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Use of Zebrafish Embryos for Small Molecule Screening Related to Cancer

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The introduction of mechanism-based targeted therapies to treat human cancers is fruit of decades of research into the molecular basis of cancer pathogenesis. Despite the growing knowledge about the molecular mechanisms governing its causes and progression, there is a lack of effective treatments for many types of cancer. The expensive and time-consuming preclinical pipeline for testing molecules slows the discovery of new therapies. Therefore, it is important to consider alternative methodologies both for accelerating therapeutic discovery and reducing costs. In that regard, zebrafish is becoming an attractive model for fast and efficient drug screening. Its use has expanded to many disease research areas, and the postgenomic era has led to the progression of functional studies and boosted the development of general databases, such as ZFIN, and the emergence of more specialized ones, including several catalogues of transgenic reporter screens. Taken together, they provide to the scientific community many tools that could be used for drug discovery. The use of zebrafish in cancer drug screenings could help to economize time and resources even more if we rationalize its use: we could use embryonic screens to identify drugs that address general hallmarks of cancer, and use adults for finding molecules that target specific cancer models. *Developmental Dynamics 242:97–107, 2013*.

Key words: zebrafish; cancer; drug screening; preclinical analysis

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INTRODUCTION

Cancer is the common name given to a multifactorial illness that encompasses different syndromes. It is considered multifactorial because the contributions of inheritance, infection, age, gender, pollution, or habits will together provide the right environment (genetic instability and inflammation) in which genetic mutations are allowed to promote carcinogenesis, tumor formation, and metastasis (Hahn and Weinberg, 2002).

The syndromes grouped under the name of cancer will have a diverse prognosis depending on their tumor location (breast, lung, skin, etc; Jemal et al., 2011). Nevertheless, when cell transformation occurs, any given syndrome shares common hallmarks that are the necessary steps beyond cancer progression: sustained cell proliferation, protection against apoptosis, de novo vascularization (angiogenesis), abnormal activation of oncogenes/repression of tumor suppressor genes, initiation of cell invasion molecular programs, evasion from the immune system and changes in metabolic homeostasis (Hanahan and Weinberg, 2011). These shared features are the major phenotypes targeted in developing a treatment for cancer. Therefore, drugs detected during preclinical studies that affect any of these hallmarks are very attractive candidates to proceed on human clinical trials.

Recent studies have identified extensive overlap between the molecular mechanisms controlling embryonic development and cancer (Wodarz and Nathke, 2007; de Beco et al., 2012; Geissler and Zach, 2012). Understanding their role during development may, therefore, provide new insights into the nature of cell transformation (Martinez-Arias, 2001). In fact, basic research in developmental biology, and its associated generation of genetic tools, has opened the door to successful drug screening assays in search of new

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cancer therapies, examples of which will be described below. The point of view we share is that drugs identified through chemical screenings performed with model animals, which affect basic cellular mechanisms (cell proliferation, apoptosis, etc.), developmental processes (angiogenesis, neural crest differentiation, etc.), or modulate distinct developmental signaling pathways (Notch, TGFβ, Wnt, FGF, Shh, RA) could serve to control these same mechanisms, processes or pathways when they happen to be missregulated during cancer progression (Fig. 1). Assuming the aim of any therapy is the treatment of the phenotype, we believe this approach can be very useful, and it could help to find suitable therapies for human cancer.

ZEBRAFISH AS A TOOL FOR DRUG SCREENING

Traditionally, the pipeline for preclinical drug discovery includes a first step of in silico and biochemical affinity assays, which aim is to sort out drugs regarding their binding to target molecules (Zhu and Cuozzo, 2009; Tuffery and Derreumaux, 2012). This step is followed by cell culture assays designed to address how efficient are these molecules when confronted to the target biological process, i.e., angiogenesis, inflammation, etc. (Evensen et al., 2010). Both procedures help to reduce the number of initial molecules based fundamentally on their possible biological function. In a later stage, it is essential to use mammalian models, primarily rodents, to fully understand the properties of new drugs and avoid any possible adverse effects on humans (Jackson-Grusby, 2002). However, this conventional pipeline has certain disadvantages: first, some molecules brought forward from the in vitro stage have serious toxicity effects when administered to mammals; and second, by focusing only on the affinity of the screened drugs to target molecules, other compounds with interesting properties might get discarded. Thus, it becomes clear the need for new model organisms such as *D. melanogaster*, *C. elegans*, or *D. rerio* in the preclinical pipeline to fill the gap between in vitro assays and expensive screenings using mammals (Lieschke and Currie, 2007; Giacomotto and Segalat, 2010; Hampson and Wyatt, 2011). By using these model organisms, safety and function of new drugs can be tested faster and cheaper than in mammals. Importantly, the gathering of biological information will be more complete than the obtained by in vitro assays, leading eventually to a better exploitation of drug libraries. Accordingly, previous reviews have explored the potential of zebrafish as a model system to assess drug toxicology (Parng, 2005; Rubinstein, 2006; Barros et al., 2008; Sipes et al., 2011), but also to fight infection (Hamm and Ballard, 2007; Meijer and Spaink, 2011), cardiovascular diseases (Chico et al., 2008; Rocke et al., 2009; Chan and Mably, 2011), neural disorders (Flinn et al., 2008; Gerlai, 2010), or find new cancer therapies (Stoletov and Klemke, 2008; Liu and Leach, 2011).

