Original Research

Targeting the Wnt pathway in zebrafish as a screening method to identify novel therapeutic compounds

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Abstract

Activating mutations in the Wnt signaling pathway account for the initiation of greater than 90% of all colorectal cancers and this pathway has been implicated in numerous other diseases. Therefore, identifying small molecule inhibitors of this pathway is of critical importance towards identifying clinically relevant drugs. Numerous screens have been employed to identify therapeutic reagents, but none have made it to advanced clinical trials, suggesting that traditional screening methods are ineffective at identifying clinically relevant targets. Here, we describe a novel *in vivo* screen to identify small molecule inhibitors of the Wnt pathway. Specifically, treatment of zebrafish embryos with LiCl inhibits GSK3 kinase function, resulting in hyperactivation of the signaling pathway and an eyeless phenotype at 1 day post fertilization. Using the small molecule XAV939, a known inhibitor of Wnt signaling, we rescued the LiCl induced eyeless phenotype, confirming efficacy of the screen. We next tested our assay with 400 known small molecule kinase inhibitors, none of which should inhibit Wnt signaling below the level of GSK3 based on their known targets. Accordingly, none of these small molecules rescued the eyeless phenotype, which demonstrates the stringency of the assay. However, several of these small molecule kinase inhibitors did generate a non-Wnt phenotype in accordance with the kinase they targeted. Therefore, combining the efficacy, sensitivity, and stringency of this preliminary screen, this model will provide an alternative to the traditional *in vitro* screen, generating potentially clinical relevant drugs in a rapid and cost-effective way.

Keywords: Cancer, zebrafish, small molecules, Wnt signaling, LiCl, eyeless

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with over 1.2 million new diagnoses each year. What makes CRC unique among cancers is its dependence on a single signaling pathway. A recent comprehensive study reports that greater than 90% of all colon cancers are caused by activating mutations in genes within the Wnt signaling pathway.[1–3] In parallel research, it is now well established that cancer stem cells (CSC) are the source for both the initiating tumor and recurrent malignancies that are therapy-resistant.[4–7] Wnt signaling drives CSCs making this pathway the definitive signaling cascade that drives tumorgenesis in colon and many other cancers.[8–16] Thus, the Wnt pathway in CSC is an excellent therapeutic target for a myriad of human cancers, particularly of the colon.[5,9,10,17] To this end, there have been numerous small molecule screens for Wnt inhibitors, but these typically utilized cell lines with an artificial reporter. While such screens have had some success in identifying Wnt inhibitors, none have advanced to stage III clinical trials.

To further our understanding of CRC development, we have developed an innovative strategy to use the *in vivo* stem cells of the zebrafish blastula in a semi-high-throughput screen for small molecules that target the Wnt pathway. We take advantage of the highly conserved Wnt signaling pathway,[18] which is active in the stem cells of the early zebrafish blastula stage embryo.[19–22] Zebrafish are rapidly becoming the preferred model for whole animal toxicity screens in the pharmaceutical industry[23,24] as they are a well-established genetic tool for understanding the function of human genes in development and disease.[25–27]

Wnt signaling pathway

In its resting state, in which there is no Wnt ligand (Figure 1(a)), an intracellular, constitutively active, destruction complex consisting of axin, adenomatous polyposis coli (APC) (both scaffolding proteins) and GSK3 (a kinase) binds

to and phosphorylates β-catenin, resulting in ubiquitin-mediated degradation of β-catenin. The presence of Wnt ligand binding to its receptors initiates activation of the pathway (Figure 1(b)), resulting in deactivation of the destruction complex.[28–30] This deactivation results in accumulation of cytoplasmic β-catenin, which translocates into the nucleus where it interacts with TCF/LEF transcription factors to activate transcription of target genes. Wnt signaling transcriptional targets are context-dependent and can be involved in proliferation, migration, and differentiation.[30–34] However, in vertebrates there appears to be two potentially universal and obligate targets of Wnt signaling: Nkd1 and Axin2.[35–40]

