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Gastrulation Screening to Identify Anti-metastasis Drugs in Zebrafish Embryos

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Abstract

Few models exist that allow for rapid and effective screening of anti-metastasis drugs. Here, we present a drug screening protocol utilizing gastrulation of zebrafish embryos for identification of anti-metastasis drugs. Based on the evidence that metastasis proceeds through utilizing the molecular mechanisms of gastrulation, we hypothesized that chemicals interrupting zebrafish gastrulation might suppress the metastasis of cancer cells. Thus, we developed a phenotype-based chemical screen that uses epiboly, the first morphogenetic movement in gastrulation, as a marker. The screen only needs zebrafish embryos and enables hundreds of chemicals to be tested in five hours by observing the epiboly progression of chemical-treated embryos. In the screen, embryos at the two-cell stage are firstly corrected and then developed to the sphere stage. The embryos are treated with a test chemical and incubated in the presence of the chemical until vehicle-treated embryos develop to the 90% epiboly stage. Finally, positive 'hit' chemicals that interrupt epiboly progression are selected by comparing epiboly progression of the chemical-treated and vehicle-treated embryos under a stereoscopic microscope. A previous study subjected 1,280 FDA-approved drugs to the screen and identified adrenosterone and pizotifen as epiboly-interrupting drugs. These were validated to suppress metastasis of breast cancer cells in mice models of metastasis. Furthermore, 11β-hydroxysteroid dehydrogenase 1 (HSD11β1) and serotonin receptor 2C (HTR2C), the primary targets of adrenosterone and pizotifen, respectively, promoted metastasis through induction of epithelial-mesenchymal transition (EMT). Therefore, this screen could be converted into a chemical genetic screening platform for identification of metastasis-promoting genes.

Keywords: Anti-metastasis drugs, Metastasis, Gastrulation, EMT, Phenotyping screening, Zebrafish

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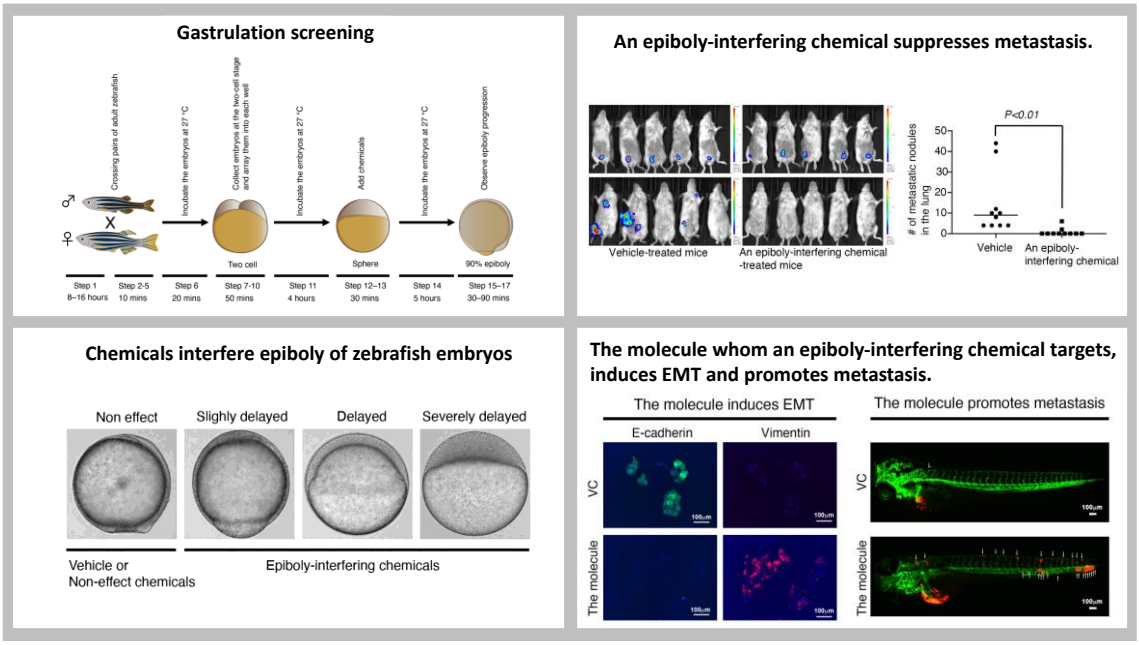
Graphical abstract:



Background

Cancer research using zebrafish as a model has attracted attention because it offers many unique advantages that are not readily provided by other animal models. Furthermore, the zebrafish system has also been increasingly recognized as a chemical screening platform because it provides the advantage of high-throughput screening in an in vivo vertebrate setting with physiologic relevance to humans (Zon and Peterson, 2005; MacRae and Peterson, 2015; Nakayama et al., 2020; Nakayama and Makinoshima, 2020; Nakayama and Gong, 2020).

