Published in final edited form as:

Methods Mol Biol. 2010 ; 636: 317–327. doi:10.1007/978-1-60761-691-7_20.

Zebrafish small molecule screen in reprogramming/cell fate modulation

Kathleen M. Munson and Jing-Ruey J. Yeh *

Developmental Biology Laboratory, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA; Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.

Abstract

Embryonic zebrafish have long been used for lineage tracing studies. In zebrafish embryos, the cell fate identities can be determined by whole-mount *in situ* hybridization, or by visualization of live embryos if using fluorescent reporter lines. We use embryonic zebrafish to study the effects of a leukemic oncogene AML1-ETO on modulating hematopoietic cell fate. Induced expression of AML1-ETO is able to efficiently reprogram hematopoietic progenitor cells from erythroid to myeloid cell fate. Using the zebrafish model of AML1-ETO, we performed a chemical screen to identify small molecules that suppress the cell fate switch in the presence of AML1-ETO. The methods discussed herein may be broadly applicable for identifying small molecules that modulate other cell fate decisions.

Keywords

chemical screen; zebrafish; hematopoiesis; AML; leukemia; reprogramming; cell fate; *in vivo*; erythroid; myeloid

1. Introduction

Many leukemic oncogenes, including AML1-ETO, contribute to leukemogenesis by modulating hematopoietic stem/progenitor cell differentiation. Embryonic zebrafish is a powerful model to study hematopoietic cell fate. Within the first day post-fertilization, zebrafish embryos develop two localized pools of hematopoietic progenitor cells (HPCs). The anterior blood island expresses *pu.1*, and will give rise to the myeloid cells (1). On the other hand, the posterior blood island expresses *gata1*, and will differentiate into the erythroid cells (1). These synchronized pools of HPCs are useful for studying the signaling pathways that underlie or affect hematopoietic cell fate determination.

We have shown that expressing AML1-ETO, an oncogene frequently associated with acute myelogenous leukemia, leads to a rapid and efficient cell fate switch in the posterior blood island of zebrafish embryos (Fig. 1). This fate switch is characterized by downregulation of *gata1*, suggesting suppression of erythropoiesis, and upregulation of *mpo*, indicating conversion into the granulocytic cell fate (2). Furthermore, to identify candidate small molecules that can reverse AML1-ETO's effects and the mechanisms by which AML1-ETO reprograms hematopoietic cell fate, we conducted a chemical suppressor screen using zebrafish embryos. From this screen we have identified several classes of compounds that restored

*To whom correspondence should be addressed. jyeh1@partners.org, Tel: (617)726-6469, Fax: (617)726-5806.

gata1 expression in the presence of AML1-ETO (3). The chemical suppressors of AML1-ETO identified from the *in vivo* zebrafish screen may provide not only new insights into AML1-ETO-mediated hematopoietic differentiation but also new means to block AML1-ETO's effects in the clinical settings.

For the chemical suppressor screen of AML1-ETO we used a transgenic zebrafish line, Tg (*hsp:AML1-ETO*), in which AML1-ETO expression is controlled by a zebrafish heat shock *hsp70* promoter. Thus, AML1-ETO expression can be induced by incubating zebrafish embryos at 37–42°C as compared to the regular embryo culture temperature at 24–28.5°C. Tg (*hsp:AML1-ETO*) embryos were arrayed into 96-well screening plates. Compounds from the chemical library were also added to the screening plates, and the plates were subjected to the heat treatment to induce AML1-ETO expression. Subsequently, the embryos were fixed and processed for whole-mount *in situ* hybridization of *gata1* through both manual and automated procedures.

Conceivably, the present method could also be adapted for investigation of other cell fate decisions by using other zebrafish lines and cell markers. The combination of facile detection of cell fates and high-throughput *in vivo* small molecule screening surely will make the embryonic zebrafish a unique model system to study reprogramming/cell fate modulation.

2. Materials

2.1 Zebrafish

1. Adult wild-type and transgenic Tg(*hsp:AML1-ETO*) zebrafish, males and females (see **Note 1**).
2. Mating cages for the zebrafish that have divider slots in the middle.
3. E3 buffer: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄.
4. Petri dishes.
5. Egg strainers for collecting zebrafish embryos.
6. Incubators at 24°C and 28.5°C.