Wnt signaling in early development

Wnt signaling is one of the first pathways to be activated upon fertilization of the egg, patterning the dorsal-ventral axis and subsequently the anterior-posterior neuraxis.[41,42] Mutants in this pathway underscore the importance of Wnt signaling in development: mutations in the central signaling component β-catenin results in the absence of dorsal organizer formation and defects in the neuroectoderm.[43] In contrast, hyperactivation of the pathway is a consequence of mutations in the Wnt antagonists *masterblind* (*axin*) and *headless* (*tcf7l1*) and, as their names suggest, these embryos develop without eyes.[44–46] Consistent with these phenotypes, ectopic activation of Wnt signaling also results in an eyeless embryo.[20,22,47,48]

Wnt signaling in cancer

In the majority of CRCs, mutations in APC, Axin2 or β-catenin itself, prevent the destruction complex from degrading β-catenin.[1] This leads to activation of Wnt target genes in the absence of a Wnt ligand (Figure 1(c)). The current hypothesis is that aberrant Wnt signaling in the colonic crypt stem cells leads to the formation of adenomas. Mutations in other signaling pathways, such as EGF or K-RAS, transition these adenomas into adenocarcinomas and these tumors consist of a heterogeneous population of cells including differentiated cells and CSC. It is believed that CSC within the adenoma and tumor are refractory to chemotherapy and are the source for recurrent tumor formation. Thus, if therapies could be targeted specifically to the Wnt pathway in these CSC, it would halt the formation of the adenomas and/or their progression into more malignant tumors.[49,50]

Previous Wnt antagonist screens

There have been several screens over the past eight years that have identified inhibitors acting at different levels in the Wnt signaling cascade (Figure 1(c)).[51] Three screens identified small molecules that inhibit the β-catenin-TCF activation of Wnt target genes.[52–54] In two independent screens, two different molecules were identified (IWR-1 and XAV939) that inhibit tankyrase function, leading to increased stability of Axin and thus increased degradation of β-catenin.[55,56] What is common to these *in vitro* screens is