Metastasis is responsible for approximately 90% of cancer-associated mortality. It proceeds through multiple steps: invasion, intravasation, survival in the circulatory system, extravasation, colonization, and metastatic tumor formation in secondary organs with angiogenesis (Nguyen et al., 2009; Chaffer and Weinberg, 2011; Welch and Hurst, 2019). Dissemination of cancer cells is an initial step of metastasis, and its molecular mechanism involves local breakdown of basement membrane, loss of cell polarity, and induction of epithelial-mesenchymal transition (EMT) (Tsai, J. H. and Yang, 2013; Lu, W. and Kang, 2019). These cellular and biological phenomena are also observed during vertebrate gastrulation in that evolutionarily conserved morphogenetic movements of epiboly, internalization, convergence, and extension cooperate to generate germ layers and sculpt the body plan (Solnica-Krezel, 2005). In zebrafish, the first morphogenetic movement, epiboly, is initiated at approximately four hours post fertilization (hpf) to move cells from the animal pole to eventually engulf the entire yolk cell by 10 hpf. These movements are governed by molecular mechanisms that are induced by temporally and spatially regulated gene expression; these mechanisms and changes in gene expression are partially observed in metastatic progression (White et al., 2017).

At least 50 common genes are involved in both gastrulation and metastasis progression (Table 1) (Yang, J. and Weinberg, 2008; Thiery et al., 2009; Nieto et al., 2016; Dongre and Weinberg, 2019; Yang, J. et al., 2020). These genes are expressed in *Xenopus* or zebrafish embryos, and genetic inhibition of each gene in these embryos interferes with gastrulation progression. Conversely, the same 50 genes are ectopically expressed in metastatic cancer cells and confer metastatic properties to cancer cells; genetic inhibition of each of these genes suppresses metastasis progression. This evidence led us to hypothesize that chemicals that interfere with zebrafish gastrulation might suppress metastasis progression of cancer cells. Based on the hypothesis, we developed a phenotype-based chemical screen that uses epiboly, the first morphogenetic movement in gastrulation, as a marker. This screen measures the

suppressor effect of each test chemical by observing epiboly progression of chemical-treated embryos (Figures 1 and 2).

Table 1. A list of the 50 common genes that are involved in gastrulation and metastasis progression.

