

Effects of Valproic Acid on Somitic Development and Chondrogenesis in *Danio rerio*

Bryan Graye '01 and Jeff Mindel '02
Spring 2001

Revised by Jake Beckman, Spring 2004

Experimental Introduction and Summary:

The Pax-1 gene has been shown to be an important regulator of axial skeletal patterning and is specifically implicated in the formation and maturation of the distinct somite sclerotome that gives rise to the axial skeleton as vertebrae, intervertebral discs, ribs, and neural arches (Smith & Tuan, 1995). Studies also suggest that Pax-1 function is required for the transition from the mesenchymal stage to the onset of chondrogenesis. (Wallin J. et. al. 1994)

The chemical valproic acid is known to be a powerful teratogen that inhibits the expression of the gene Pax-1 in some vertebrates. When tested on chicken embryos, valproic acid affected Pax-1 expression, causing the disorganization or fusion of somites (Barnes et. al., 1996). Other studies showed that rats treated with valproic acid demonstrated similar results of fused ribs, missing vertebrae, and kinky tails (Vorhees, 1987).

Valproic acid is an anticonvulsant drug used to treat epilepsy. Since its release for human use in 1967, it has been found to cause a number of congenital birth defects in the children of women who use the drug. Such defects include facial malformations and skeletal abnormalities (Lammer et. al., 1987).

We examine the effect of valproic acid on somite patterning and cartilage formation in Zebrafish embryos by incubating developing embryos in varying concentrations of valproic acid and then staining for somite boundaries and cartilage. Normal zebrafish somite development begins at approximately 10-11 hours after fertilization. Our incubation valproic acid concentrations will be 1.5M, 0.1M, 0.05M, and 0.025M.

It is our hypothesis that we will see an increase in somite abnormalities and cartilage malformation proportionally related to valproic acid concentration. We can classify the somite abnormalities according to the guidelines set out by Smith and Tuan (1996).

Materials Required:

Mature zebrafish on 10 hr./14 hr.-dark/light cycle
Valproic acid

Procedure:

1. Collect *Zebrafish embryos* at the 16 or 32-cell stage, prior to the mid-blastula transition and store in Zebrafish embryo media.

2. At seven hours after fertilization (the time of first somitogenesis), Divide embryos into three groups containing approximately the same number of embryos each.

Incubation solutions:

1. ZES (control)
2. 0.05M valproic acid
3. 0.1M valproic acid

3. Prepare two solutions of valproic acid and Zebrafish Embryonic Solution (ZES). Use ZES without valproic acid as a control.

4. Pour solutions into three separately labeled dishes and add each group of embryos.

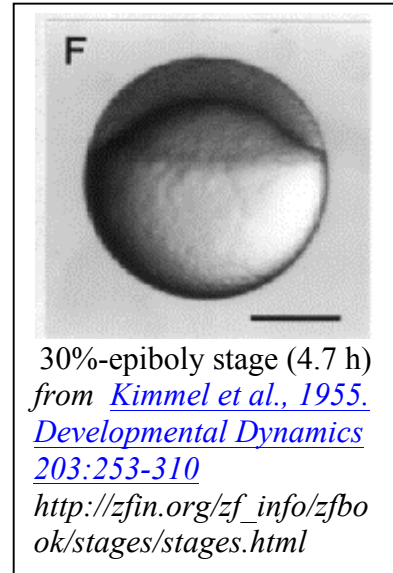
5. Incubate embryos at 18-20°C for 20 hours.

6. Remove half the embryos from each group (call them Set A). Set A will be stained with F6, an antibody that stains somite boundaries. The remaining embryos (set B) will grow until they are four days old, at which point they will be stained with Alcian Green in order to observe any anomalies in cartilage formation.

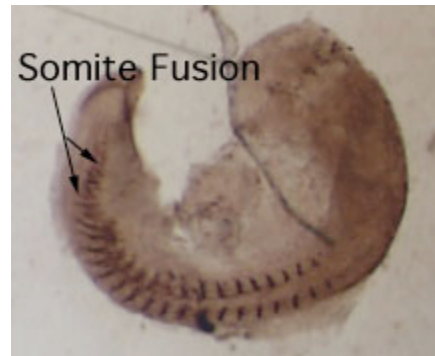
7A. With Set A, follow F6 antibody staining protocol given on last page.

