Variation in Gene Expression at Precisely-Timed Stages in *Drosophila melanogaster*

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Variation in gene expression has been found between isolates of many species, however current literature fails to control for heterogeneity, staging, and differences in environmental condition. In looking at a precisely timed, narrow window of development of four *Drosophila melanogaster* isolates, we controlled for these differential factors to explore whether the observed differences can be attributed to intrinsic differentiation in expression. We used qPCR to measure expression levels of the transcriptional factor EVE during the Blastoderm stage, which is crucial to normal development of *D. melanogaster.* Results obtained contradicted our hypothesis, by showing large differences in expression of the gene EVE between the four isolated strains, suggesting that variation is intrinsically different between isolates and that embryogenesis is robust against variation differences.

Project introduction and objectives

Expression variation refers to and is calculated by the percentage of genes between two organisms that show a significant difference in expression level. Previous research looking at expression variation has firmly established that between isolates of the same species there is extensive variation in adults and developed organisms. For example, 7% of genes were found to be expressed differentially between four cultured yeast isolates (Townsend et al., 2003), 18% of genes between two wild populations of *Fundulus sp* (Oleksiak et al., 2002), and 27% of genes between four isolates of *Drosophila melanogaster* (Baker et al., 2007). What these studies failed to consider was whether such genetic expression difference can be attributed to intrinsic differentiation in expression. Indeed, we know that genetic expression in adult organisms can be affected by the extent to which their environment interacts with their genome, a phenomenon known as epigenetics. Every one of the aforementioned studies looked between isolates that were not controlled for heterogeneity, staging, or environmental condition. Therefore, these studies cannot successfully conclude that genetic expression variation inherently exists within different isolates, as the variation observed could be a product of environment or age, for example. In our study, we therefore hypothesized that the expression level of an essential developmental gene during a narrow window of embryogenesis would in fact be highly conserved among genetic isolates. The organism we chose was *Drosophila melanogaster*. We hypothesized this because developmental genes are crucial to the formation of the body plan, and as the body plan of all *D. melanogaster* isolates are identical, we would expect that there would be very little room for variation in the manner by which this critical developmental feature occurs.

We decided to look at the expression variation of even-skipped (EVE) among four D. melanogaster strains, South Africa (A), Hawaii (H), Australia (A), and Malawi (M). EVE codes for a transcriptional repressor protein that binds to DNA, and is highly expressed during development beginning in the Blastoderm stages (4-6) of embryogenesis. EVE has a pair-rule pattern expression ensuring unique cell identity, segmentation, and polarity of the blastoderm (FlyBase). It is therefore a fundamental and primary determinant of organism development, and is highly conserved within the species. The first part of our project therefore focused around accurate identification of the Blastoderm stage in which EVE is known to be expressed, and subsequent confirmation of the precise post-fertilization timing that corresponded to this narrow developmental window. The Blastoderm stage is traditionally split into three stages - the syncytial stage, the mid-blastoderm transition (MBT), and the cellular stage - based on

clear morphological distinctions. The syncytial stage describes nuclei migration to the perimeter of the egg (Figure 1, left), MBT refers to when the cell membrane invaginates around each nucleus (Figure 1, right), and the cellular stage is when each cell has been fully encapsulated by its own cellular membrane (Figure 3). We confirmed, using a confocal microscope and DAPI staining (Figures 1-2), that all embryos collected between 2.5 and 4hr post-fertilization visually corresponded to one of these three stages, and that this was uniform across all isolates. We were then able to quantify the expression level of EVE using qPCR, in the knowledge that each embryo of each strain was in the Blastoderm developmental stage.

Methodology used

Embryo Collection

Four replicate egg laying cages per strain of *D. melanogaster* - M, A, S, and H – were acquired, each with a base egg-laying plate. The plates were changed 45 minutes before sunset, and subsequently collected 45 minutes after sunset, the period over which the reproductivity of *D. melanogaster* peaks. The collected plates were allowed to further develop for another 2.5hrs, to ensure that the age of the embryos laid were between 2.5 to 4hrs old.

RNA extraction

The embryos were grinded in the collection tube. RNA was extracted from each sample using the RNeasy minikit, and following the manufacturer's protocol (QIAGEN, RNeasy, TissueRuptor (QIAGEN Group) 1067551 01/2011). The RNA products were saved at -20°C.

cDNA production

Genomic DNA was removed from RNA products using a DNase treatment. Per sample, a sample master mix (16μl RNA sample, 4μl gDNA Wipeout Buffer, 8μl RNase-free H_20), and two reverse transcription (RT) master mixes, one -RT (4.4μl Quantiscript RT Buffer, 1.1μl RT Primer Mix, 1.1μl RNase-free H_20) and one +RT (4.4μl Quantiscript RT Buffer, 1.1μl RT Primer Mix, 1.1μl Quantiscript Reverse Transcriptase), were generated. After the samples were incubated for 2 minutes in 42°C water bath and then put back on ice, 6μl of one of the reverse transcription master mix and 14μl of the sample master mix was combined into each sample. The combined samples were incubated for 15 min × 42°C, and then 3 min × 95°C. The final cDNA products were stored at -20°C.