The technical advantages of using an animal model such as zebrafish for drug screening are numerous: (i) drugs can be administered directly in the swimming water (Peterson et al., 2000). This feature has two main advantages: it is quicker and easier than injecting drugs into mice, and it could eventually help to determine how a molecule behaves in terms of ADME (Absorption, Distribution, Metabolism and Excretion) when exposed to a whole living animal. But it has also two caveats: first, some molecules are not water-soluble and this might have a direct impact in the stability and amount of absorbed drug by the treated embryos. To enhance solubility, compounds are first dissolved in organic solvents or carriers such as DMSO, methanol, acetate or cyclodextrin (Maes et al., 2012). Second, a universal ADME profiling has proven difficult to perform in zebrafish embryos; however, advances in the detection of radio and fluoro-labeled molecules, combined with organ and cell sorting procedures, plus a higher knowledge on zebrafish drug metabolism might contribute to reach this important goal (Peterson and MacRae, 2012). (ii) The prolific egg laying and small size of zebrafish embryos allow the parallel and reproducible testing of several drugs and dosages in simple multiwell plates. (iii) Zebrafish has a high genetic conservation with higher vertebrates (Woods et al., 2000), also analogous organs (heart, liver, pancreas, brain) and many important aspects of human physiological processes (Bakkers, 2011; Gestri et al., 2012); however, it is important to notice that some organs with importance in cancer studies such as lung, prostate or breast are absent. But clearly, zebrafish shares with mammals most of the molecular mechanisms governing embryonic development (Eyal-Giladi, 1997). (iv) Zebrafish embryos are transparent, which combined with a growing battery of fluorescent tissue-specific transgenic lines (Tsang, 2010), and novel advances in imaging capture and analysis (Vogt et al., 2009a; Pardo-Martin et al., 2010), allow the visualization and analysis in vivo of the effects of drugs in groups of cells or whole tissues. In that regard, useful information on genetic tools and gene expression patterns can be found in ZFIN [www.zfin.org]. Moreover, the implementation of the Tol2 transposon technology (Balciunas and Ekker, 2005) has allowed the generation of several gene and enhancer trap reporter screenings, which have produced hundreds of transgenic lines available for the scientific community through public databases: zTRAP [www.kawakami.lab.nig.ac.jp/ztrap/] (Kawakami et al., 2010), Fliptrap [www.fliptrap.org/static/index_new.html] (Trinh le et al., 2011) and ZETRAP 2.0 [www.plover.imcb.a-star.edu.sg/webpages/home.html] (Choo et al., 2006). The full exploitation of these resources could be the basis for new drug screenings relying in fluorescent lines.

Some of the advantages of using zebrafish embryos (small size, high number of progeny, easy drug administration or high-throughput analysis) are comparable to the benefits of using invertebrates. However, zebrafish is a vertebrate, making it a more suitable candidate to fill the gap between "easy, but incomplete" in vitro/in silico screenings and "necessary, but costly and time consuming" mammalian drug screens. Nevertheless, given the complexity of the zebrafish genome, compared with the more compact and simpler invertebrate genomes, the ideal would be to use a mix of both