Genes	Gastrulation Defects	Reference	Effects in Metastasis	Reference
BMP	Convergence and extension	(Kondo, 2007)	EMT	(Katsuno et al., 2008)
WNT	Convergence and extension	(Tada and Smith, 2000)	Migration and invasion	(Vincan and Barker, 2008)
FGF	Convergence and extension	(Yang, X. et al., 2002)	Invasion	(Nomura et al., 2008)
EGF	Epiboly	(Song et al., 2013)	Migration	(Lu, Z. et al., 2001)
PDGF	Convergence and extension	(Damm and Winklbauer, 2011)	EMT	(Jechlinger et al., 2006)
CXCL12	Migration of endodermal cells	(Mizoguchi et al., 2008)	Migration and invasion	(Shen et al., 2013)
CXCR4	Migration of endodermal cells	(Mizoguchi et al., 2008)	Migration and invasion	(Shen et al., 2013)
PIK3CA	Convergence and extension	(Montero et al., 2003)	Migration and invasion	(Wander et al., 2013)
YES	Epiboly	(Tsai, W. B. et al., 2005)	Migration	(Barraclough et al., 2007)
FYN	Epiboly	(Sharma et al., 2005)	Migration and invasion	(Yadav and Denning, 2011)
MAPK1	Epiboly	(Krens et al., 2008)	Migration	(Radtke et al., 2013)
SHP2	Convergence and extension	(Jopling et al., 2007)	Migration	(Wang, F. M. et al., 2005)
SNAI1	Convergence and extension	(Ip and Gridley, 2002)	EMT	(Batlle et al., 2000)
SNAI2	Mesoderm and neural crest formation	(Shi, J. et al., 2011)	EMT	(Medici et al., 2008)
TWIST1	Mesoderm formation	(Castanon and Baylies, 2002)	EMT	(Yang, J. et al., 2004)
TBXT	Convergence and extension	(Tada and Smith, 2000)	EMT	(Fernando et al., 2010)
ZEB1	Epiboly	(Vannier et al., 2013)	EMT	(Spaderna et al., 2008)
GSC	Mesodermal patterning	(Sander et al., 2007)	EMT	(Hartwell et al., 2006)
FOXC2	Unclear, defects in gastrulation	(Wilm et al., 2004)	EMT	(Mani et al., 2007)
STAT3	Convergence and extension	(Miyagi et al., 2004)	Migration	(Abdulghani et al., 2008)
POU5F1	Epiboly	(Lachnit et al., 2008)	EMT	(Dai et al., 2013)
EZH2	Unclear, defects in gastrulation	(O'Carroll et al., 2001)	Invasion	(Ren et al., 2012)
EHMT2	Defects in neurogenesis	(Lin, F. et al., 2005)	Migration and invasion	(Chen et al., 2010)
BMI1	Defects in skeleton formation	(van der Lugt et al., 1994)	EMT	(Guo et al., 2011)
RHOA	Convergence and extension	(Zhu, S. et al., 2006)	Migration and invasion	(Yoshioka et al., 1999)
CDC42	Convergence and extension	(Choi and Han, 2002)	Migration and invasion	(Reymond et al., 2012)
RAC1	Convergence and extension	(Habas et al., 2003)	Migration and invasion	(Vega and Ridley, 2008)
ROCK2	Convergence and extension	(Marlow et al., 2002)	Migration and invasion	(Itoh et al., 1999)
PAR1	Convergence and extension	(Kusakabe and Nishida, 2004)	Migration	(Shi, X. et al., 2004)
PRKCI	Convergence and extension	(Kusakabe and Nishida, 2004)	EMT	(Gunaratne et al., 2013)
CAP1	Convergence and extension	(Seifert et al., 2009)	Migration	(Yamazaki et al., 2009)
EZR	Epiboly	(Link et al., 2006)	Migration	(Khanna et al., 2004)
EPCAM	Epiboly	(Slanchev et al., 2009)	Migration and invasion	(Ni et al., 2012)
ITGB1/ITA5	Mesodermal migration	(Skalski et al., 1998)	Migration and invasion	(Felding-Habermann, 2003)
FN1	Convergence and extension	(Marsden and DeSimone, 2003)	Invasion	(Malik et al., 2010)
HAS2	Dorsal migration of lateral cells	(Bakkers et al., 2004)	Invasion	(Kim et al., 2004)
MMP14	Convergence and extension	(Coyle et al., 2008)	Invasion	(Perentes et al., 2011)
COX1	Epiboly	(Cha et al., 2006)	Invasion	(Kundu and Fulton, 2002)

PTGES	Convergence and extension	(Speirs et al., 2010)	Invasion	(Wang, D. and Dubois, 2006)
SLC39A6	Anterior migration	(Yamashita et al., 2004)	EMT	(Lue et al., 2011)
GNA12/13	Convergence and extension	(Lin, F. et al., 2005)	Migration and invasion	(Yagi et al., 2011)
OGT	Epiboly	(Webster et al., 2009)	Migration and invasion	(Lynch et al., 2012)
CCN1	Cell movement	(Latinkic, 2003)	Migration and invasion	(Lin, J. et al., 2012)
TRPM7	Convergence and extension	(Liu et al., 2011)	Migration	(Middelbeek et al., 2012)
MAPKAPK 2	Epiboly	(Holloway et al., 2009)	Migration	(Kumar et al., 2010)
B4GALT1	Convergence and extension	(Machingo et al., 2006)	Invasion	(Zhu, X. et al., 2005)
IER2	Convergence and extension	(Hong et al., 2011)	Migration	(Neeb et al., 2012)
TIP1	Convergence and extension	(Besser et al., 2007)	Migration and invasion	(Han et al., 2012)
PAK5	Convergence and extension	(Faure et al., 2005)	Migration	(Gong et al., 2009)
MARCKS	Convergence and extension	(Iioka et al., 2004)	Migration and invasion	(Rombouts et al., 2013)

