Vivian Siegel
Editor, Cell

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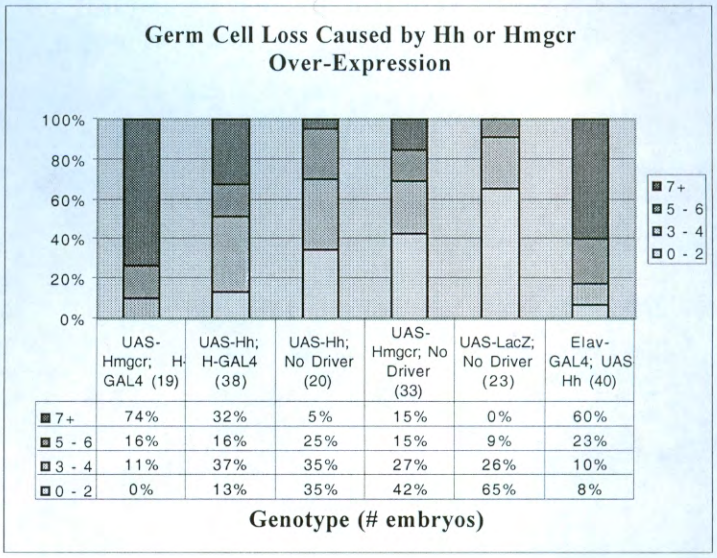
Updated March 28, 2002 Updates are in bold italic*

Dear Vivian,

I have now conducted some experiments to examine the question of whether Hedgehog (Hh) might be an attractant for pole cells during their migration to the somatic gonad in *Drosophila*. To accomplish this in as timely a manner as possible, it is impossible to be "complete"; yet, I feel I have accomplished enough to report to you what I see, and on which you might base your next decision. Please consider this preliminary, as I want to finish up one more experiment (that of a CNS GAL4 driver (Elav-GAL4), which is currently underway). ****I now have some data on this point (see below, both Chart & text)*.***

Ectopic Expression:
I did one complete "experiment", which entailed driving Hh expression using Hairy-GAL4 and examining the fate of Anti-Vasa-labeled pole cells in late stage embryos. As a positive control for migration defects, I drove HMG-CoA reductase (Hmgcr; the product of the Columbus gene) as examined (and defined in) Van Doren et al (1998). Three further controls included examining embryos containing a UAS-LacZ but no driver, a UAS-Hh but no driver, or a UAS-Hmgcr but no driver. All crosses and ageings were carried out at 25°C. The data is charted.



	UAS-Hmgcr; H-GAL4 (19)	UAS-Hh; H-GAL4 (38)	UAS-Hh; No Driver (20)	UAS-Hmgcr; No Driver (33)	UAS-LacZ; No Driver (23)	Elav-GAL4; UAS Hh (40)
7+	74%	32%	5%	15%	0%	60%
5 - 6	16%	16%	25%	15%	9%	23%
3 - 4	11%	37%	35%	27%	26%	10%
0 - 2	0%	13%	35%	42%	65%	8%

A final column presents data using a distinct Gal4 driver, Elav-GAL4. This experiment was done only for Hh.

Some information follows, that might be important to the parties involved, and to you. To complete these experiments in a timely manner, I could not harvest as many embryos at the appropriate

stage that one would like (I could not expand the stocks to collect more virgins for all the crosses, etc, and get you results inside of a month). Nevertheless, the numbers of embryos I examined, I believe is significant, and leads to significant results (see below).

In addition, to get the collections done on a schedule that I could deal with, and still have enough embryos to score, the crosses involved some flies that are heterozygous for the H-GAL driver:

H-GAL4 / TM3 x UAS-Responder / UAS-Responder

Thus, although all embryos have one copy of the responder, some embryos will not have a driver. Since all crosses were done this way, the effects should be quite roughly the same for the UAS-Hh and the UAS-Hmgcr cross. As you will see below, there is no way for me to guess which embryos have both the driver and responder, and which have only a responder. This is because, in my hands, embryos having only a responder appear to have germ cell loss defects (see below).