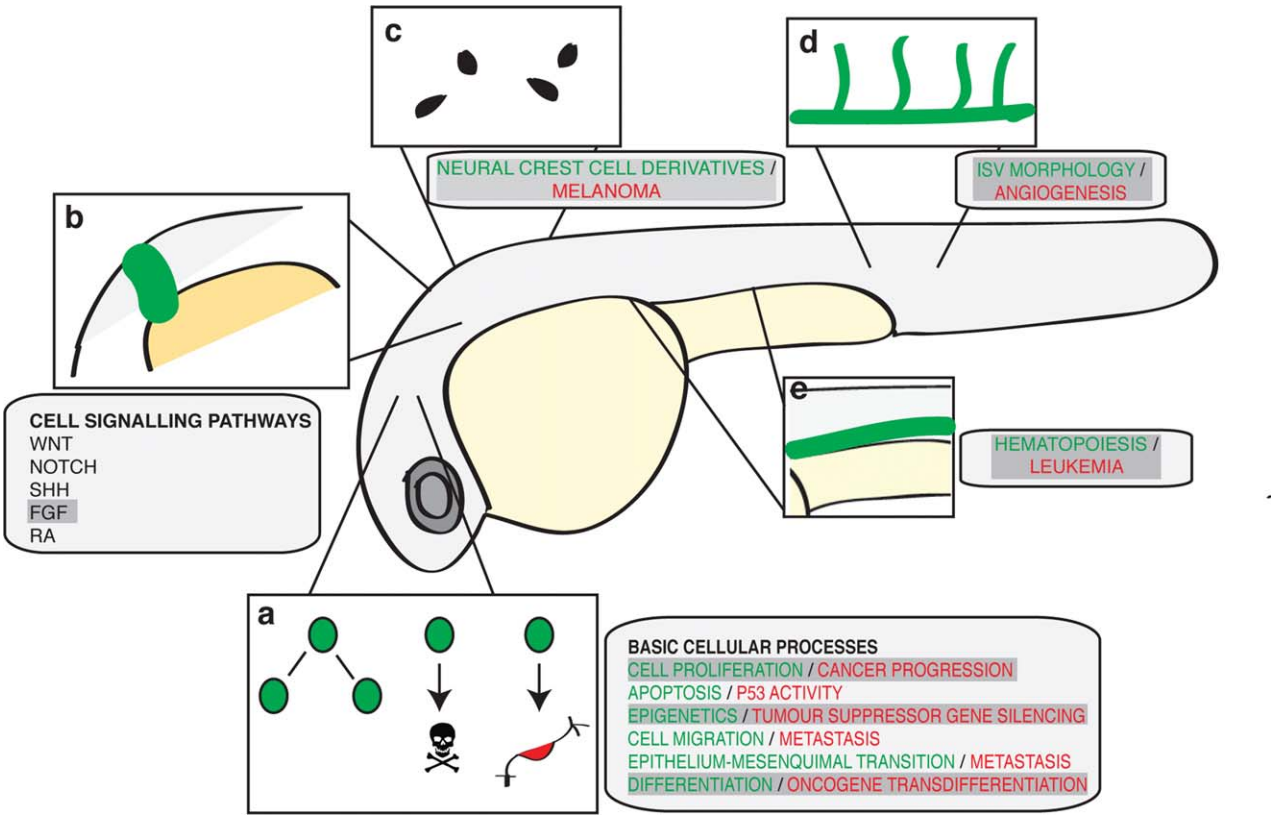

Fig. 1. Embryonic developmental processes that share common cellular mechanisms with cancer can be used as biological models for cancer related drug screenings. Schematic diagram of a zebrafish embryo with anterior to the left. Adjoining box: developmental process (green) and related cancer hallmark (red). Successful drug screens for targeted cancer pathways are shadowed in their corresponding box. **a-e:** Specific embryonic structures or cellular events have been zoomed. **a:** Basic cellular processes that take place during embryonic development. **b:** Key developmental signaling pathways that play important roles during cancer. **c:** Studies in neural crest cells derivatives could be used to model melanoma. **d:** ISV outgrowth models angiogenesis. **e:** Hematopoiesis can be a good model for leukemia.

invertebrates and zebrafish in the drug-screening pipeline before entering studies with mammals.

CANCER DRUG SCREENINGS USING ZEBRAFISH EMBRYOS

The aim of this review is to give an overview on some validated screens that have used zebrafish embryos to identify new compounds with possible therapeutic uses in treating cancer. In addition, we want to propose alternative approaches to some of the described studies: currently available molecular and genetic tools implemented in zebrafish can be further exploited to improve the efficiency and efficacy of the screens and, therefore, save resources.

We will focus on tests performed in embryonic zebrafish as opposed to adults for two main reasons: (i) there are already several good reviews whose principal scope is the use of adult zebrafish as cancer models for drug screening (Stern and Zon, 2003; Feitsma and Cuppen, 2008; Stoletov and Klemke, 2008; Liu and Leach, 2011), and (ii) we fully acknowledge the importance of generating and studying adult zebrafish models that mimic human cancer types, such are melanoma (Patton et al., 2005) or acute lymphoblastic leukemia (ALL) models (Langenau et al., 2003). They provide us with a unique understanding of the tumor microenvironment and its genetic basis. Therefore, adult models are invaluable tools for finding tailor-made treatments. However, we believe that by using zebrafish embryos one can tackle in a faster and easier manner some of the general hallmarks described for cancer progression, such as the control of apoptosis or angiogenesis. In fact, it could be a good strategy to perform some of the initial tests in embryos as a way to narrow down the number of molecules to test later in adults. On the other hand, it is important to consider that some of the screens described below will rely on drug-induced morphological changes that could eventually be due to unspecific teratogenic toxicity. We support the idea that the combination of a controlled drug treatment (high n and accurate timing and drug concentration, with an appropriate training on zebrafish developmental processes) should allow the sorting out of these drugs promoting the searched primary phenotype from those that only promote toxicity.

As mentioned above, most cancer types share common molecular mechanisms to promote transformation and progression. Many of these hallmarks have been studied in detail during zebrafish embryonic development. This expertise has allowed the design of the specific assays described below (see also Table 1).