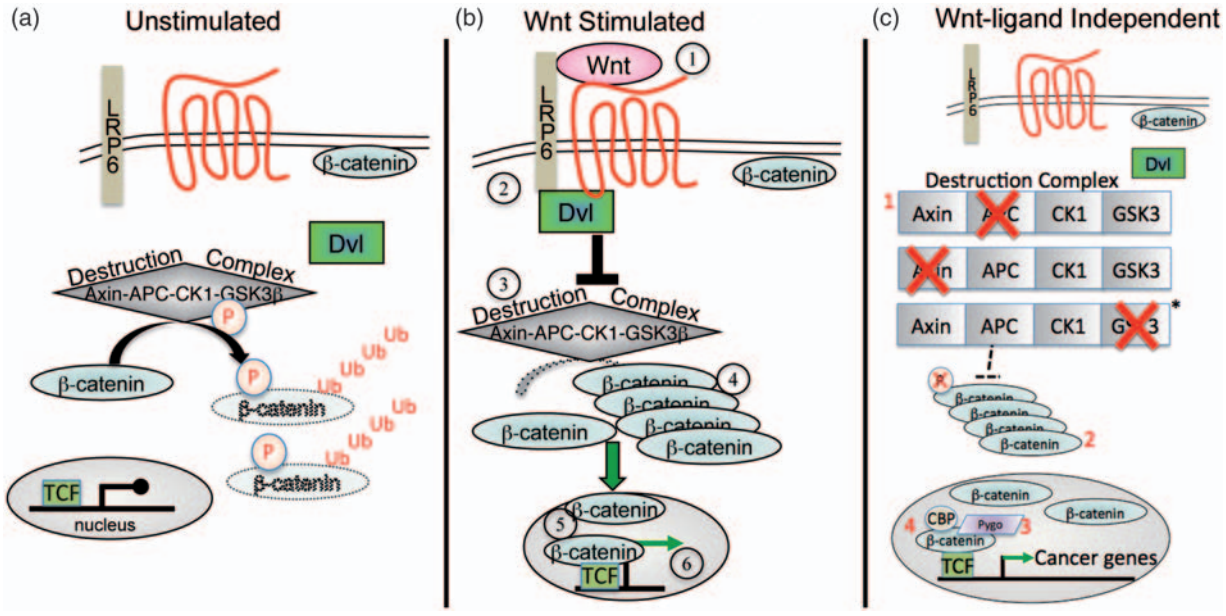

Figure 1 Wnt signaling cascade. In its resting, unstimulated state, in which there is no Wnt ligand (a), an intracellular, constitutively active, destruction complex consisting of Axin, APC, GSK3, and CK1 binds to and phosphorylates β-catenin, resulting in ubiquitin-mediated degradation of β-catenin. (b) The presence of Wnt ligand binding to its receptors, Frizzled and LRP6 (1), initiates activation of the pathway, resulting in activation of the scaffolding protein Dvl (2). Activated Dvl inhibits the destruction complex (3). This results in accumulation of cytoplasmic β-catenin (4), which translocates into the nucleus (5) to activate transcription of target genes (6). (c) In the vast majority of colon cancers mutations are found in the APC gene, disabling the destruction complex. This results in accumulation of cytoplasmic β-catenin and translocation into the nucleus where it maintains cells in a cancer stem cell state. Other mutations, such as in Axin, or in the phosphorylation sites of β-catenin, also result in accumulation of β-catenin and signaling.* Mutations in GSK3 or inhibition of GSK3 have not been observed in colorectal cancers but are included here because inhibiting GSK3 with LiCl mimics the molecular events that occur in colon cancer. The numbers refer to *in vitro* screens that have identified small molecules that inhibit Wnt signaling at various positions within the pathway. Numbers 1–4 refer to the following references: 1: [55,59]; 2: [53]; 3: [60]; 4: [52,54,58,64]. (A color version of this figure is available in the online journal.)

the use of an artificial GFP or luciferase reporter assay in cell lines such as HEK293T, which have 'normal' Wnt signaling not reflective of the oncogenic signaling that occurs in cancer.[52–55,57–59] In one particularly novel screen that diverges from the typical cell culture and reporter scheme, *Xenopus* cellular extract was used to measure the ratio of β-catenin to Axin in the presence of different small molecules. This screen revealed that the FDA-approved drug pyrvinium (used to treat pinworm infection) was effective at reducing the activation of Wnt target genes.[60] Importantly, other groups screened this same library and identified other Wnt antagonist compounds, but not pyrvinium.[59] Thus, different models clearly have different responses to the same small molecules.

It should be noted that several of these screens have included *Xenopus* and/or zebrafish models to validate putative molecules, which has greatly informed the efficacy, specificity, and toxicity of these drugs.[53–57,60] *Xenopus* and zebrafish embryos are amenable to these tests because of the high conservation of the Wnt pathway and the known effects of Wnt signaling in development.[55,56] Recently, Hao *et al*.[61] have used zebrafish dorsoventral patterning as a platform to screen for novel small molecules that target the Wnt pathway. However, that screen was not designed specifically to identify Wnt inhibitors. Here, we describe a novel strategy for specifically identifying small molecules that counteract aberrant Wnt signal activation in zebrafish embryos.

Materials and methods

Approximately 600 healthy wild-type embryos at mid-blastula stage were placed in a mesh basket to facilitate transfer between solutions. At 4.5 h post fertilization (dome – 30% epiboly stage), embryos were immersed in a solution of 300 mM LiCl (Sigma) for exactly 10 min at room temperature with agitation approximately every minute. Embryos were washed three consecutive times, 20 s each, in room temperature water and then incubated at 28.5°C for 15 min. Necrotic embryos were removed and five to six