I counted the total number of germ cells that were "outside" (i.e., significantly away from) coalescing or formed gonads in an appropriately staged embryo. The number of embryos that happened to be of the correct ages for me to score is listed in parentheses for each genotype in the chart (e.g.: "UAS-Hmgcr; H-GAL4 (19)"). I grouped embryos into classes, similar to the chart you provided to me from the Lehmann work. What is charted then, for example, is the percent of the embryos with, say, 7 or more lost germ cells. As a specific example, 14 out of the 19 embryos scored of the genotype "UAS-Hmgcr; H-GAL4" had 7+ lost pole cells, whereas 12 out of 38 embryos scored of the genotype "UAS-Hh; H-GAL4" had 7+ lost pole cells.

As to the data. First, note that in what are essentially wild-type embryos, some pole cells go astray (UAS-LacZ; No Driver). This has been previously noted, and is expected.

Note also that in embryos carrying either UAS-Hmgcr or UAS-Hh but without a driver more pole cells go astray than observed in wild-type embryos. To my mind, this is not unexpected. Transgenes based on the UAS-GAL4 system are believed to have "basal", GAL-4-independent expression; this could vary from construct to construct, of course. Presumably, there is some amount of ectopic expression without a driver for both the UAS-Hh and the UAS-Hmgcr construct. Note that this expression would be assumed to be generally "unpatterned", although there could certainly be peaks and troughs. The fact that there is some effect without a driver does complicate things, and I will return to this point below. It is worth noting here that, again in the interest of time, for the controls that involved examining embryos containing a UAS-Hh but no driver, or a UAS-Hmgcr but no driver, I had to examine embryos containing two copies of the UAS construct. The "experimentals" contained one copy of responder (and one of the driver). The degree to which one rather than two copies of UAS-responder might reduce the driver-independent germ cell loss is unknown.

In the positive control, UAS-Hmgcr; H-GAL4, there appears to be a significant increase in stray pole cells as compared to the strain without the driver. This is especially so, given that I am comparing to a strain without the driver, but having two copies of UAS-Hmgcr.

Finally, for the experimental, UAS-Hh; H-GAL4, there also appears to be an increase in the number of lost pole cells, and this appears to be significant compared to UAS-LacZ; No Driver embryos. This is especially so, given that I am comparing to a strain without the driver, but having two copies of UAS-Hh. ***Numbers of lost germ cells in the H-GAL4; UAS-Hh background are not reported by Lehmann et al, submitted, so I cannot compare this data to theirs.****

Updated: In my hands, there does appear to be germ cell loss associated with expression of Hh by Elav-GAL4, as indicated by data plotted in the last column. This contrasts strikingly with the data plotted in Lehmann et al, submitted, and in their Figure 3). I do not know the reason for this discrepancy.

Details (important): male Elav-GAL4 / Y x virgin female UAS-Hh / UAS-Hh. Thus, all embryos should have one copy of the UAS-Hh responder, but only half of the embryos have the Elav-GAL4 driver. As with my other crosses, I did not unambiguously genotype the embryos. I did not repeat a determination of the germ cell loss rate with UAS-Hh alone, in parallel with this particular experiment. However, the striking affects with the Elav-GAL4 driver would certainly suggest a solid germ cell loss phenotype.

Please note that I obtained UAS-Hh virgins for this cross from Girish Desphande; I did verify by eye color (a marker for the UAS transgene) that the flies sent had an appropriate yellowish-orange eye color (this matches the UAS-Hh flies I have, and, for example, does not match the UAS-Hmgcr flies I have. Thus, I have no reason to suspect that these flies were not UAS-Hh).*

However, the crux of the mater is whether pole cells are attracted to sites of (ectopic) Hh expression, as they appear to be to sites of (ectopic) Hmgcr expression (Van Doren, et al, 1998). Given the fact that pole cells do go astray in embryos containing either UAS-Hmgcr or UAS-Hh (but no driver), one has to carefully discriminate between pole cells gone astray and those "targeting" to sites of ectopic expression.