2.2 Chemical Screening in Zebrafish Embryos

1. A dissecting microscope.
2. A desiccator.
3. Pronase, which is a mixture of proteinases available from various commercial sources.
4. Pipette Pump™, 10 ml (#378980000, Scienceware®).
5. Glass Pasteur pipettes with a large bore size.
6. MultiScreen-Mesh Filter plates with 96-well receiver plates (MANMN6010, Millipore). Each set of the plates includes a cover, a 96-well nylon mesh (60 µm) plate, a 96-well receiver plate, and a single-well reservoir tray.
7. A multi-channel pipette.
8. Chemical libraries — Any small molecule libraries may be used. The compounds in the chemical libraries are generally dissolved in dimethyl sulfoxide (DMSO) and stored in a 96 or 384-well format.
9. Dimethyl sulfoxide (DMSO).

10. Foil plate seals.

2.3 Incubation and Fixation of Compound-treated Zebrafish Embryos

1. A water bath at 39°C for heat shock treatment.
2. 4% paraformaldehyde in 1X phosphate buffered saline (PBS), (4% PFA/PBS): 4g PFA in 100 mL 1X PBS. Dissolve at 65°C. Alternatively, dilute 37% formaldehyde solution to 4% with 1X PBS. Store in foil-wrapped Falcon tubes at 4°C.

2.4 Digoxigenin Probe Labeling

1. Plasmid DNA pBS-ZG1 (4).
2. *Xba*I restriction enzyme.
3. QIAquick PCR Purification kit (#28104, Qiagen).
4. T7 RNA polymerase (#P2075, Promega).
5. 0.1 M dithiothreitol (DTT).
6. RNasin® Ribonuclease Inhibitor (#N2115, Promega).
7. DIG RNA Labeling Mix (#11277073910, Roche).
8. RNase-free DNase (#M6101, Promega).
9. mini Quick Spin RNA Columns (#11814427001, Roche).
10. A 37°C incubator.

2.5 Whole-Mount In Situ Hybridization

1. 10X PBS: 800 mL RNase-free water, 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, 2.4 g KH₂PO₄, adjust pH to 7.4 with HCl and add RNase-free water to 1 liter. Store at room temperature.
2. 1X PBS: Dilute 1 part of 10X PBS with 9 parts of RNase-free water.
3. PBST: 1X PBS with 0.1% Tween-20. Store at room temperature.
4. 20X SSC: 175.3 g NaCl, 88.2 g sodium citrate, adjust pH to 7.0, and add RNase-free water to 1 liter. Store at room temperature.
5. Hyb(−) solution: 50% formamide, 5X SSC, 0.1% Tween-20. Use RNase-free water. Add 1 ml of 1 M citric acid per 100 ml of hyb(−) solution. Store at −20°C.
6. Hyb(+) Solution: Hyb(−) solution with 500 µg/mL Torula Yeast RNA and 50 µg/mL heparin. Store at −20°C.
7. Maleic acid buffer: 0.1 M maleic acid, pH 7.5, 0.15 M NaCl.
8. Blocking solution: Maleic acid buffer with 10% calf serum.
9. Antibody solution: Diluted anti-digoxigenin-AP antibody (#11093274910, Roche) 1:5000 in the blocking solution.
10. NTMT: 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂, 0.1% Tween-20.
11. NBT: 4-nitro blue tetrazolium chloride solution (100 mg/ml).
12. BCIP: 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (50 mg/ml).

13. BioLane™ HTI (Holle & Huttner AG), which is an automated liquid handling platform that can be programmed for various applications.
14. A 68°C incubator.
15. A dissecting microscope.

3. Methods

3.1 Collection of Zebrafish Embryos

1. **Day 1:** Set up matings of wild-type and transgenic fish pairs using the mating cages with dividers in the middle to separate males and females.
2. **Day 2:** Pull the dividers in the morning. Two hours later, collect embryos using the egg strainers and place each clutch of the embryos in a 10-cm Petri dish with E3 buffer. Incubate dishes with the embryos at 28.5°C for 5 hours and then at 24°C overnight (see **Note 2**).