7B. With set B, follow the Alcian Green staining protocol given on the last page.

8. Photograph stained embryos. Score somite stained embryos into two classes as formulated by Smith and Tuan (1996). Class I anomalies refer to a discrete fusion of an adjacent pair of somites or a mis-segmentation of a single somite. Class II anomalies refer to regions devoid of new somite formation or containing disorganized or "scrambled" somites (Smith & Tuan, 1996).



Images from previous experiment:



F6 antibody stain for somites in an embryo incubated in 0.05 M valproic acid. Shows Class I anomalies.



F6 antibody stain shows normal somite patterning in 28 hour control embryo



F6 antibody stain for somites in an embryo incubated in 0.1 M valproic acid. Shows Class II anomaly

Additional Protocols

F6 Whole mount antibody staining

Prep- divide 0.5 M and 1.0 M groups into two halves. The first half will be incubated in both the primary antibody and the secondary; the second group will be incubated in only secondary antibody.

	F6 primary	TgG+ secondary
1st half	+	+
2nd half		+

1. Dechorionate 26 hr embryos (pharyngula stage) carefully with two fine forceps. Transfer to fixative (1% formaldehyde in PBS). Fix for 1 hour rocking at 4 degrees.
2. Wash with 5 ml 0.1% BSA in PBS for 10 minutes. Wash 3X with 5 ml PBS, 10 minutes each.

3. Incubate 1st half overnight at 4 degrees (cold room) in 0.5 ml primary antibody in 0.2% saponin in PBS. Use F6@ 1/500 (somite boundaries). Place other half in cold room.
4. Wash for a minimum of 2 hours with several changes of 0.2% saponin in PBS (can store in cold room at this point).
5. Incubate both halves overnight with 0.5 ml secondary antibody conjugated to peroxidase (HRP) (Goat anti- mouse IgG+IgM-HRP) @ 1/500at 4 degrees.
6. Wash for a minimum of 2 hours with several changes of 0.2% saponin in PBS (can store in cold room at this point).
7. Wash with 1 ml HRP substrate buffer.
8. Develop with HRP substrate (metal-enhanced DAB). Monitor under dissecting microscope after 5 minutes. When brown color is clearly visible, stop reaction by washing with PBS.
9. Clear embryo by soaking in 1:1 glycerol:CMFET, followed by 1:4 glycerol:CMFET. Observe with dissecting microscope, and mount with depression slides to observe with compound microscope.

Alcian green stain for cartilage:

Alcian green stain binds to cartilage, but not calcified bone. This allows studying the early skeletal formation.

1. Fix embryos in 5% TCA for 1 hr. at room temp.
2. Wash 3 times with PBS.
3. Immerse in Alcian green stain for 4 hours with gentle agitation.
4. Wash 3 times with 70% ethanol.
5. Wash with 85%, 95%, and 100% ethanol.
6. Clear embryos by washing 1-3 times with methyl salicylate.

----->Alcian green stain:0.1g Alcian green
(100 ml)70 ml100% ethanol
1 ml conc. HCl
29 ml dH2O

Literature Cited

Barnes, G. L., Hsu, C. W., Mariana, B. D., and Tuan, R. S. 1996. Valproic acid-induced

somite teratogenesis in the chick embryo: Relationship with Pax-1 gene expression. *Teratology*. 54: 93-102.

Gilbert, Scott F. 2000. Developmental Biology. 6th ed. Sinauer Associates: Sutherland, Massachusetts.

Lammer, E. J., Sever, L. E., and Oakley, G. P. 1987. Teratogen update: Valproic acid. *Teratology*. 35: 465-473.

Smith, C. A., and Tuan, R. S. 1995. Functional involvement of Pax-1 in somite development: Somite dysmorphogenesis in chick embryos treated with Pax-1 paired-box antisense oligodeoxynucleotide. *Teratology*. 52: 333-345.

Vorhees, C. C. 1987. Teratogenicity and development toxicology of valproic acid in rats. *Teratology*. 35: 195-202.

Wallin J, Wilting J, Koseki H, Fritsch R, Christ B, Balling R. 1994. The role of Pax-1 in axial skeleton development. *Development*. May;120(5):1109-2