Perhaps the best way to do this experiment is to examine embryos simultaneously expressing ectopic (or ectopic Hmgcr) and LacZ, so one can mark the cells expressing Hh ectopically. One would then score Vasa-positive cells near/touching/adjacent to LacZ-positive cells. I did not have time to construct the appropriate stocks for this experiment. And, in fact, neither lab appears to have done it this way (neither Deshpande, et al 2001, nor Van Doren et al, 1998). No matter; there are two ways around this. One is to use a different driver, expressed in, say, the nervous system (like Elav-GAL4), and pay careful attention to whether the lost pole cells now "migrate" to that tissue. This was reported in both Van Doren et al, 1998 and Deshpande, 2001, using Elav-GAL4, but is a point of

contention between Lehmann and Desphande. I am currently doing this experiment examining for affects caused by ectopic Hh expression.

In the meantime, using the H-GAL4 driver data, there is another way to approach whether pole cells are attracted to sites of ectopic expression with some measure of confidence. One can count only those "lost" pole cells that are in regions where H-GAL4 is expressed. The H-GAL4 pattern is known, and I also stained embryos expressing both H-GAL4 and UAS-LacZ to remind myself of that pattern.

During development, as germ cells emigrate from their endodermal pocket, the as generally located near "posterior" mesoderm. Thus, germ cells simply "lost" in posterior portions of the embryo are not necessarily being attracted to H-GAL4 sites of expression. In wild-type embryos, the coalesced gonad forms at about a region spanning the posterior part of segment 5 and the beginning portion of segment 6. You may recall that Hairy (H-GAL4 is a GAL4 enhancer trap near the Hairy gene) is a "pair-rule" gene. As a consequence of this, there is no or little H-GAL4 expression in posterior part of segment 6 and the beginning portion of segment 7. However, there is substantial H-GAL4 expression more posterior to this, spanning the posterior part of segment 7 and the beginning portion of segment 8. Van Doren et al, 1998 used this information to help in their documentation that Hmgcr ectopic expression directed pole cell migration.

From another set of slides, I scored embryos and lost pole cells again. I tallied up total lost pole cells (see Table: "Total Lost"), but now also specially noted the subset of the total that appeared to be "directed" near this site of ectopic expression (see Table: "Directed"). As I see H-GAL4 UAS-LacZ expression in both ectodermal and some mesodermal derivatives at these positions, I did not discriminate between germ layers.

	UAS-LacZ; No Driver (23)[a]		**UAS-Hh; H-GAL4 (20)**		**UAS-Hmgcr; H-GAL4 (16)**	
	Directed	Total Lost	*Directed*	Total Lost	*Directed*	Total Lost
	18	36	54	95	74	114
Mean	0.8	1.6	2.7	4.8	4.6	7.1
Std. Dev.	0.95	1.9	1.9	2.7	1.8	2.2

[a] The number of embryos scored.

Are the numbers of lost pole cells significantly different among the genotypes? Are the numbers of lost, but "directed" pole cells significant? I did some rudimentary statistics (a t test; frankly, I might have gotten "one" and "two-tailed" tests mixed up – but I think it does not matter, with these numbers).

First, considering total germ cell loss:

Comparing UAS-LacZ vs UAS-Hmgcr, 3.3218E-09

Comparing UAS-LacZ vs UAS-Hh, 4.2588E-05

Comparing UAS-Hmgcr vs UAS-Hh, 1.2899E-05

So, it seems germ cell loss is significant in Hh-expressing embryos. However, it is also different in quantity from Hmgcr-expressing embryos.

Next, considering germ cells "directed" to posterior A7 – anterior A8:

Comparing UAS-LacZ vs UAS-Hmgcr, 5.4067E-08

Comparing UAS-LacZ vs UAS-Hh, 0.00016914

Comparing UAS-Hmgcr vs UAS-Hh, 0.00182503

Conclusions: Ditto, the above.