TABLE 1. Cancer-Related Drug Screenings Using Zebrafish Embryos

Targeted molecules	Detection method	Alternative method	References
Cytotoxic molecules	TUNNEL assay/*p21* and *mdm2* *in situ* hybridization	Ubiquitous SecA5-YFP	(Langheinrich et al., 2002; van Ham et al., 2010)
Cell cycling modulators	Phenotypic observation/pH3 antibody staining	Tissue-specific Fucci Fish	(Moon et al., 2002; Murphey et al., 2006; Sugiyama et al., 2009)
DNA methyltransferase inhibitors	Phenotypic observation/5-methylcytosine antibody staining	Silenced 14xUAS GFP	(Goll et al., 2009; Ceccaldi et al., 2011)
Oncogene trans- differentiation inhibitors	*gata1*, *mpo* and *crestin* in situ hybridization	Hematopoietic and neural crest cell reporter lines	(Long et al., 1997; Yeh et al., 2009)
Anti-angiogenic molecules	ISV patterning showed by Tg[Fli:GFP]	None	(Cross et al., 2003; Tran et al., 2007; White et al., 2011)
Development and cancer signaling pathways modulators	Fluorescence reporters of: Notch, Wnt, TGFβ, Shh, Retinoic Acid and FGF activity	None	(Perz-Edwards et al., 2001; Molina et al., 2007; Parsons et al., 2009; Schwend et al., 2010; Laux et al., 2011; Moro et al., 2012).

Cytotoxic/Pro-apoptotic Molecules

The escape from apoptosis is a necessary hallmark to allow the maintenance and steady growth of tumor cells. *p53* is a well-known tumor suppressor and pro-apoptotic gene (Yonish-Rouach et al., 1991; Rowan et al., 1996), which is absent or down-regulated in more than 50% of human tumors (Hollstein et al., 1991). Of interest, the p53 pathway is conserved between human and zebrafish (Cheng et al., 1997; Gerety and Wilkinson, 2011). Therefore, zebrafish is a suitable organism to find cytotoxic molecules that promote apoptosis and could target cells where p53 has been repressed specifically, such as cancer cells (Pyati et al., 2007).

As a proof of principle, Langheinrich and colleagues showed precisely that. They incubated zebrafish embryos from 24 to 48 hours postfertilization (hpf) with various compounds known to promote apoptosis through p53 activation (Tishler et al., 1993). They detected increase in cell death by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling) assay, and the activation of known transcriptional p53 targets, such as *p21* and *mdm2* (Langheinrich et al., 2002).

Cell Cycle Inhibitors

Cancer cells display sustained proliferation compared with healthy somatic cells, where cell division is tightly controlled. Thus, targeting proliferation is an important way to stop cancer progression. There have been some studies using zebrafish embryos to identify drugs that control cell proliferation. Here, we will highlight two.

In the first study, a screen of a 16k library of small molecules was performed during 16 weeks. They assessed their effect on cell proliferation by incubating zebrafish embryos from 24 to 30 hpf with the given molecules, and determined the number of dividing cells by antibody staining against pH3, which is a known marker of mitosis (Mahadevan et al., 1991). They confirmed their pH3 results by flow cytometry and identified 14 novel compounds that changed the mitotic rate (Murphey et al., 2006).

Many known cell cycle inhibitors are tubulin-binding agents such as Taxol or Vinka alkaloids, some of them in standard chemotherapy use or in clinical phase trials as anticancer drugs (Altmann, 2001). Hence, in the second study described here, Moon and colleagues looked specifically for microtubule binding molecules. They tested more than 100 triazine derivatives. To assay the effect of compounds they took advantage of a known microtubule disruption phenotype: delayed epiboly (Jesuthasan and Stahle, 1997; Jesuthasan, 1998). Thus, the treatment was performed on early zebrafish embryos to allow the assessment of morphological changes after drug treatment. This test allowed them to isolate 3 novel compounds with microtubule binding activity (Moon et al., 2002).

DNA Methyltransferases Inhibitors (DNMTi)

Another hallmark in carcinogenesis is the silencing of tumor suppressor genes. One molecular mechanism to achieve silencing is through epigenetics, which is implicated in many biological processes, such are embryonic development and memory consolidation (Kiefer, 2007; Day and Sweatt, 2011). Of interest, epigenetic changes have also been shown to be involved in cell transformation. Particularly, the activity of methyltransferase enzymes promote the silencing of many tumor suppressor genes in cancer cells by hyper-methylation of their promoters (Portela and Esteller, 2010). In agreement with this, there

are several DMNTi drugs in clinical trials (Yoo and Jones, 2006).

So far, only one drug screening that uses zebrafish to search for molecules preventing methylation, as a way to find tumor suppressor derepressors during cancer progression, has been described. A combination of morphological features (gastrulation and somite and tail defects due to perturbed methylation; Martin et al., 1999) and methyltransferase activity analysis (changes in 5-methylcytosine antibody staining) have been used. Using these known phenotypes, they could confirm several hits that inhibited methyltransferase enzymatic activity (Ceccaldi et al., 2011).

