Quantifying the interaction between IWR-1 and fulvestrant in the migration of the primordium in zebrafish: independent project final report

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Introduction

The primordium in zebrafish is a migratory epithelial structure which makes its way down the body of the fish during development, depositing neuromasts along the way, a sensory organ for detecting changes in water flow consisting of a hair cell surrounded by support cells with the potential to replace it if damaged. This system, called the lateral line, is of interest to neuroscientists both because it is an instance of hair cell regeneration, which is relevant to hearing loss in humans, and because the primordium migrates via a signaling pathway that is also exploited by human cancer cells; the differential activation of the chemokine receptors *cxcr4b* and *cxcr7b* by SDF-1 (Gamba et al., p. 1). Activation of *cxcr4b* makes cells "leading end," flattening them, and activation of *cxcr7b* makes them "trailing end," unflattening them and making them organize into rosettes (clumps of cells which will later be deposited as neuromasts). SDF-1 runs in equal amounts down the horizontal myoseptum, so it is being bound by both of these receptors constantly – the question is to which ones it binds more. In order to create a gradient between the level of activation of these receptors, the morphogen Wnt, which pools in the anterior end of the fish, represses *cxcr7b* in the leading edge – indirectly promoting *cxcr4b* – by modulating FGF in the anterior. FGF, in turn, directly upregulates *cxcr7b* expression from the posterior end. Aman and Piotrowski found that when Wnt is inhibited, primordia migrate to the tip of the tail without depositing any rosettes, presumably due to *cxcr7b* being expressed everywhere along the primordium; they came to this conclusion after heat-shocking zebrafish embryos at different times to induce expression of the Wnt-repressor *dkk1*, finding that, the later they did this, the later the primordium stopped depositing neuromasts (Aman and Piotrowski, 2008, figures 4J and 4E). Migration at large, however, remained unaffected, as the lateral nerve was still visible; they do not mention whether the speed of migration was affected (ostensibly not, as they do mention speed in cases where it does differ). There is a possibility that *cxcr4b* was not fully repressed in this experiment, and that a small amount of *cxcr4b* activity in the front of the primordium was enough to lure it forward. In the event that FGF alone is inhibited, the primordium ceases to migrate after the awkward deposition of a single rosette (Ma and Raible, 2009, pp. 2 and 3). This result indicates that either the gradient was destroyed as a result of a lack of *cxcr7b* in the trailing end, or the loss of the rosette formation function alone made migration impossible regardless of whether or not there was a *cxcr7b-cxcr4b* gradient. In summary, morphogens tightly regulate the differential expression of SDF-1 receptors in the primordium, causing cells in the leading end of the primordium to express more *cxcr4b* than *cxcr7b*, and vice versa in the trailing end.

Gamba et al.'s 2010 paper demonstrated that ESR-1 loss-of-function leads to ectopic *cxcr4b* expression (including the trailing region) and total repression of *cxcr7b* in zebrafish, similar to when FGF is inhibited but with the added effect of *cxcr4* promotion, slowing down the migration of the primordium (Gamba et al., 2010, figure 2b and c). They initially thought that ESR-1 was controlling *cxcr4* expression here by interacting with Wnt and having it express everywhere along the primordium, but found that injecting fish with an ESR-1 morphogen had no effect on the expression pattern of *lef1*, a Wnt effector, meaning that ESR-1 must repress *cxcr4* independent of it. This makes sense in light of Ma and Raible's claim that Wnt only

promotes *cxcr4b* by inhibiting FGF. However, Gamba et al. think that Wnt itself might repress ESR-1 in the leading edge, though they don't test it by combining their ESR-1 morpholino treatment with Wnt-inhibition or upregulation.