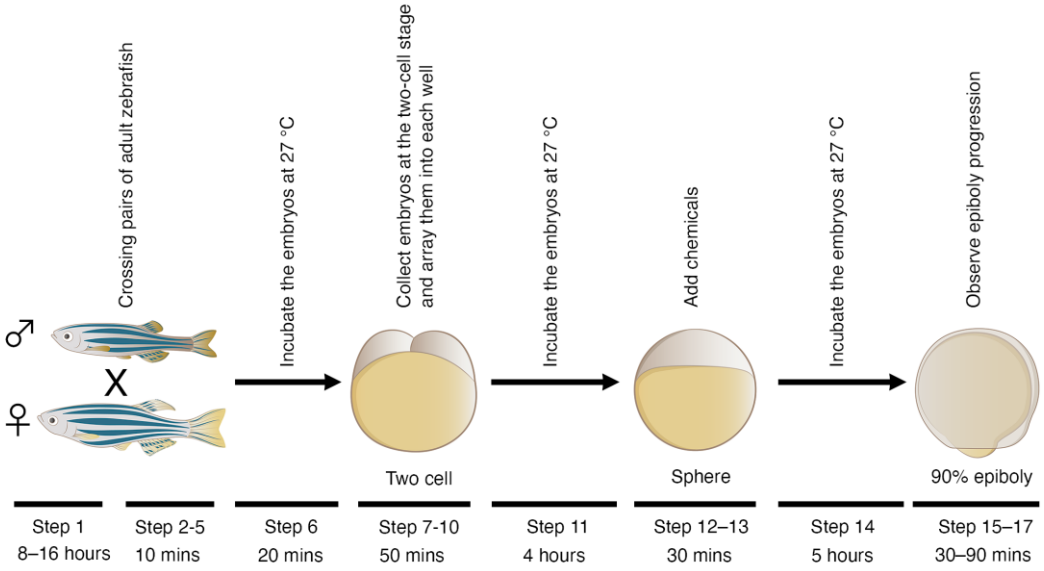


Figure 1. Schematic diagram of a phenotype-based chemical screen using zebrafish embryos.
Pairs of adult zebrafish are crossed, and their embryos are collected at the two-cell stage and arrayed into individual wells of a 24-well plate. Different chemicals are added to each well when the embryos develop to the sphere stage. Epiboly progression of each chemical-treated embryo is compared to DMSO-treated embryos under a stereoscopic microscope when DMSO-treated embryos develop to the 90% epiboly stage.

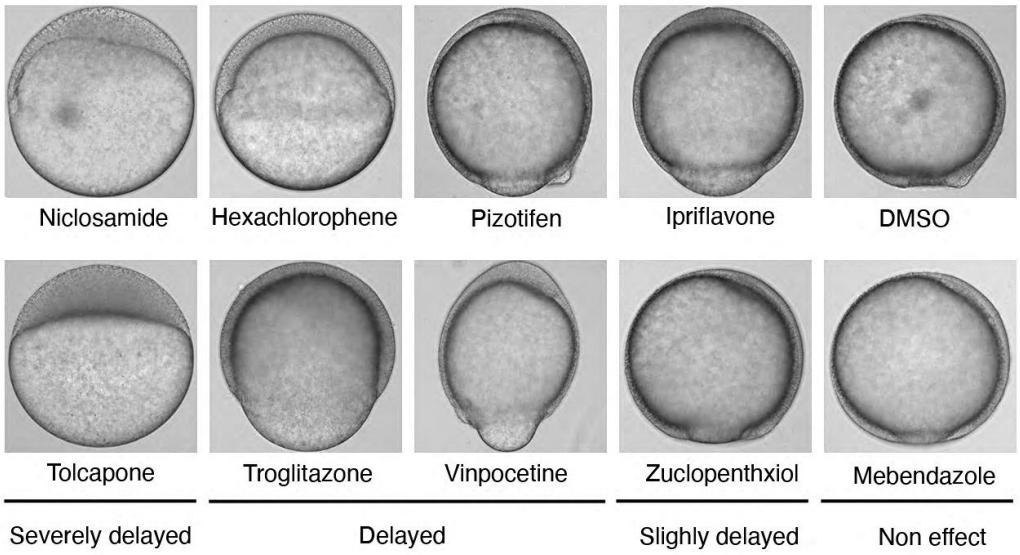


Figure 2. Representative samples of chemical-treated embryos.
Embryos at the sphere stage were treated with 10 μM of each indicated chemical. Niclosamide-treated embryos served as positive controls, while DMSO-treated embryos served as negative controls. Epiboly progression of each chemical-treated embryo was compared with that of DMSO-treated embryos under a stereoscopic microscope, when DMSO-treated embryos developed to the 90% epiboly stage.