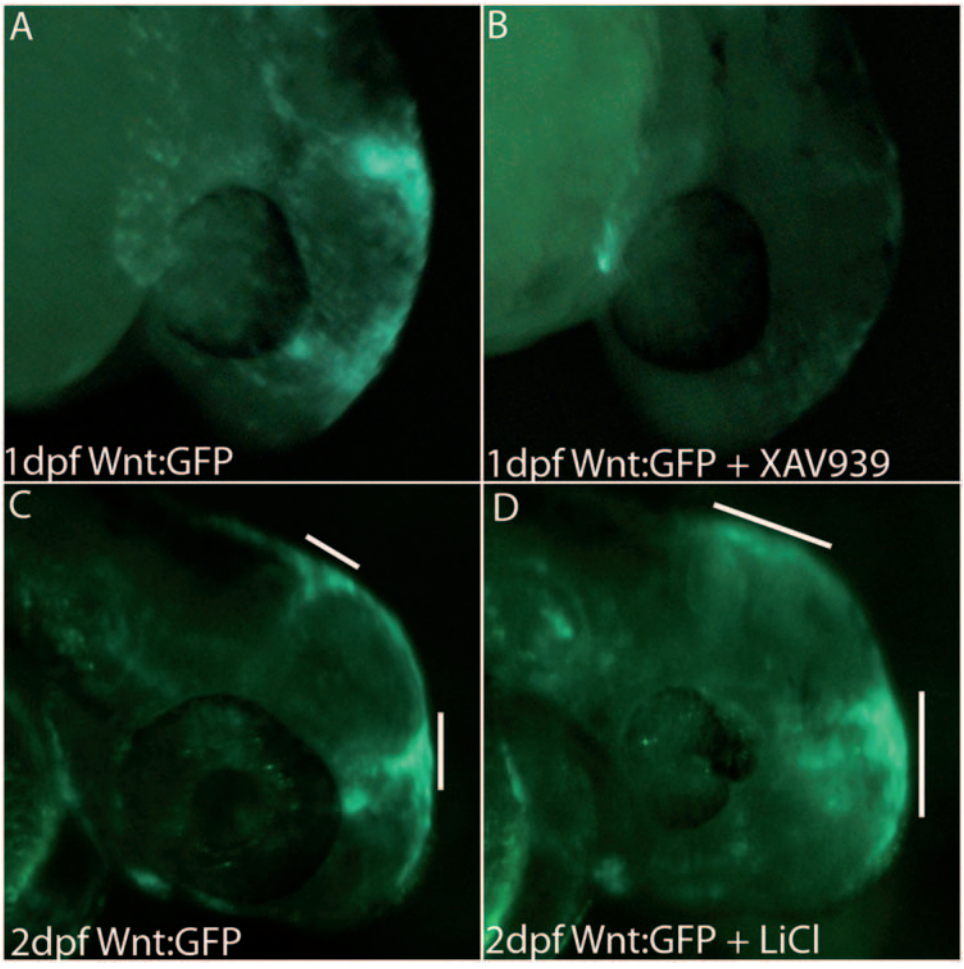

Figure 2 Response of Wnt: GFP transgenic zebrafish to agonists and antagonists to Wnt signaling. Wnt reporter transgenic zebrafish display GFP in known Wnt responsive regions of the developing nervous system at 1 dpf (a) and 2 dpf (c). (b) Treatment with 1 μM of XAV939 dramatically reduces the expression of the Wnt: GFP reporter. (d) Treatment with 300 mM LiCl results in expanded Wnt signaling domains in the developing nervous system at 2 dpf (bottom; compare white bars). Note the dramatically reduced eye in LiCl-treated embryos. This phenotype is critically dependent on the LiCl treatment procedure. Incomplete mixing of embryos in LiCl will result in variable expressivity and penetrance of the eyeless phenotype as seen here, resulting in a smaller, but not absent, eye. (A color version of this figure is available in the online journal.)