In this experiment, I will test that hypothesis by incubating fish with different combinations of IWR-1, a Wnt inhibitor, and fulvestrant, an ESR-1 inhibitor 22 hours post-fertilization, and observing the pattern of migration of their primordia with claudin as they age. If Wnt indirectly promotes *cxcr4* by downregulating *cxcr7b* and repressing ESR-1, a *cxcr4b* repressor, at the leading edge, treating fish with IWR-1 as well as fulvestrant could rescue the control phenotype, or at least produce something closer to it than the fish treated only with IWR-1 and/or only with fulvestrant; since ESR-1 is a *cxcr4b* inhibitor, repressing it will lessen the impact to *cxcr4b* expression from inhibiting Wnt, and, conversely, since Wnt, like fulvestrant, is potentially an ESR-1 inhibitor, repressing it should lessen whatever effect it has in conjunction with fulvestrant in the fulvestrant-only fish. Specifically, the IWR-1+Fulvestrant fish should show normal deposition and migration, the IWR-1-only fish should show normal migration and no deposition, and the fulvestrant-only fish should show disrupted migration and deposition. That being said, if the effects of drug incubation take place globally, changing total *cxcr4b* expression levels but ultimately not affecting the *cxcr7b*-*cxcr4b* gradient, there is a chance that nothing will change besides the speed of migration from the overall activation rate of this pathway. After all, since Wnt activity is already directional because it only pools on the anterior end by default, removing it and replacing it with a globally-acting *cxcr4b* promoter might not produce a rescue of the control, and vice versa with fulvestrant. Adding only IWR-1 should result in the primordium making all the way down the fish without depositing any rosettes, as found by Aman and Piotrowski. Because adding only fulvestrant might lead to ectopic *cxcr4b* expression, we would expect it to produce the same results that Gamba and company show in figures 2b and c of their paper, disrupted migration and deposition. The dependent variable is the length of the pLL at 36 hpf, as well as any qualitative vicissitudes in migration, such as observable disruption in deposition.

Overall, this experiment tests both the impact of Wnt and estrogen receptor activation on the expression of *cxcr4b* and the importance of the spatial distribution of morphogens and receptors in the development of the posterior lateral line.

Materials and Methods

Fish strain. I used forty −8.0cldnb:lynEGFP zf106 zebrafish, ten per experimental group, incubated in a slow-development chamber at 25° C.
Drugs. I incubated the fish with IWR-1 (diluted 10 mM IWR-1/DMSO to a final concentration of 10 μM) and/or fulvestrant (diluted 10 mM fulvestrant/DMSO to a final concentration of 10 μM) (Gorelick et al., 2014) at 22 hpf. Photos were taken at 42 hpf with our inverted fluorescence microscope "Betty" at 10x magnitude.

Results

The control group, surprisingly, exhibited the longest pLL, not the IWR-treated group, which in fact had an average marginally *lower* than the control's (**$F = 0.0012$, $p = 0.972588$**). Treatment with fulvestrant, however, significantly slowed the progress of the pLL (**$F = 13.3409$, $p =$**

0.002612). Despite its low sample size, the interaction between fulvestrant and IWR was significant, bringing the phenotype much closer to the IWR-treated phenotype than the fulvestrant-treated one (**F = 6.3006, p = 0.024974**). Thus, though IWR had little to no effect on its own, it interacted significantly with fulvestrant to produce a phenotype much closer to that of IWR-only fish and controls than to that of fulvestrant-only fish.

Table 1. Average length of pLL per drug treatment.

Drug treatment	Mean distance from cloaca to primordium (µM)	Standard error of mean	n
Control	1040.428	162	5
Fulvestrant	-399.383	357	5
IWR	492.304	162	5
Fulv+IWR	311.194	169	3