Anti-angiogenic Molecules

Angiogenesis is a tightly regulated physiological process needed, during normal embryonic development, to provide oxygen and nutrients to growing tissues. In common with that, tumor cells require angiogenesis as a necessary step to achieve growth and metastatic potential (Carmeliet and Jain, 2011). Therefore, drugs that affect this process are attractive candidates in preventing the growth and spread of transformed cells (Cao, 2008).

The goals of the zebrafish drug screenings exemplified here were to search for molecules that prevent angiogenesis during development as a mean to find inhibitors of tumor vascularization. Several articles share the method of phenotype assessment based on the absence or defective development of Inter-Segmental Vessels (ISV; Chan et al., 2002; Cross et al., 2003; Tran et al., 2007; Crawford et al., 2011; Camus et al., 2012; Radi et al., 2012). These vessels are visualized either by fluorescent microangiography (Weinstein et al., 1995) or by using the Tg[Fli:GFP] fish line in which green fluorescent protein (GFP) is expressed in the embryonic vasculature (Lawson and Weinstein, 2002).

The latter method is a clear example of how the zebrafish strengths (transparency, size, and fluorescent reporters) combined with cutting-edge imaging and algorithms for automated image processing, can lead to fast and cheap quantitative and automated high-throughput drug screening. Tran and colleagues exemplified them: they screened a 1280 molecule library (LOPAC1280) and they got three hits, two of them were known anti-angiogenic molecules (SU4312 and AG1478), while the third one (IRO) was a novel anti-angiogenic compound (Tran et al., 2007).

Oncogene Trans-differentiation Inhibitors

An additional cancer hallmark is the abnormal activation of oncogenes. In that regard, there are two relevant examples that use zebrafish to find molecules targeting this issue.

The first screening focuses on drugs that inhibit the oncogene AML1-ETO, which overexpression promotes acute myelogenous leukemia (AML). Oncogenes can promote leukemia by deregulating both proliferation and differentiation of hematopoietic cells (Lam and Zhang, 2012). Current treatments for leukemia focus primarily on proliferation, but a large percentage of patients experience recurrence after remission (Redaelli et al., 2004). This suggests that targeting cell proliferation may not be sufficient for eradicating leukemia. Thus, therapies that could reverse the differentiation effects of oncogenes in leukemic stem cells (LSC) are promising complements to cell proliferative repressors or cytotoxic agents. Accordingly, the induced overexpression in the zebrafish hematopoietic lineage of the human oncogene AML1-ETO converts erythropoietic cells to granulopoietic cells. This anomalous trans-differentiation was confirmed by transcriptional changes in *gata1* and *mpo*, known markers of these distinct cell populations. Hence, the overexpression of the human AML1-ETO oncogene in zebrafish embryos phenocopies one crucial step in the initiation and progression of AML in human patients (Yamasaki et al., 1995; Yeh et al., 2008). Based on this information, the same authors designed a high-throughput drug screen to look for molecules that prevented the abnormal LSC differentiation as a mean to find leukemia differentiation repressors. To do so, they sought molecules that reverted the transcriptional changes in *gata1* and *mpo* after the induced overexpression of AML1-ETO, and 15 hits of two thousand screened molecules were identified (Yeh et al., 2009).

The second screening described here looked for drugs that target melanoma. The BRAF oncogene is the most frequently mutated gene in human melanomas (Davies et al., 2002). In agreement to that, the screening was based in several observations made on ^{p53} mutants where the human oncogene BRAF(V600E) was ectopically expressed in the melanocyte lineage (White et al., 2011). The authors found that embryonic neural crest cells from those animals failed to terminally differentiate; adults from that genetic background developed melanoma within 4–12 months; and last, the transcriptional profile of those induced melanomas was very similar to that of wild-type neural crest precursor cells. All these observations suggested that adult melanomas might derive from neural crest progenitors that were kept undifferentiated after embryogenesis. Therefore, drugs suppressing neural crest development might also suppress melanoma appearance and growth. They treated wild-type embryos with a 2000 molecules library looking at the absence of neural crest derivatives by different means: in situ hybridization to detect *crestin* (an early marker of neural crest cells; Rubinstein et al., 2000), direct visualization of pigmented melanocytes, and fluorescence detection on two different neural crest cells derivatives reporters, one that marks melanocyte progenitors (Tg[mitf:GFP]) and another for Schwann cells (Tg[mbp:mCherry]). They found that NSC210627, a chemical inhibitor of dihydroorotate dehydrogenase (DHODH), suppresses both neural crest development and melanoma growth (White et al., 2011).

Both assays are nice examples of zebrafish embryos as good animal models for drug screening. First, they show the strengths of zebrafish transgenesis by using different kinds of transgenic lines to ectopically express oncogenes and to report the expression of different genes. And second, they illustrate that zebrafish embryos could also be appropriate for modeling specific diseases, such as AML or

melanoma, and not only for targeting general cancer hallmarks.