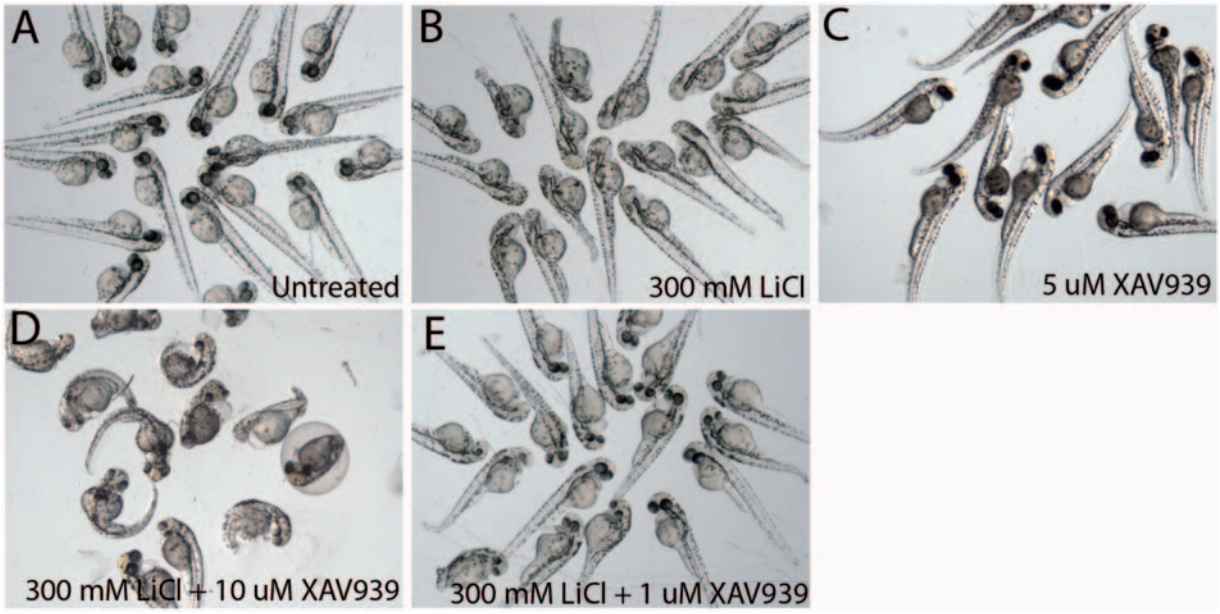

Figure 3 XAV939 rescue of LiCl treatment. (a) Untreated embryos at 2 dpf. (b) LiCl treatment eliminates the eye in 100% of embryos, but otherwise development proceeds relatively normally. Treatment of embryos with 5 μM of XAV939 has no gross effect on zebrafish development (c). Treatment of zebrafish first with LiCl followed by 10 μM of XAV939 results in rescue of the eye but also other defects such as heart edema and truncated/twisted axis development (d). Reducing the concentration of XAV939 to 1 μM XAV939 to LiCl-treated embryos suppresses the eyeless phenotype in 95% of LiCl-treated embryos (e). Zebrafish are 2 dpf for clarity of the eye phenotype

embryos were placed in each well of a 96-well plate and excess water removed.[62] Using a multi-channel pipettor, 160 uL of water was added to each well of the 96-well plate followed by 40 uL of 50 μM compound containing 5% DMSO. Note that column 1 of the compound plate contains 5 μM XAV939 (Reagents Direct) in 5% DMSO and column 12 contains just 5% DMSO in water. Therefore, each 96-well plate contains 80 testable compounds. Compounds were a gift from the Ontario Institute for Cancer Research (OICR) and contain small molecule inhibitors of known kinases.[63] The final concentration of all small molecules (with the exception of XAV939) was 10 μM in 1% DMSO, consistent with *in vitro* screens. Plates were incubated at 28.5°C for a further 20 h and scored visually for the presence or absence of an eye and general toxicity. All eye phenotypes were compared to controls consisting of untreated (negative control), LiCl treated ($n \approx 45$ positive control) and LiCl plus 1.0 μM XAV939 ($n \approx 45$ rescue control).

Results and discussion

This screen takes advantage of the known effects of ectopic Wnt activation on zebrafish development. Ectopic activation of Wnt signaling in zebrafish blastula stem cells with transient LiCl treatment results in a robust eyeless phenotype at 1 day post fertilization (dpf) (Figure 3(b)).[65] LiCl inhibits GSK3 function, which in turn deactivates the destruction complex, resulting in accumulation of β-catenin and activation of its transcriptional targets (Figure 2). This time-limited over-activation of Wnt signaling effectively reprograms the late blastula stage stem cells to become posterior neural tissue at the expense of anterior neural tissue during early gastrulation.[20,22] The net result is an eyeless zebrafish at 1 dpf. Importantly, the effect of LiCl on GSK3 function mimics mutations found in CRC (Figure 1(c)). Thus, an eyeless phenotype in zebrafish is similar at the molecular level to the initiation of CRC in humans. Therefore, if we can identify inhibitors of the eyeless phenotype, that is, rescue the LiCl induced eyeless phenotype, then these compounds represent excellent therapeutic targets.

First, we determined whether a known small molecule Wnt inhibitor XAV939 (see above)[55] was sufficient to rescue the LiCl-induced eyeless phenotype. Treatment with LiCl alone results in 100% of the zebrafish embryos being completely eyeless at 1 dpf (Figure 3(b)). Treatment with 1–10 μM XAV939 alone has no obvious phenotype (Figure 3(c) and not shown). Embryos first treated with LiCl and then with 10.0 μM of XAV939 resulted in ~95% of the embryos with obvious eyes, consistent with published reports on XAV939.[55] However, these embryos also had other developmental defects, such as heart edema and a distorted axis (Figure 3(d)). Reducing the dose of XAV939 to 1 μM also resulted in ~95% of the embryos with obvious eyes but without the other defects (Figure 3(e)). Therefore, we used 1 μM of XAV939 as a control for the study. These results show that the addition of a small molecule can rescue the eyes in zebrafish treated with LiCl, validating the viability of this screen.