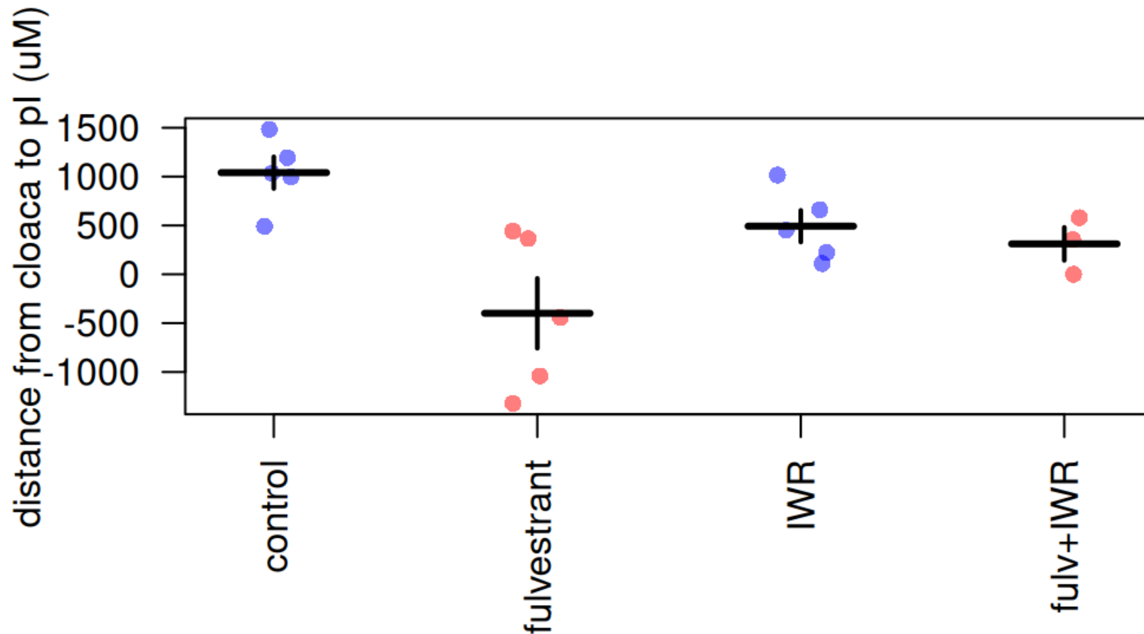
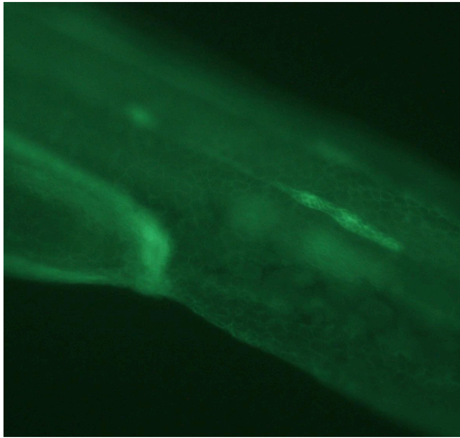
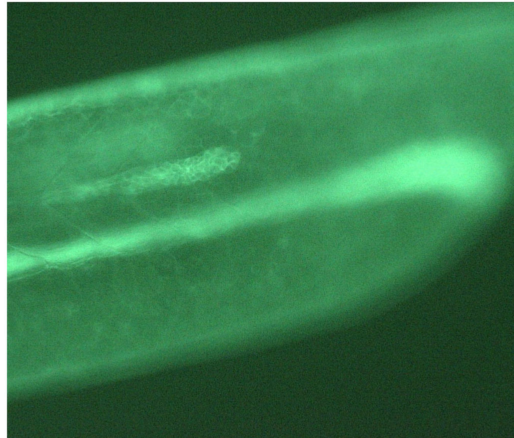


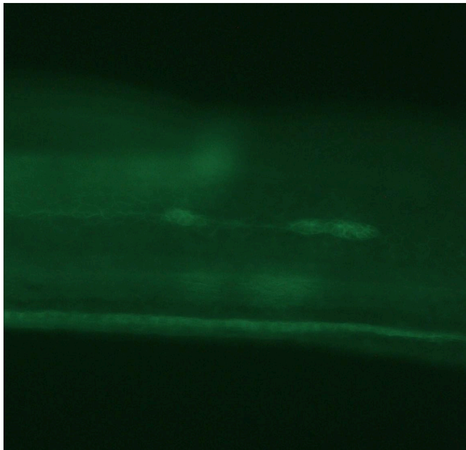
Figure 1. Average length of pLL per drug treatment, measured by distance from cloaca to the primordium. Fish treated with only fulvestrant had significantly shorter pLLs than the rest, including those treated with both drugs.