3.2 Arraying the Embryos and Administering the Chemicals

1. **Day 3:** Set water bath temperature at 39°C.
2. Thaw compound plates from –80°C in a desiccator (see **Note 3**).
3. Discard dead embryos and stage embryos under a dissecting microscope, pooling clutches of similarly staged embryos. Embryos should be younger than 14-somite stage.
4. Reduce the E3 volume in the Petri dish just to the point at which the embryos can move freely in the dish when swirled. Add 0.5 µg of pronase to each ml of E3 buffer. Swirl Petri dishes to mix. Monitor the embryos, swirling often to promote the dechoriation of the embryos. Once all of the embryos come out of the chorions, rinse at least 5 times in E3 to remove chorion debris and any remaining pronase (see **Note 4**).
5. Assemble the screening plate with one 96-well mesh plate, one 96-well receiver plate, and one single-well reservoir tray. Add 250 µl of E3 to each well of the screening plate using a multi-channel pipette. Make sure that the solution flows through the mesh (see **Note 5**).
6. Using a Pipette Pump with a glass Pasteur pipette, transfer 5 dechorionated embryos to each well of the screening plate (see **Note 6**):
 - a. In many chemical libraries, Columns 1 and 12 of the compound plates are empty. Thus, these wells can be used for positive and negative controls.
 - b. Add wild-type embryos to wells in Column 1 to serve as positive controls. These embryos should stain positive with *gata1* probe.
 - c. Add transgenic embryos to the remaining wells including Column 12.
 - d. Add 0.5 µl DMSO to wells in Column 12. These wells should stain negative with *gata1* probe and will serve as negative controls for the assay.
7. Add 0.5 µl of each individual compound from the compound plate to the corresponding wells in Column 2–11 of the screening plate. Use the pipette tip to gently mix the solution while adding the compound (see **Note 7**).
8. Reseal the compound plate with foil seal when finished. Store the chemical libraries at –80°C.

3.3 Heat Shock Treatment and Fixing the Embryos

1. Put the lid back on the screening plate and let stand at 25–28.5°C for at least one hour before heat shock. The embryos should not exceed 18-somite stage before heat shock.
2. Remove the single-well reservoir tray from the rest of the screening plate and float the screening plate (including the lid, 96-well mesh plate and 96-well receiver plate) in a 39°C water bath for 1 hour to heat-shock the embryos.
3. Remove the screening plate from the water bath, replace the reservoir tray to the bottom of the screening plate and incubate embryos at 28.5°C for 90 minutes.
4. Remove the reservoir tray and pour 30 ml of 4% PFA/PBS solution into it. Lift the mesh plate, let drain of the solution and place the mesh plate directly into the reservoir tray containing the fixative. Make sure all of the embryos are covered in the solution. Store the screening plate overnight at 4°C.
5. Clean the 96-well receiver plate for future use (**3.5 Whole-Mount In Situ Hybridization** step 7).

3.4 Digoxigenin Labeling of Antisense RNA Probe

1. Digest 10 µg of pBS-ZG1 with *Xba*I. This will yield linearized plasmid DNA. Run one hundredth of the sample on an agarose gel to confirm that the digestion is complete.
2. Purify the linearized DNA with Qiagen PCR Purification kit. Alternatively, the DNA may be purified by phenol/chloroform 1:1 extraction followed by ethanol precipitation.
3. Mix the following components in an RNase-free microcentrifuge tube:
 - 1 µg linearized DNA
 - 8 µl 5X transcription buffer
 - 4 µl 0.1 M DTT
 - 4 µl DIG RNA Labeling Mix
 - 1 µl RNase inhibitor (40 U/µl)
 - 2 µl T7 RNA polymeraseAdd RNase-free water to 40 µl and incubate in a 37°C water bath for 2 hours to overnight.
4. Following the reaction, add 2 µl of RNase-free DNase I to the reaction mix and incubate for additional 30 minutes in the 37°C water bath.
5. Use mini Quick Spin RNA Columns from Roche to purify the probe. Follow the manufacturer's protocol (see **Note 8**).
6. Dilute the purified probe from one 40-µl reaction into 16 ml of hyb(+) solution. This is enough for one 96-well screening plate. Store the probe/hyb(+) solution at –20°C.

3.5 Whole-mount In Situ Hybridization

1. (continued from **3.3 Heat Shock Treatment and Fixing the Embryos**) **Day 4:** Lift the mesh plate with the embryos, let drain of the solution, and pour 4% PFA/PBS from the reservoir tray to a waste container. Replace with 30 ml of 1X PBS, put the mesh plate back into the reservoir tray and incubate for 5 minutes at room temperature.