We next tested for specificity of the screen using a small molecule library of 400 known kinase inhibitors from OICR.[63] Of these 400, none rescued the eyeless phenotype. It is important to note that the targets of these kinase inhibitors are known, such as Gleevac, which targets Brc-Abl,

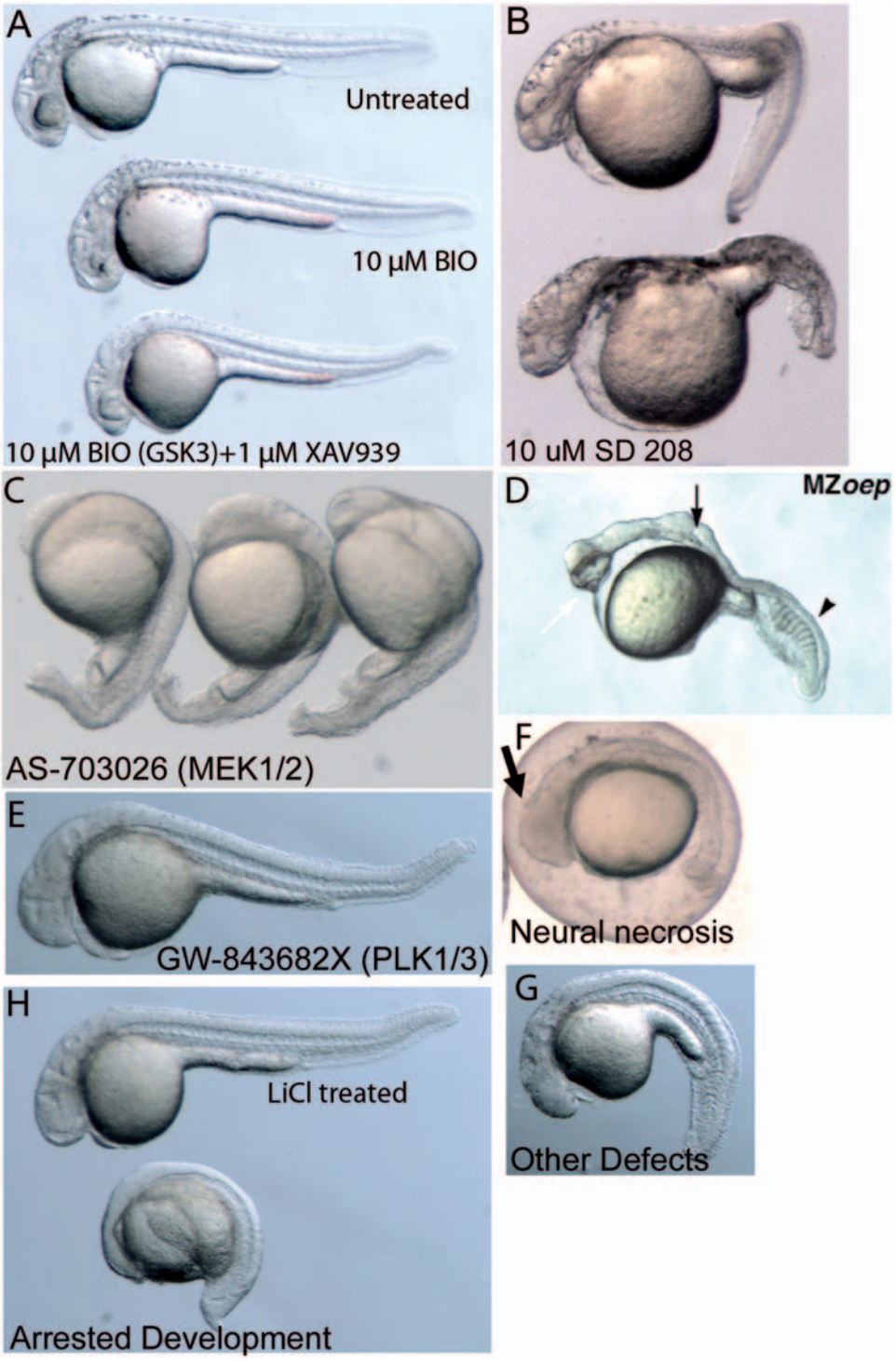

Figure 4 Small molecule kinase inhibitors result in predictable and novel phenotypes. The GSK3 inhibitor BIO also induces an eyeless phenotype in zebrafish, which can be rescued by 1 μM XAV939 (a). Inhibition of TFGβ signaling with SD 208 (b) results in a classical maternal-zygotic one-eyed-pinhead (MZoep) phenotype (d, reprinted by permission from Bamford et al.[69]). For clarity these embryos were not treated with LiCl. Embryos first treated with LiCl and then SD208 have a similar phenotype, but lack eyes altogether (not shown). (c) The MEK1/2 inhibitor AS-703026 results in truncated tail development, while inhibition of PLK1/3 by GW-843682X results in dysmorphology of the developing central nervous system (e). Other phenotypes observed in the screen included neural necrosis (f) arrested development (h), and other general defects (g). Arrow in (f) identifies the neural necrosis. (A color version of this figure is available in the online journal)

PDGFR and c-KIT kinases, and that none of the compounds are expected to rescue the LiCl-induced eyeless phenotype based on their known targets. In this library there are eight GSK3 inhibitors. Treatment of zebrafish embryos with these inhibitors (without LiCl) resulted in only one of these (BIO) generating an eyeless phenotype at 1 dpf at 10 μM, which was rescued by the addition of 1 μM XAV939 (Figure 4(a)). This suggests that not all small molecules will be effective,

possibly due to their permeability or lack of sufficient homology between zebrafish and human target sites. Nonetheless, the fact that XAV939 is effective against two GSK3 inhibitors supports the validity of the screen. Taken together, this suggests that the false positive rate is very low, but the false negative rate may be high.

Of the 400 kinase inhibitors we observed toxicity in 45 of these at 10 μM, which resulted in death or arrested development in all of the embryos within the well. Several of these were cell cycle kinase inhibitors (Cdc, Ckd, cyclin). We re-tested these at 1 μM, and while no longer toxic, none rescued the eye or had other observable defects.

