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Expression of Genes Involved in *Drosophila* Wing Morphogenesis and Vein Patterning Are Altered by Spaceflight

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INTRODUCTION

Imaginal wing discs of *Drosophila melanogaster* (fruit fly) defined during embryogenesis ultimately result in mature wings of stereotyped (specific) venation patterning. Major regulators of wing disc development are the epidermal growth factor receptor (EGF), Notch, Hedgehog (Hh), Wingless (Wg), and Dpp signaling pathways. Highly stereotyped vascular patterning is also characteristic of tissues in other organisms flown in space such as the mouse retina and leaves of *Arabidopsis thaliana*. Genetic and other adaptations of vascular patterning to space environmental factors have not yet been systematically quantified, despite widespread recognition of their critical importance for terrestrial and microgravity applications. Here we report changes in gene expression with space flight related to *Drosophila* wing morphogenesis and vein patterning. In addition, genetically modified phenotypes of increasingly abnormal ectopic wing venation in the *Drosophila* wing[1] were analyzed by NASA's VESSel GENeration Analysis (VESGEN) software[2]. Our goal is to further develop insightful vascular mappings associated with bioinformatic dimensions of genetic or other molecular phenotypes for correlation with genetic and other molecular profiling relevant to NASA's GeneLab and other Space Biology exploration initiatives.

METHODS

Gene Expression Analyses from Drosophila. Spaceflight-reared larvae and adult samples were collected, processed and analyzed as described previously by Marcu et al[3]. Briefly, the Gal4-UAS transgenic line of *Drosophila melanogaster* that expresses two copies of eGFP under the control of the hemolectin promoter was used in all experiments. RNA samples were processed and hybridized to Drosophila 2.0 Affymetrix® arrays. Six sets of larval arrays and 3 sets of adult arrays were used to provide repeats for statistical validation. The False Discovery Rate (FDR) criterion by Benjamini and Hochberg was applied to *p*-values.

VESGEN Mapping and Quantification. Binary vascular patterns extracted from grayscale images published by Johannes and Preiss[1] of the *Drosophila* wing (Figure 1) were analyzed by automated, user-interactive VESGEN software to generate parameters that include vessel diameter (D_v), fractal dimension (D_f) and densities of vessel area (A_v), length (L_v), number (N_v), and branch point (Br_v) as described previously[2] (Figure 2).