Development and Cancer Signaling Pathways Modulators

Signaling pathways are essential molecular mechanisms controlling cell communication and coordination during development. When deregulated they can also contribute to cancer initiation and progression (Martinez-Arias, 2001; Massague, 2008; Batlle and Wilkinson, 2012). Thus, they are putative cancer therapeutic targets.

Due to important advances, transgenesis in zebrafish has been made extremely efficient (Kawakami et al., 2004; Balciunas et al., 2006) and allowed the generation of an enormous battery of tools, ranging from reporters to gain-of-function tools (Halpern et al., 2008; Distel et al., 2009). Not surprisingly, this technology has also permitted the creation of custom-made activity reporters for the principal signaling pathways involved in both development and cancer such as: Notch (Parsons et al., 2009), Wnt (Moro et al., 2012), TGFβ (Laux et al., 2011), Shh (Schwend et al., 2010), Retinoic Acid (Perz-Edwards et al., 2001), or FGF (Molina et al., 2007) pathways. All these tools have been validated by the expression pattern of the reporter when compared with the expression of target genes. And significantly, their specific activity has been confirmed by confronting them with known chemical inhibitors that target the respective signaling pathways they report. This latter fact is the proof of principle needed to show that these transgenic lines are suitable tools for chemical screenings.

One of the best examples of this strategy is the work performed by the Tsang laboratory in their search for molecules that modulate FGF signaling (Molina et al., 2007, 2009; Vogt et al., 2009b; Saydmohammed et al., 2011). They used a sensible reporter transgenic line Tg[Dusp6:EGFP] and implemented with custom cognition network algorithms, the use of state of the art equipment that detect, capture, and analyze the levels of GFP in single embryos arrayed in multiwell plates. This methodology allowed them to screen vast chemical libraries to find molecules that specifically regulate FGF signaling. Of interest, the knowledge acquired on FGF function during development has let them to confirm their hits also by the morphological defects they promote in zebrafish embryos (i.e., dorsalization).

Remarkably, signaling pathway reporter strategies can have a broader scope than simply finding cancer therapies, because they can also be used to tackle other diseases, such as diabetes. In this regard, Rovira et al. showed how the zebrafish Notch reporter line they previously reported (Parsons et al., 2009) was used to screen a drug library in search for molecules promoting the differentiation of insulin-producing β-cells in vivo, as a way to generate new insulin producing cells in diabetic patients (Rovira et al., 2011).

COMPLEMENTARY DRUG SCREENING STRATEGIES

As we have emphasized before, the future of using zebrafish embryos in drug screening will rely on the further development and usage of fluorescent reporter lines. However, there are some drawbacks to this type of screens: (i) some fluorescent lines do not recapitulate the whole pattern they intend or display fluorescence perdurance that can affect the final readout. Therefore, as part of the screening strategy, it is important to carefully adjust the incubation time and the spatial reading parameters. (ii) Multiwell high-throughput fluorescent imaging has some complications, mainly the fact that the orientation and centering of every embryo in its given well is stochastic. Taking into account that manual orientation is not feasible for high-throughput aims, alternatives need to be found to gain full optical access on every treated embryo. To this end, available multiwell fluorescent readers (Tecan Infinite M1000, ImageXpress Ultra) can be adapted to zebrafish high-throughput imaging upon adjustment of some parameters (liquid volume, well shape, etc.; Vogt et al., 2010; Walker et al., 2012). An alternative method called Vertebrate Automated Screening Technology (VAST) has been developed. VAST couples a confocal microscope with a system of valves and capillaries that allows the automated extraction of embryos from their specific well. Every embryo is then positioned inside a rotating and transparent capillary to be imaged under the confocal microscope in a way its orientation is carefully controlled (Pardo-Martin et al., 2010; Yanik et al., 2011; Chang et al., 2012). Both approaches (multiwell fluorescent readers and VAST) can be combined with image recognition algorithms that detect and interpret changes in fluorescence levels, thus allowing for high-throughput fluorescent quantification of embryos arrayed in multiwell plates (Fig. 2).

We have already drawn attention to a few drug screen assays (angiogenesis, FGF signaling and melanoma assays) that use fluorescent transgenic lines to measure drug-inducing changes in gene activity or cell differentiation. Some available transgenic lines, combined with any of the approaches explained above for imaging fluorescence, could extend this methodology to the other screens we have described earlier.

Cytotoxic/Pro-Apoptotic Molecules

Van Ham et al. demonstrated that the ubiquitous overexpression of the fusion protein SecA5-YFP (secreted Annexin-5 fused to YFP) specifically marked apoptotic cells in vivo without causing any morphological defects during development. To prove this, they promoted apoptosis by chemical and ultraviolet radiation treatment and compared the output of this genetic tool with traditional methods of cell death detection. They concluded with high reliability that SecA5-YFP was detectable only in apoptotic cells (van Ham et al., 2010). Given that the visual output of this tool is in vivo fluorescence, its use could save time in the processing of embryos, presenting itself as a powerful alternative to the expensive reagent kits used to detect apoptotic cells.