I note that my numbers are slightly different (lower) than those reported for H-GAL4;UAS-Hmgcr in Van Doren et al, 1998. They report "7.4 germ cells lost in A7 (+/- 3.1)", compared with my 4.6 (+/- 1.8). I do not ascribe any significance to this difference.*

I still think it is important to repeat the Elav-GAL4 UAS-Hh experiment, and determine whether indeed germ cells now find their way near the CNS.

I did this experiment now. I tallied total lost Vasa-positive cells (germ cells), and also tallied those near the ventral nerve cord. I scored any cells within about two cell diameters as "near" the ventral cord. Most often, these cells were located lateral or dorsal to the cord.

I was not able to do Elav-GAL4; UAS-Hmgcr in parallel, as a positive control, myself.

I compare the data with Elav-GAL4; UAS-Hh to that I derived before with H-GAL4;UAS-Hh. That is, I went back and scored my H-GAL4;UAS-Hh now for germ cells lost "near the CNS" (there should not be any pronounced preference for this given the H-GAL4 pattern; this seemed as good a control as any I could do in this time frame). Most of my Elav-GAL4; UAS-Hh embryos on the slide were stage 14 and older.

	UAS-Hh; Elav-GAL4 (35) [a]		**UAS-Hh; H-GAL4 (22)**	
	Directed near CNS	Total Lost	*Directed near CNS*	Total Lost
	75	300	1	123
Mean	1.9	7.9	0.05	5.6
Std. Dev.	2.1	4.0	0.2	2.6

[a] The number of embryos scored

I indeed found Vasa-positive cells (germ cells) near the CNS, and in an apparently quantitatively significantly different manner than I observed for H-GAL4; UAS-Hh. Comparing the number of cells directed near the CNS using Elav-GAL4 vs H-GAL4, a t-test shows that the results appear significantly different at7.5375E-07

I should note that I did not see many embryos quite like those shown in Van Doren et al, 1998, Figure 4d. That is, embryos where six or more germ cells appeared nestled on top of the ventral nerve cord (I am assuming their report shows them associated ventrally with the nerve cord). Rather, as I indicated above, I found germ cells near the nerve cord, and usually dorsal (or lateral) to it. Perhaps my observations are similar to that shown in Deshpande et al, 2001, but I cannot tell for sure from their photo (Figure 2, Elav-GAL4/ UAS-Hh panel).

I did note many lost germ cells apparently within the gut in my Elav-GAL4; UAS-Hh embryos. I do not know the significance of this, or if it differs from Van Doren et al, 1998, since they do not mention this (neither does Deshpande, et al 2001). I raise this point because although there is striking germ cell loss in Elav-GAL4; UAS-Hh, the number "directed" to the CNS is the essential contested point here.

Whether my observations will resolve the issue of contention between the parties, I leave for them to decide.*

Smoothened mutants:

You asked in an email whether I might repeat one of the loss-of-function studies. I agree that this needs to be done. I could not (can not) grow up the flies from our stocks, and expand them enough to really do this in any reasonable time frame (I do have other commitments).

I did check our frozen stocks of embryos, and found I had done some germ-line clones of *smoothened* some time ago. I thought I would try to help you out and stain these with Anti-Vasa. Basically, I think it is a "no-go", however.

The point of contention between the two camps is that *smoothened* mutants show a phenotype at early-ish stages, and not later. Most of my embryos turned out to be of later stages. In scoring those, indeed, I do not think there is any significant germ cell "loss" phenotype. (If this no-go experiment eventually gets passed on to the labs concerned, please note a crinkle in the experiment: my embryos were derived actually *smo wg* doubly mutant germline clones, due to the specifics of the experiment I was doing at that time. Thus, it is not exactly a pure *smo* germline clone experiment).

In any event, even for the few embryos I had that were at appropriate early stages, I did not double label these in a way where I could unambiguously determine their genotype. (Some embryos on my slide are both maternally and zygotically mutant for *smoothened* – this affects germ cell migration for likely uninteresting reasons. The relevant embryos, according to Deshpande, are zygotically rescued *smoothened* maternal mutants, but – I could not genotype these, and then score their pole cell phenotype.) Sorry.

Respectfully,

Steve DiNardo