2. Lift the mesh plate, let the solution drain, and pour out the PBS in the reservoir tray. Replace with 30 ml of methanol, put the mesh plate back into the reservoir tray and incubate for 5 minutes.
3. Repeat methanol washes 3 times for 5 minutes each, leaving final wash. Store embryos for a minimum of 2 hours in methanol at -20°C . Alternatively, the plates may be stored long-term at -20°C .
4. **Day 5:** Wash plate as described above for 5 minutes each of 3:1, 1:1, 1:3 methanol:PBST solutions to rehydrate the embryos at room temperature. Use 30 ml of solution per plate for all washes (see **Note 9**).
5. Wash plates two times quickly in PBST, followed by 4 more washes of PBST for 15 minutes at room temperature. During these washes, heat hyb(-) solution and probe/hyb(+) solution in a 68°C incubator.
6. Replace final PBST wash with warm hyb(-) buffer and incubate at 68°C for 30 minutes to 2 hours.
7. At the end of step 6, add 160 μl of probe/hyb(+) solution to each well of the 96-well receiver plate.
8. After hyb(-) incubation, lift the mesh plate from the reservoir tray and let the solution drain. Place the mesh plate into the 96-well receiver plate. Make sure that all of the embryos are covered with probe/hyb(+) solution. Place the screening plate including the lid, the mesh plate and the receiver plate in a humidified chamber and incubate at 68°C overnight. Clean the reservoir tray for later use (step 10).
9. Keep hyb(-) solution at 68°C . Prepare 3:1, 1:1, 1:3 hyb(-):2X SSC solutions, 80 ml per plate, 2X SSC, 80 ml per plate, and 0.2X SSC, 160 ml per plate. Store at 68°C for **Day 6**.
10. **Day 6:** Pour 30 ml of hyb(-) solution into the reservoir tray. Transfer the mesh plate from the 96-well receiver plate to the reservoir tray and incubate at 68°C for 5 minutes.
11. Collect the probe/hyb(+) solution from the 96-well receiver plate into a 50-ml Falcon tube. Solution can be used once again. Bring up to 16 ml with hyb(+) and store at -20°C .
12. Pour warm 3:1 hyb(-):2X SSC solution into the tray of the BioLane™ HTI machine. Transfer the mesh plate from the reservoir tray to BioLane™ HTI. Up to four plates may be processed at once using BioLane™ HTI (see **Note 10**). Clean the reservoir tray for later use (step 16).
13. Set up the program in BioLane™ HTI according to Table 1. Connect the solutions to the corresponding ports of the machine. The volume required per wash per plate is 80 ml. Start the program.
14. **Day 7:** Prepare NTMT solution. Reserve 25 ml NTMT per plate and connect remaining NTMT to the correct port on BioLane™ HTI.
15. Add 56.25 μl of NBT (100 mg/ml) and 87.5 μl of BCIP (50 mg/ml) substrates per 25 ml of reserved NTMT solution.
16. At the end of the final NTMT wash, pour NTMT with substrates into the reservoir tray and remove the mesh plate from the machine into the reservoir tray. Cover the screening plate with the lid, wrap in aluminum foil, and incubate at room temperature.
17. Check staining periodically using a dissecting microscope. Check the staining of the positive and negative control embryos (Fig. 2). The development is complete when

the positive control embryos show strong purple staining in the posterior blood island, or the intermediate cell mass (ICM). Stop the reaction if the negative control embryos start to show any staining in the ICM or when the background staining becomes obvious.

18. To stop the reaction, lift the mesh plate and change the solution in the reservoir tray into PBST. Replace the mesh plate and incubate for 5 minutes at room temperature. Repeat PBST wash once more.
19. Inspect the staining of each embryo under a dissecting microscope and log the results into 8×12-grid Excel spread sheets. Embryos can remain in PBST to be photographed using a light microscope and camera either as separate wells of the mesh plate (Fig. 2) or as individual embryos on the lid of petri dish. The embryos can be stored within the plate in PBST at 4°C for several weeks, although the color of the staining will fade over time.

4. Notes

1. In this experiment, we use Tg(*hsp:AML1-ETO*) zebrafish to identify chemical modifiers of AML1-ETO function. Other lines of zebrafish may be used for different experimental designs.
2. The use of dividers ensures the synchrony of embryo stages, which is crucial to this experiment. Transferring embryos from 28.5 to 24°C slows down the embryonic development, so that they will be at the desired stage on the next day.
3. The chemical libraries should be aliquoted into several copies to prevent frequent freeze-thaw cycles.
4. In general, it takes about 15 to 30 minutes to dechorionate embryos with pronase. Prolonged incubation with pronase or insufficient rinsing after pronase incubation will cause destruction of the embryo, as evidenced by disintegration of the embryo during later steps of the *in situ* hybridization protocol. Once dechorionated, embryos are fragile and have a tendency to stick to dry plastic surface. Handle with care.
5. If the solution does not flow through the mesh right away, suck the solution back up with the multi-channel pipette and expel the solution again. Once the mesh is wet, the solution should flow through easily.
6. To transfer embryos without increasing the volume of the solution in each well, simply hold the dial on the pump (to prevent expelling the solution) and gently tap the tip of the glass pipette in the solution of each well. The embryos will naturally come out of the glass pipette and sink into the well.
7. The volume of the chemical libraries to use is determined by the concentrations of the compound stocks and the desired effective dose range. We performed this screen at 20 µM concentration. Compounds may be added using a multi-channel pipette or a 96-pin transfer device. If using a pin transfer device, the device should be cleaned between different compound plates. This is done by dipping the pins into a DMSO bath and an ethanol bath, and by briefly flaming the device to remove any residual solution.
8. The quality of the RNA probe synthesized may be monitored on a denaturing agarose gel. The yield of the RNA probe may be quantified using a spectrophotometer. We typically obtain around 10–20 µg per 40-µl reaction.
9. The steps from rehydration to hybridization may also be done using BioLane™ HTI. However, the volumes of the solutions required for each step will need to scale up to