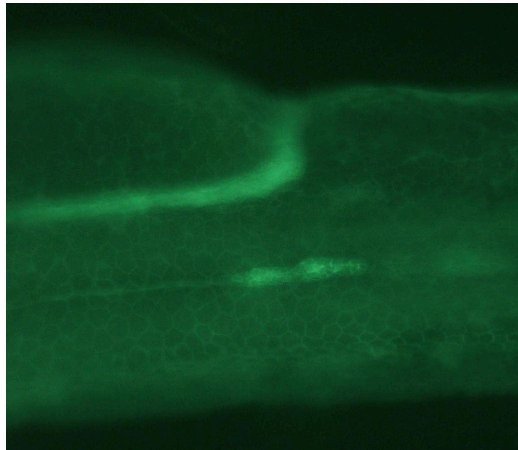
Control



Fulvestrant



IWR-1



Fulvestrant+IWR-1

Figure 2. Blue-light photos of claudin-stained posterior lateral lines from different zebrafish experimental groups. Left = anterior, right = posterior. In the control, the primordium has already migrated past the cloaca. In the fulvestrant-treated fish, it has not reached the cloaca. In the IWR-1 fish, the primordium is past the cloaca. Finally, in fish treated with both drugs, the primordium is marginally past the cloaca.

Discussion

Fish treated with fulvestrant had significantly shorter pLLs than controls, IWR-treated fish, and IWR-and-fulvestrant-treated fish. No differences in deposition were observed. This is likely because 10 mM fulvestrant / IWR-1 are weaker treatments than the 0.35 mM ESR-1 morpholino used by Gamba et al. and the heat-shock-induction of *dkk1* used by Aman and Piotrowski, respectively. It is likely that their experiments altogether eliminated *cxcr4b* or *cxcr7b* expression levels, while mine merely reduced them. Thus, small differences in *cxcr4b*/*cxcr7b* expression lead to differences in migration speed, whereas totalising, all-*cxcr4b*/all-*cxcr7b* differences affect deposition as well.

The fact that only the fulvestrant fish had slower migration, and that the two drugs exhibited a significant interaction in the IWR-1+Fulvestrant rescues, indicates that ESR-1 does not express differently and cause ectopic *cxcr7b* expression in the absence of Wnt, but that Wnt does cause ectopic *cxcr4b* expression in the absence of ESR-1. This could mean that, opposite to Gamba et al.'s hypothesis, the Wnt pathway might be repressed by ESR-1 activation, and that ESR-1 represses *cxcr4b* only indirectly by inhibiting Wnt. Inhibiting Wnt in addition to ESR-1 produces a phenotype closer to the control because the IWR-1 treatment is making up for the lack of Wnt-repression from ESR-1. The remaining question is what stops ESR-1 from activating in the leading end, if not Wnt. One possibility is that the pLL receives a gradient of estrogen; another is that FGF signaling is required for ESR-1 expression.

These results also carry some implications about what 'leading end' and 'trailing end' fates mean. Because ectopic *cxcr4b* expression causes slower migration, it is safe to say that *cxcr7b* activation 'trailing end' identity is the main driver behind migration. Given Aman and Piotrowski's findings, it is likely that the point of being a 'leading end' cell is only to trigger rosette-formation and deposition. That being said, the marginal difference in migration speed between controls and the IWR-1 and IWR-1+Fulvestrant groups indicates that small differences in *cxcr4b* expression at the leading end could matter for migration speed; there might be a synergistic effect wherein the repression-battle between Wnt and FGF stimulates both of the pathways to express more in their respective domains than they would in the absence of the other morphogen.

Future directions

To see if the differences between these results and the previous literature really are due to differences in strength of treatment, different concentrations of the two drugs could be tested. Additionally, in-situ hybridization not only for ESR-1 would inform whether or not my prediction that ESR-1 does not express differently in the absence of Wnt-signaling is correct. In-situ hybridization for *cxcr4b* and *cxcr7b* would also inform whether or not the small differences in migration between control groups and IWR-1 and IWR-1+Fulvestrant groups were due to the presence of a small *cxcr4b* gradient. Finally, an estrogen treatment that would lead to ectopic ESR-1 activation would inform whether or not the endocrine system is responsible for bridling ESR-1 activation in the absence of Wnt.

References

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