PCR Confirmation

The quality of cDNA and primers were tested using PCR followed by running PCR samples on agarose gels. The primers were prepared 1:2 from the stock and all samples and reagents kept on ice. To test the quality of the cDNA, the housekeeping gene RP49 was employed in a PCR mix (0.5μl of cDNA template (+RT) or DNase control (-RT), 1μl dNTP, 10μl Phusion buffer, 1μl RP49 forward primer, 1μl RP49 reverse primer, 0.5μl DNA polymerase, 36μl RNase-free H_20) that was added to each tube. To test the eve primers, the same PCR mix composition per tube was used, just with the RP49 primers replaced with equivalent amounts of the forward and reverse EVE primers. The samples underwent PCR Programme (I Cycler) for 15min ×42°C, and then 3 min × 95°C, before finally being stored at 4°C. The result of PCR was examined by agarose gel electrophoresis. The samples and primers that exhibited bright bands were deemed to contain an appropriate amount of cDNA and were chosen for further analysis, with each strain having three biological replicates for qPCR.

qPCR Analysis

Into each tube, a composition of 15μl sample master mix (7μl cDNA product, 28μl RNase-free H_20) and 60μl target master mix (212.5μl SYBR Green PCR master mix, 8.5μl forward primer, 8.5μl reverse primer, 111μl RNase water) was dispensed. 23.5μl of the resulting mixture in the tube was dispensed into each well of a 48-well plate. The 48-well plate was then centrifuged (4000 rpm ×2min), and expression of EVE then analyzed by qPCR, with RP49 chosen as the expression reference. The qPCR cycler ran the following stages: Holding stage (10minutes x 95°C), 35 cycles of cycling stage (15 seconds × 95°C and 1 min × 60°C per cycle), Melt Curve stage (15 sec × 95°C, 1 min × 60°C, 15 sec × 95°C). The Ct values of target genes were compared to those of the reference genes to obtain relative expression level of EVE. Statistical analysis of the ΔC_T values was performed in GraphPad Prism 7.03.

Results obtained

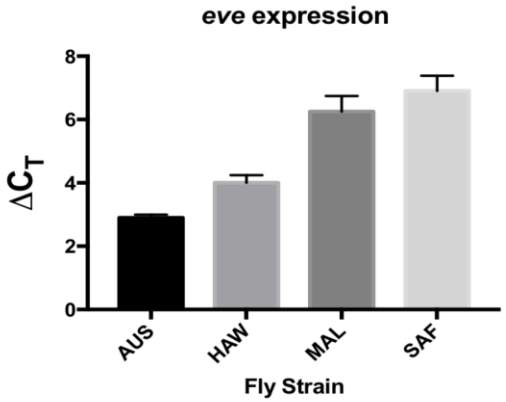

Figure 1. Expression for EVE differed significantly among four Drosophila isolates (ANOVA, p value = 0.008); EVE is expressed significantly higher in A than in M and S; EVE is expressed significantly higher in H than in M and S

Expression for EVE varied dramatically among the four *D. melanogaster* isolates. Specifically, both strains A and H had significantly higher eve expression that strains M and S ($p < 0.05$), although neither A and H nor M and S significantly different from each other. The expression level of A was in fact 16-fold higher than the expression level in S, an astounding quantification that describes an extremely significant level of gene expression variation.

Significance and interpretation of results

The results contradict our original hypothesis, as it shows that despite the precisely timed crucial developmental window and identical environmental conditions, and despite the vital role of EVE in the formation of the *D. melanogaster*, gene expression variation between isolates does seem to be innate to this species. The implications of this are that Drosophila embryogenesis seems to be extremely robust against variation in gene expression level in that it can still produce identical body plans between isolates.

Further research may focus on this innate differential gene expression at a molecular level, looking to see where exactly differences arise, and how changing the level of expression past a specific point may eventually affect embryogenesis and normal development.

Figures

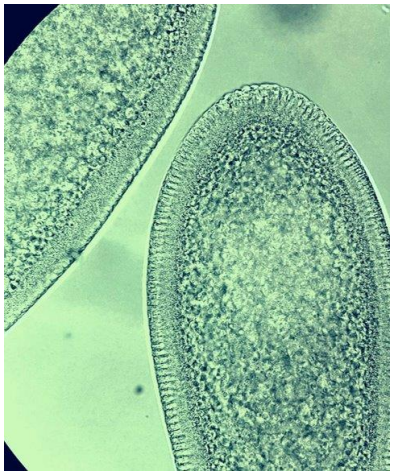

Figure 1. The left embryo depicts the syncytial stage of the Blastoderm stage with nuclei clearly visible along the inside edge of the embryo. The right embryo depicts the MBT stage of Blastoderm stage with invagination visible but not complete as cell membrane has not yet entirely surrounded each nucleus.

Figure 2. This embryo is in the cellular stage of the Blastoderm stage, with cell membrane visibly encapsulating each nucleus along the inside edge of the embryo.

Acknowledgements and references

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EVE information found on FlyBase retrieved from http://flybase.org/reports/FBgn0000606.html, accessed 01/05/18.

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