80 ml per plate. In addition, all solutions containing Tween-20 should not be stored for long-term use. Stocks may be stored for long term without Tween-20.

10. All of the following steps may also be done manually by changing the solutions in the reservoir tray.

Acknowledgments

The authors would like to thank Dr. Randall T. Peterson for his advice and support during the development of this project. J.-R. J. Yeh is supported by a Career Development Award (AG031300) from the National Institute of Aging. This work was supported by RO1 CA118498 and the Claflin Distinguished Scholar Award.

REFERENCES

1. Davison AJ, Zon LI. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene* 2004;23:7233–7246. [PubMed: 15378083]
2. Yeh J-RJ, Munson KM, Chao YL, Peterson QP, MacRae CA, Peterson RT. AML1-ETO reprograms hematopoietic cell fate by downregulating scl expression. *Development* 2008;135:401–410. [PubMed: 18156164]
3. Yeh J-RJ, Munson KM, Elabgib KE, Goldfarb AN, Sweetser DA, Peterson RT. Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nat. Chem. Biol* 2009;5:236–243. [PubMed: 19172146]
4. Detrich HW, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, Zon LI. Intraembryonic hematopoietic cell migration during vertebrate development. *PNAS* 1995;92:10713–10717. [PubMed: 7479870]

Figure 1. Expression of AML1-ETO in embryonic zebrafish reprograms hematopoietic cell fate

At 15-somite stage, erythroid cell markers such as *gata1* are strongly expressed in the intermediate cell mass (ICM) of zebrafish embryos (indicated as a red stripe in the ventral side of the trunk). In the absence of AML1-ETO (top scheme), the hematopoietic progenitor cells in the ICM will continue to express erythroid cell markers and will eventually become red blood cells. Only a small number of cells in the ICM express myeloid cell markers (indicated as green dots). However, induced expression of AML1-ETO (bottom scheme) leads to rapid downregulation of *gata1* in the ICM and eventually the accumulation of myeloid cells at 2 days post fertilization (dpf). Red stripes and red dots, cells expressing erythroid cell markers; green stripe and green dots, cells expressing myeloid cell markers.

Figure 2. The *gata1* staining in the positive and negative control embryos

Strong staining of *gata1* probe appears in the ICM region of the heat-treated wild-type embryos (A) but not the heat-treated Tg(*hsp:AML1-ETO*) embryos (B). Five embryos were arrayed into each well of the screening plate. The *gata1* staining was performed as described in the Methods.

Table 1

Program for whole-mount *in situ* hybridization using BioLane™ HTI.

Step	Solution	Time	Temperature
1	3:1 hyb(-):2X SSC	15 min	68°C
2	1:1 hyb(-):2X SSC	10 min	68°C
3	1:3 hyb(-):2X SSC	10 min	68°C
4	2X SSC	10 min	68°C
5	0.2X SSC	30 min	68°C
6	0.2X SSC	30 min	68°C
7	3:1 0.2XSSC:PBST	5 min	22°C
8	1:1 0.2XSSC:PBST	5 min	22°C
9	1:3 0.2XSSC:PBST	5 min	22°C
10	Blocking solution	2 hours	22°C
11	Antibody solution	2 hours	22°C
12	PBST	10 min	22°C
13	PBST	10 min	22°C
14	PBST	4 hours	4°C
15	PBST	4 hours	4°C
16	PBST	4 hours	4°C
17	PBST	4 hours	4°C
18	PBST	10 min	22°C
19	PBST	10 min	22°C
20	PBST	4 hours	22°C
21	NTMT	10 min	22°C
22	NTMT	10 min	22°C
23	NTMT	10 min	22°C