This screen enables hundreds of chemicals to be tested in only five hours. Our study subjected 1,280 FDA-approved drugs to this screen and identified adrenosterone and pizotifen as epiboly-interfering drugs. These were further validated to suppress metastasis of breast cancer cells in mouse models of metastasis (Figure 3) (Nakayama et al., 2021a, 2021b). This screen can also measure suppressor effect of crude drugs. We subjected 120 herbal medicines to this screen and identified cinnamon bark extract as an epiboly-interfering drug. Cinnamon bark extract was validated to suppress metastatic dissemination of breast cancer cells in a zebrafish xenograft model (Nakayama et al., 2022). Moreover, this screen can be converted into a chemical genetic screening platform for identification of metastasis-promoting genes. We demonstrated that 11β-hydroxysteroid dehydrogenase 1 (HSD11β1) and serotonin receptor 2C (HTR2C), the primary targets of adrenosterone and pizotifen, respectively, induced EMT and promoted metastasis of breast cancer cells (Nakayama et al., 2021a, 2021b) (Figure 4).

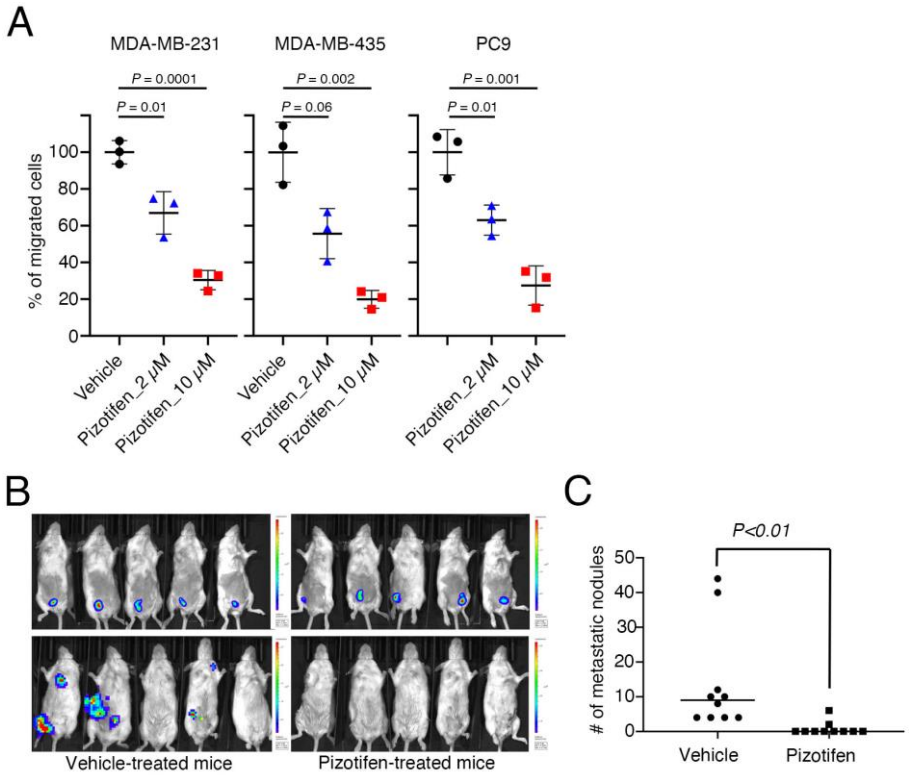


Figure 3. Pizotifen, one of the epiboly-interrupting drugs, suppresses metastatic progression of breast cancer cells in vitro and in vivo.

(A) Effect of pizotifen on cell motility and invasion of MBA-MB-231, MDA-MB-435, and PC9 cells. Either vehicle- or pizotifen-treated cells were subjected to Boyden chamber assays. Fetal bovine serum (1% v/v) was used as the chemoattractant in both assays. Each experiment was performed at least twice. **(B)** Representative images of primary tumors on day 10 post-injection (top panels) and metastatic burden on day 70 post-injection (bottom panels) taken using an IVIS Imaging System. **(C)** Number of metastatic nodules in the lungs of vehicle- and pizotifen-treated mice.