Cell Cycle Inhibitors

The Fucci fish is a transgenic line that allows the detection of the different cell cycle states by the expression

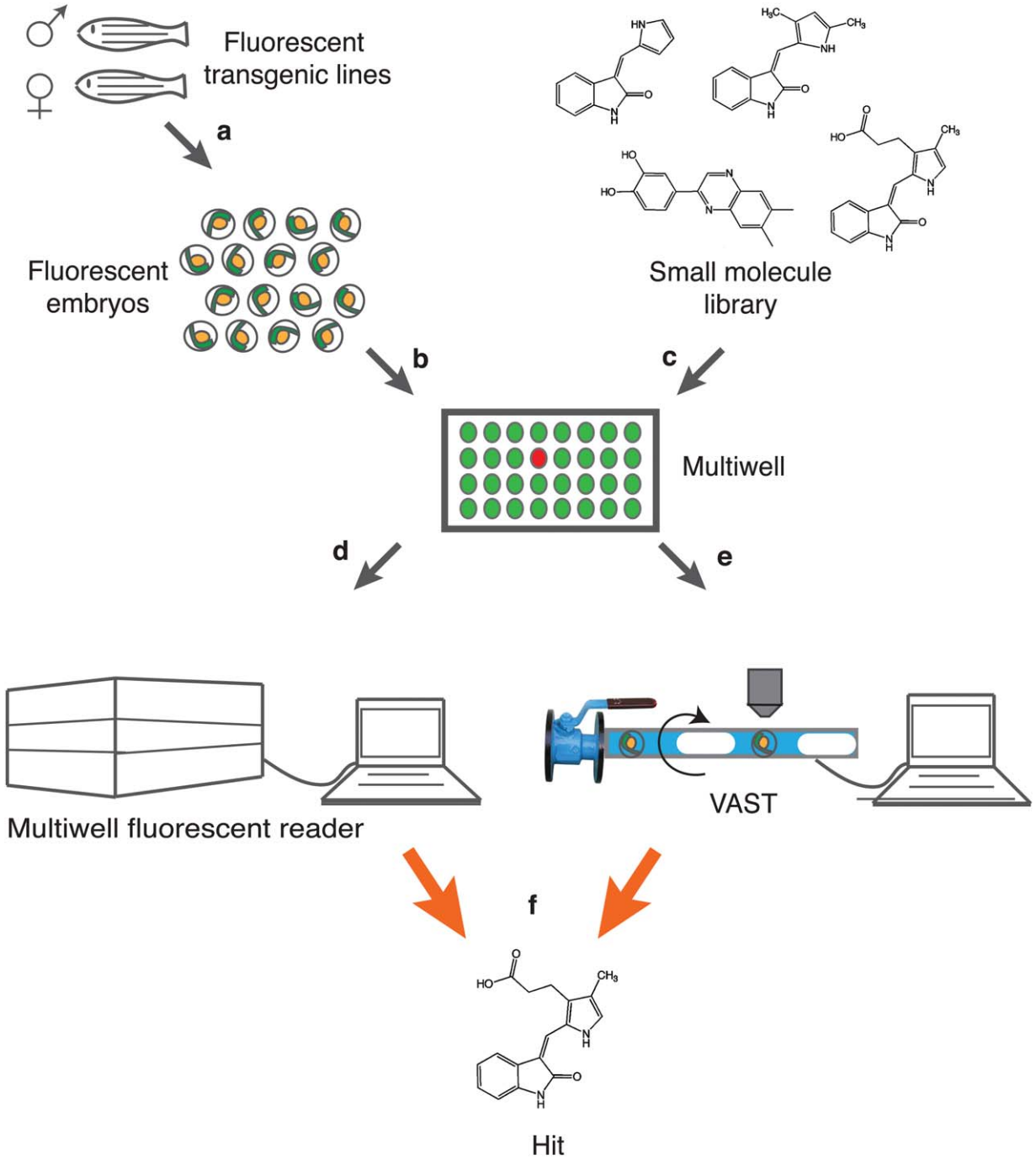

Fig. 2. High-throughput drug screening strategy using fluorescent zebrafish embryos. The pipeline would be the following: transgenic zebrafish adults are crossed (**a**), embryos are collected and distributed into multiwell plates in equal numbers (**b**). **c:** Single compounds or different combinations of them are added to the water of each well where zebrafish embryos have been placed, and embryos are incubated the desired time. Then either plates are placed: in a multiwell fluorescent reader and changes in fluorescence extension and/or intensity are measured by the use of specific image recognition algorithms (**d**), or in the VAST system (**e**), which will extract automatically every embryo from each well to place it in a transparent and rotating capillary under a confocal microscope. The embryos are moved through the system by a series of valves that generates air bubbles to keep them apart from each other. Changes in fluorescence extension and/or intensity are measured by the use of specific image recognition algorithms. **f:** The new compound is identified and selected for further research.

