and death within the inner ear is a major cause of hearing loss. In an innovative set of experiments, the Raible lab performed chemical and genetic suppressor screens to identify drugs and genetic pathways capable of conferring protection from aminoglycoside-induced hair cell death (Owens *et al.* 2008). In the chemical screen, zebrafish embryos were pre-treated with library of 10 960 compounds, and then subjected to treatment with neomycin. They identified two compounds, both benzothioephene carboxamides, which were capable of blocking neomycin-induced hair cell death, without interfering with neomycin's antibiotic activity. The active compounds were also capable of protecting against neomycin-induced cell death in murine utricle cells *in vitro*. The authors performed an F3 genetic screen to identify genetic modifiers of neomycin-induced hair cell toxicity. They identified several mutants, one of which, *sentinel* (*cc2d2a*, coiled-coil and C2 domain containing 2a), is protective against neomycin-induced hair cell death but not against cell death caused by cisplatin. Starting from a phenotype that has relevance in medicine these studies in zebrafish uncover novel drugs and genes, which may lead to new treatments for drug-induced ototoxicity.

North *et al.* (2007) performed a small molecule screen to identify chemicals capable of inducing or repressing HSC formation in the zebrafish embryo. Remarkably, they found that prostaglandin E₂ (PGE₂) increased HSC formation and PGE₂ antagonists, such as celecoxib, decreased HSC formation. PGE₂ also increased HSC formation in murine stem cell assays. This study led to a phase I clinical trial in which cord

blood stem cells are treated *ex vivo* with PGE₂ in an effort to expand HSCs before transplantation (Lord *et al.* 2007). This is the first example of a discovery in zebrafish that led directly to a clinical trial in human subjects.

In a recent study from the Zon lab, White *et al.* (2011) performed a chemical screen to identify drugs that can suppress cells of neural crest lineage. They found that leflunomide, an FDA approved drug for treatment of rheumatoid arthritis, inhibited multiple neural crest derivatives including melanocytes from the developing embryo. Treatment of melanoma xenografts with a combination of PLX4720 (a *BRAF*^{V600E} inhibitor) and leflunomide led to greater growth inhibition than treatment with either drug alone, and in 40% of animals it led to near complete tumor regression. By focusing on the embryonic lineage of melanocytes and using zebrafish embryos to discover lineage specific chemical inhibitors, White *et al.* (2011) uncovered an inhibitor of melanoma. Leflunomide may have therapeutic utility in other cancers arising from the neural crest such as pheochromocytoma and medullary thyroid cancer.

Conclusion

The combination of chemical and genetic approaches in zebrafish can uncover specific drug–gene effects, which have medical relevance. There are many creative ways to design genetic and chemical screens in zebrafish by utilizing existing mutants or markers of specific cell types. Chemical screens performed in zebrafish embryos have the advantage of studying the effects of compounds in the whole animal. In these screens, the outcome of treatment can therefore be due to interactions between tissues, which would be absent in screens performed on homogenous cell populations. Chemical screens in zebrafish are informationally rich, and the hits are often reproducible when tested in higher vertebrates. We see potential for chemical screens in zebrafish to address specific problems relevant to endocrine oncology. Zebrafish embryos could be used to screen for compounds capable of modulating iodine uptake, increasing the specification of pancreatic β -cells, or ablating cells that express particular oncogenes relevant in endocrine cancers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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