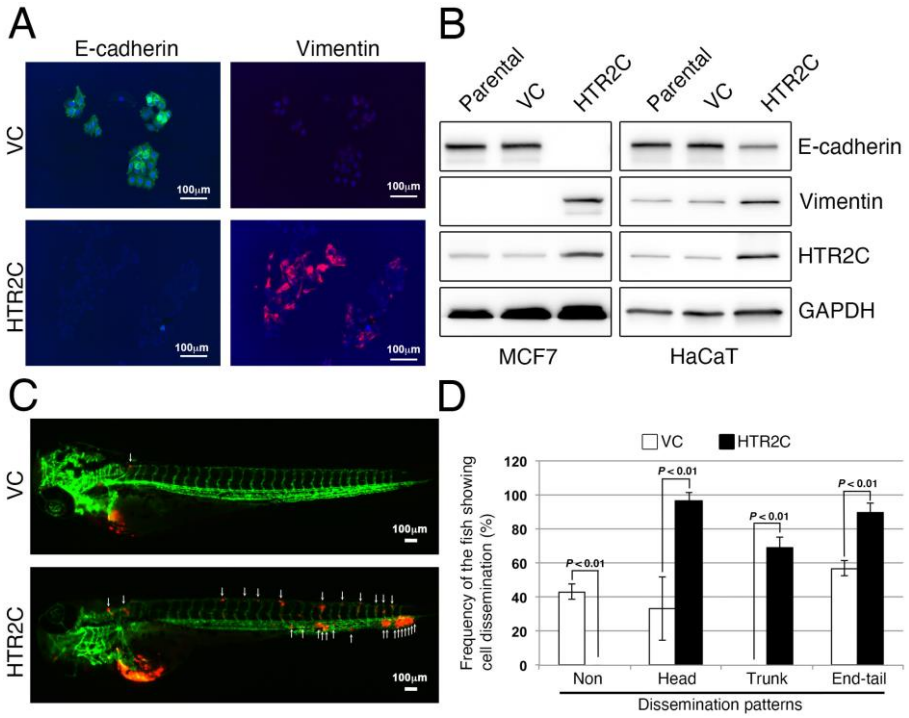


Figure 4. HTR2C, a primary target of pizotifen, induces EMT-mediated metastatic dissemination of human cancer cells.

(A) Immunofluorescence staining of E-cadherin and vimentin expressions in the MCF7 cells. **(B)** Expression of E-cadherin, vimentin, and HTR2C were examined by Western blotting in the MCF7 and HaCaT cells; GAPDH loading control is also shown (bottom). **(C)** Representative images of dissemination patterns of MCF7 cells expressing either the control vector (top left) or HTR2C (lower left) in a zebrafish xenotransplantation model. White arrowheads indicate disseminated MCF7 cells. **(D)** Mean frequencies of the fish showing head, trunk, or end-tail dissemination (right). Each value is indicated as the mean ± SEM of two independent experiments. Statistical analysis was determined by Student's *t*-test.

Materials and Reagents

1. 150 mm dish (Corning, catalog number: 430599)
2. 24-well flat bottom plastic plates (Corning, catalog number: CLS3473)
3. Wild-type zebrafish strain (AB line)
4. Tg *(kdrl:eGFP)* zebrafish (Provided by Dr. Stainier)
5. FDA-, EMA-, and other agencies-approved chemical libraries (Prestwick Chemical, Illkirch, France)
6. Niclosamide (Sigma-Aldrich, catalog number: N3510)
7. DMSO (Sigma-Aldrich, catalog number: D8418)
8. Anti-E-cadherin antibody (Cell Signaling Technology, catalog number: 14472)
9. Anti-vimentin antibody (Cell Signaling Technology, catalog number: 5741)
10. Anti-HTR2C antibody (Abcam, catalog number: ab133570)
11. Anti-GAPDH antibody (Cell Signaling Technology, catalog number: 2118)
12. Human breast cancer cell line MCF7 (ATCC, catalog number: HTB-22)
13. NaCl (Sigma-Aldrich, catalog number: S3014)
14. KCl (Sigma-Aldrich, catalog number: P9541)
15. $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, catalog number: M2773)
16. E3 medium (see Recipes)

Equipment

1. External tank, a part of zebrafish breeding tank (Tecniplast, catalog number: ZB10BTE)
2. Perforated internal tank, a part of zebrafish breeding tank (Tecniplast, catalog number: ZB10BTI)
3. Polycarbonate divider, a part of zebrafish breeding tank (Tecniplast, catalog number: ZB10BTD)
4. Polycarbonate lid (Tecniplast, catalog number: ZB10BTL)
5. Incubator (AQUALYTIC, catalog number: 2418210)
6. Stereomicroscope (Leica, catalog number: MZ75)

Procedure

Zebrafish mating setup (Day 0); 10 min

1. At the night before collecting embryos, arrange pairs of male and female zebrafish separated by a divider (Figure 5).

Figure 5. Zebrafish breeding tank with a divider.
Male and female zebrafish are separated by a divider which prevents contact for mating. The images are viewed from overhead angle (left) and from the side (right). The divider is then removed, which allows male zebrafish to contact female zebrafish for mating.