of distinct fluorophors (Sugiyama et al., 2009). The strength of this tool lies in the rapid turnover of green to red fluorescence fusion proteins when the cell changes from a cycling to a noncycling state (Sakaue-Sawano et al., 2008). This feature makes Fucci a trustworthy in vivo reporter for assessing the number of proliferating vs. nonproliferating cells, which could be detected by differences in the green/red fluorescence intensity ratio. However, to avoid statistical perturbations coming from tissues that are not proliferating when the drug is

administrated, it would be advisable to focus on a single proliferating tissue at the given developmental stage to perform the measurements. Another option would be to develop tissue specific Fucci fishes, i.e., Fucci liver. Fucci could be an invaluable alternative to pH3 antibody staining or bromodeoxyuridine (BrdU) labeling for screening molecules affecting the cell cycle.

DNA Methyltransferases Inhibitors (DNMTi)

It was shown that transgenic lines carrying 14xUAS are subjected to epigenetic changes, because their multiple repeated UAS sequences undergo methylation, hence promoting their progressive silencing after only few generations (Goll et al., 2009). This interesting observation, together with the isolation of a methylated and silenced Tg[14xUAS:GFP] line, could be the basis to design screens targeting the reversion of DNA silencing by repressive methylation.

The proposed strategy would be to cross a transgenic line carrying a 14xUAS promoter driving a fluorescent reporter, i.e., Tg[14xUAS:GFP], that has already been silenced by methylation, with a Gal4 transgenic line expressing Gal4 under the control of tissue specific regulatory elements with high reliability. Their progeny, in which the reporter will be activated only in the territories where Gal4 is present, would be incubated with chemical compounds. No fluorescence should be observed in control embryos, however if any of the tested molecules represses methylation, embryos should display fluorescence only in the tissue where the Gal4 is expressed. This screening strategy should show easy-to-detect fluorescence changes in vivo and it could, therefore, be a suitable alternative to the more tiresome and expensive phenotypic assessment and antibody staining described before (Ceccaldi et al., 2011). It is important to remark that a more sophisticated UAS promoter (4xUASnr) has been recently developed, precisely aiming to avoid silencing by methylation (Akitake et al., 2011). So, we suggest that only 14xUAS sequences will suit the proposed strategy.

Oncogene Trans-differentiation Inhibitors

Some of the methodologies previously described involve the detection of transcription by in situ hybridization. This step could be substituted by using specific reporter lines that label the same intended tissues, i.e., Tg[gata1:GFP] (Long et al., 1997). In addition, it could be advisable to check regularly the earlier described reporter databases to find other transgenic reporters for the targeted lineage. In fact, in the recently described melanoma assay (White et al., 2011), the authors made already use of fluorescent reporter lines for neural crest derivatives such Tg[mbp:mCherry] and Tg[mitf:GFP], exemplifying the usefulness in drug screenings of zebrafish transgenic lines driving reporters in specific tissues or cell types.

CONCLUDING REMARKS

We have emphasized the advantages, in terms of time, cost and efficacy, of including zebrafish embryos in the pipeline for high-throughput preclinical studies. On one hand, by being a whole vertebrate organism, it provides deeper and more complete biological information than some in vitro and invertebrate drug screening. On the other hand, the small size, transparency and growing set of transgenic lines, makes it an ideal complement to later mammal screenings, and could allow the filtering of a large number of molecules before they reach that stage in the pipeline.

This review aimed to highlight the benefits of using zebrafish embryos when the goal is the identification of molecules targeting common hallmarks of most cancer types: sustained cell proliferation, absence of apoptosis, or increased angiogenesis (Hanahan and Weinberg, 2011), vs. the study of more complex phenotypes that could be understood by using adult cancer models. However, to achieve the full potential of this animal model, more work has to be done to design screening for drugs impacting on other aspects not treated here and also related to cancer, i.e.: screens for drugs that affect ephithelial–mesenchimal transition (EMT), a key biological process controlling cell migration and delamination common to both development and metastasis (Yang and Weinberg, 2008). Or screens for compounds that could regulate telomerase activity, which in zebrafish is known to be important for ageing and regeneration (Anchelin et al., 2011), and it is also up-regulated in cancer cells to avoid cellular senescence and promote cell immortality (Finkel et al., 2007).

To summarize, all the screens described here are heavily based on extensive basic research studies, many of them focused on the understanding of the molecular mechanisms governing embryonic development, rather than understanding or curing cancer. We support the notion that the efforts made by the international scientific community in generating new tools, and understanding the biology of development, cancer and the common ground between both biological processes must be further explored for the continued successful design of cheap, fast, and reliable chemical tests. This will improve the discovery/identification of new molecules that promote a better quality of life.

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