While the screen was designed to look for zebrafish with eyes, there were some other notable results independent of the eye phenotype. First, 27 inhibitors of EGFR and its paralogs (HER2, ErbB2) had no observable effect. This suggests that EGF signaling is not functioning within the first 24 h of development, which is supported by other work from our lab (TVR, unpublished). In contrast, inhibition of TGFβR by SD 208 results in a classical TGFβ null phenotype, the one-eyed-pinhead phenotype (Figure 4(b) and (d)).[66,67] Inhibitors of other MAPK signaling kinases also had mixed results. For example, of the 13 inhibitors of MEK1/2, five displayed arrested development or other defects (Figure 4(c), (g), (h)), strongly suggesting a role for MEK1/2 activity in early zebrafish development. This is consistent with a recent report on MEK1/2 signaling interacting with TGFβ signaling.[68] However, there were no notable defects from any of the other 34 inhibitors of MAPK signaling (p38, ERK, MKK, etc). Observing no gross abnormalities at one-day of development needs to be interpreted with caution. For example, a more detailed analysis, or prolonged or delayed exposure, might reveal specific effects of these inhibitors on other systems or organs such as neural necrosis or brain dysmorphology (Figure 4(e) and (f)). Taken together, these data suggest that our screen can generate predictive responses to small molecules, and also implicate other signaling cascades in certain developmental processes.

In summary, the majority of CRCs are initiated by mutations in the Wnt pathway, making this pathway an excellent target for gene-based therapy. Unfortunately, *in vitro* screens to identify small molecules that target the Wnt pathway have fallen short of becoming clinically viable. Here, we chose to look at CRC as a stem cell disease and as such is molecularly similar to Wnt signaling in development. By manipulating the Wnt pathway in early stem cells we create a clear phenotype that can be rescued by a known inhibitor of Wnt signaling. Furthermore, in addition to this proof of principle, we have also found that the screening model is specific and can respond predictably to inhibitors of other signaling pathways as well. Identification of small molecule inhibitors of Wnt signaling from this screen will be validated in a secondary screen using human CSCs and xenographs models. This screening technique will ultimately identify putative therapies and have a significant impact in reducing the morbidity and mortality rate due to cancer.

Author contributions: All authors conducted the experiments; TVR designed and interpreted the results and wrote the manuscript.

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