Note: Young adult zebrafish (3–9 months old) should be used for the crossing. The quality of zebrafish embryos affects screening efficiency (critical step). Water temperature is 27 °C. Duration of sunshine is set from 7:00 to 19:00.

Embryo collection and distribution (Day 1); 80 min

2. Remove the divider to allow the fish to spawn in the morning (7:00–10:00).
3. Allow crossing for 10 min to obtain zebrafish embryos of the same developmental stage. For example, if more than 20 chemicals are tested, the crossing might be performed three times at three different time points (Group A: 8:30, Group B: 9:00, and Group C: 9:30).

For example, a 60-drug screening would require approximately 1,300 embryos if each drug needs to be tested at one concentration:
Group A: 20 (embryos) × 1 (concentration) × 30 (drugs) + 20 (vehicle control) + 20 (positive control).
Group B: 20 (embryos) × 1 (concentration) × 30 (drugs) + 20 (vehicle control) + 20 (positive control).
Similarly, if each drug needs to be tested at two concentrations, approximately 2,600 embryos would be required:
Group A: 20 (embryos) × 2 (concentrations) × 30 (drugs) + 40 (vehicle controls) + 20 (positive control).
Group B: 20 (embryos) × 2 (concentrations) × 30 (drugs) + 40 (vehicle controls) + 20 (positive control).

4. After 10 min, replace the divider to prevent the zebrafish from spawning.
   Note: This screen measures the suppressor effect of each chemical on the progression of epiboly in live zebrafish embryos. Therefore, epiboly proceeds while the researcher is measuring the effect under a stereoscopic microscope. If more than 20 chemicals need to be tested, the screening should be divided into more than two sessions, and each of the sessions should start at a different time point. For example, if 60 chemicals need to be tested, zebrafish should be crossed at three different time points that are over 30 min apart. That allows 30 min for measuring the effects (critical step).
5. Collect the embryos in 150 mm dishes containing E3 medium and remove dead embryos.
6. Incubate the embryos at 27 °C for 20 min.
7. Collect two-cell stage embryos under the stereoscopic microscope.
   Note: This step has limited throughput. The collection of zebrafish embryos should be completed during the two-cell stage. Thus, 900–1,200 embryos at the two-cell stage would be the upper limit for one researcher.
8. Array approximately 20 embryos into each well of a 24-well plate.
9. Remove E3 medium from each well by using a pipet.
10. Add 900 µL of E3 medium to each well.

Embryo development to the sphere stage; 4 h

11. Incubate the embryos at 27 °C until they develop to the sphere stage
    Note: The temperature of the E3 medium affects the development rate of zebrafish embryos. Higher temperatures accelerate the development rate; conversely, lower temperatures slow it down (Urushibata et al., 2021). Therefore, a non-uniform temperature in each well of a 24-well plate can cause false positives (critical step).

Addition of chemicals; 30 min

12. Prepare a 10-fold concentration of each chemical in E3 medium 30 min before adding the chemicals to the embryos.
    Note: After preparing the respective 10-fold concentration of the media, the medium should be stored at 27 °C.
13. When the embryos develop to the sphere stage, add 100 µL of the respective 10-fold media to the wells.
    For example, a 60-drug screening is divided into three groups:
    a. The first set of 20 test chemicals, as well as niclosamide and DMSO as positive and negative controls, are added into group A when embryos from group A develop to the sphere stage.
    b. The second set of 20 test chemicals, niclosamide, and DMSO are added into group B when embryos from group B develop to the sphere stage.
    c. The last set of 20 test chemicals, niclosamide, and DMSO are added into group C when embryos from group C develop to the sphere stage.

Development of DMSO-treated embryos to the 90% epiboly stage; 5 h

14. After adding the test chemicals, incubate the embryos at 27 °C for approximately 5 h.
    Note: The temperature of the E3 medium affects the development rate of zebrafish embryos. Non-uniform temperature in each well of a 24-well plate would cause false positives (critical step).

Measuring the inhibition effects of each chemical; 30 min

15. Compare the epiboly progression of chemical-treated embryos from group A and DMSO-treated embryos under the stereoscopic microscope, when the DMSO-treated embryos develop to the 90% epiboly stage.
    Note: This step has limited throughput. Comparisons should be completed before DMSO-treated embryos at the 90% epiboly stage develop to the next development stage. Thus, 20–30 chemicals would be the upper limit for one researcher.
16. Compare the epiboly progression of chemical-treated embryos from group B and DMSO-treated embryos under the stereoscopic microscope, when the DMSO-treated embryos develop to the 90% epiboly stage.
17. Compare the epiboly progression of chemical-treated embryos from group C and DMSO-treated embryos under the stereoscopic microscope, when the DMSO-treated embryos develop to the 90% epiboly stage.
    Note: Epiboly proceeds while a researcher is comparing the epiboly progression of chemical-treated and DMSO-treated embryos under the stereoscopic microscope. Therefore, measuring the effect should be completed in 30 mins (critical step). To confirm the reproducibility of whether 'hit' chemicals could interrupt the epiboly progression of zebrafish embryos, further testing on subsequent day is advised (critical step).

Data analysis

This screen measures the suppressor effect of chemicals based on the epiboly progression of zebrafish embryos. Niclosamide and DMSO can be used as positive and negative controls, respectively. Epiboly progression of chemical-treated embryos is compared with that of DMSO-treated embryos under a stereoscopic microscope, allowing positive 'hit' chemicals that interrupt epiboly progression to be selected (Figure 2) (Nakayama et al., 2021b, 2022).

Limitations

Throughput in steps 7 and 15, determine how many chemicals can be tested in one screening session. In step 7, the collection of zebrafish embryos should be completed during the two-cell stage and before the four-cell stage. Thus, 900–1,200 embryos at the two-cell stage would be the upper limit for one researcher. In step 15, comparing epiboly progression of each chemical-treated embryo with DMSO-treated embryos under the stereoscopic microscope should be completed before DMSO-treated embryos at the 90% epiboly stage develop to the next stage. Thus, 20–30 chemicals would be the upper limit for one researcher.
In addition, there are some limitations regarding chemical deliverance to zebrafish embryos. These are surrounded by the acellular chorion, which is known to be approximately 1.5–2.5 μm thick and to consist of three layers pierced by pore canals. The pore allows passage of water, ions, and chemicals. A study reported that molecules larger than 3–4 KDa fail to pass through the chorion. Therefore, this screen may not be able to measure the suppressor effect of molecules larger than 3–4 KDa (Pelka et al., 2017).

Troubleshooting

The quality of zebrafish embryos affects screening efficiency. For example, low-quality embryos show high frequencies of asymmetric cell cleavage, and their development is arrested at early cleavage stages (Yilmaz et al., 2017). If a screen used low-quality zebrafish embryos, it would generate false 'hit' chemicals since the suppressor effect of a test chemical is measured by observing the epiboly progression of chemical-treated embryos. If the number of zebrafish embryos showing morphological abnormalities correlate with the final concentration of a test chemical, those embryo abnormalities may result from the effect of the test chemical.

Anticipated results

Suppressor effects of a tested chemical on the epiboly progression of zebrafish embryos are significantly affected by the final concentration of the chemical. A previous study subjected 1,280 FDA-approved drugs to this screen and showed that 6% (78/1,280) of the tested drugs affected epiboly progression of the embryos treated with 10 µM. Out of these 78 epiboly-interrupting drugs, 25% of the drugs succeeded in suppressing cell motility and invasion of highly metastatic human cancer cells in a Boyden chamber assay. In contrast, epiboly progression was affected more severely when the embryos were treated at 50 µM, by 10.3% (132/1,280) of the tested drugs. From these, 85% (112/132) failed to suppress cell motility and invasion of highly metastatic human cancer cells in a Boyden chamber assay (Nakayama et al., 2021b).

Recipes

1. E3 medium

5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $MgSO_4$

Reagent	Final concentration	Amount
NaCl	5.0 mM	
KCl	0.17 mM	
$MgSO_4$	0.33 mM	
H_2O	n/a	1,000 mL

Acknowledgments

Figure 1 was drawn by Ami Inoue (Kyoto University of the Arts). Pictures of zebrafish mating tank with a divider were taken by Dr. Li Yan (National University of Singapore). This study was funded by grants from National Medical Research Council of Singapore (R-154000547511) and Ministry of Education of Singapore (R-154000A23112 and R154000B88112) to ZG. Experiments using mice were supported in part by research funds from the Yamagata prefectural government and the City of Tsuruoka. This screen protocol was used in previous research (Nakayama et al., 2021a, 2021b, 2022).

Competing interests

J.N., H.M., and Z.G. declare no conflict of interest.

Ethics

The study protocol using zebrafish was approved by the Institutional Animal Care and Use Committee of the National University of Singapore (protocol number: R16-1068). The study protocol using mice (protocol number: BRC IACUC #110612) was approved by A*STAR (Agency for Science, Technology and Research, Singapore).

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