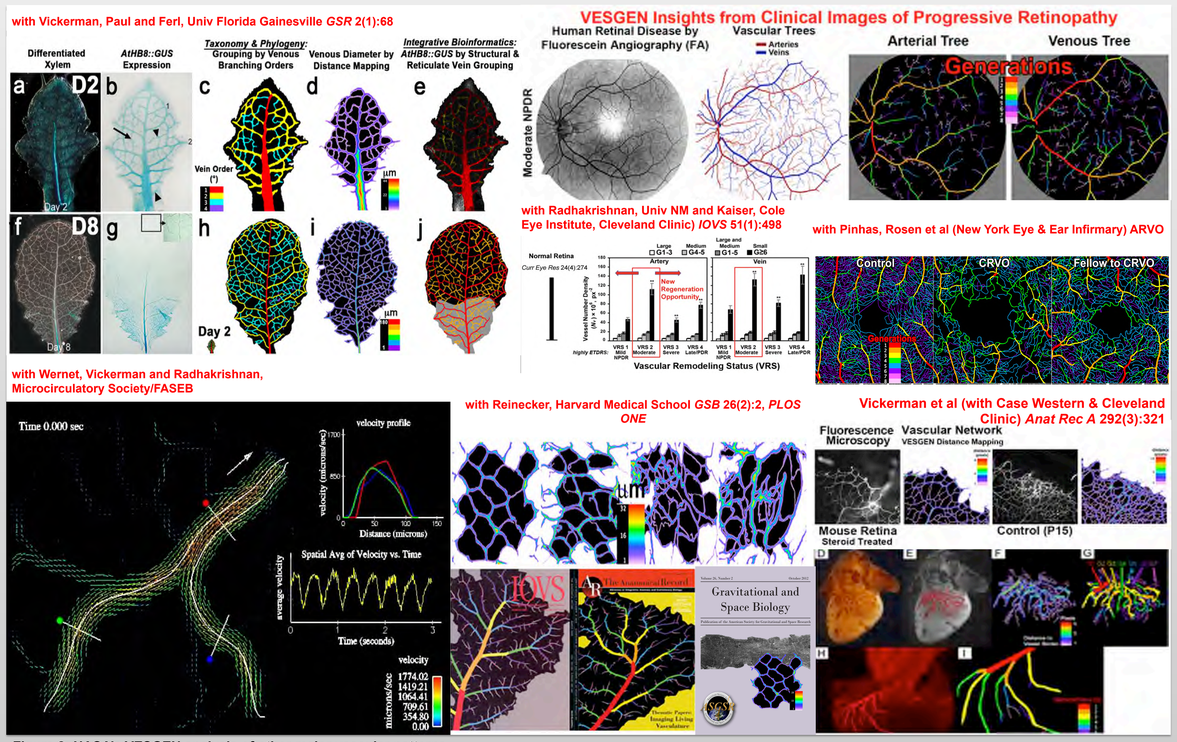

Figure 2: NASA's VESGEN analysis of other major vascular patterns
Other tissues important for space biology research and human space exploration include (clockwise) blinding vascular diseases in the human retina, where VESGEN methods are now being applied to NASA studies of astronauts and bed rest subjects; progressive inflammation in the mouse GI; a mouse model of human retinopathy of prematurity; developing coronary vessels in mouse and avian models; dependence of blood flow on vessel generation according to physiological fluid mechanics; lymphatic development and remodeling by active stem cell recruitment, and developing venation patterns in the leaves of *Arabidopsis thaliana*.

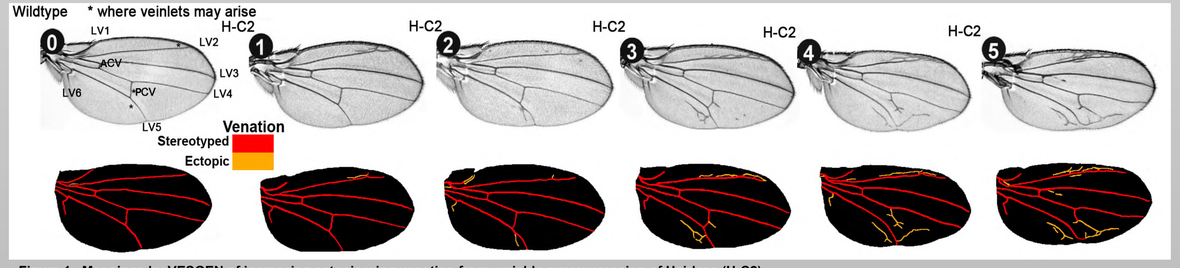

Figure 1 Mappings by VESGEN of increasing ectopic wing venation from variable overexpression of Hairless (H-C2)
(*left column*, images reproduced from Johannes and Preiss[4]) The basic aim guiding our vessel classification strategy was to differentiate between the highly stereotyped (normal) *Drosophila* venation patterning and ectopic (abnormal or additional) veins. Venation in the adult *Drosophila* wing generated by overexpression of H-C2 (Classes 1-5) are compared to wildtype (Class 0). Asterisks indicate sensitive, variable regions where ectopic veins can arise by H-C2 overexpression. Class 0 (wildtype), no ectopic veins; Class 1 (H-C2), ectopic veins in distal region of the costal cells between LV1 and LV2; Class 2 (H-C2), ectopic veins distal between LV1 and LV2 and close to LV5 in marginal cells; Class 3 (H-C2), increased branching of ectopic veins with vein dots between LV4 and LV5; Class 4 (H-C2), increased branching and detachment of posterior CV from LV5 and Class 5 (H-C2), massive network of ectopic veins and veinlets. Vascular maps generated by VESGEN compare stereotyped venation (red) with ectopic venation (orange). Hairless (H) is known to antagonize Notch signaling by binding to the Notch signal transducer, Suppressor of Hairless [Su(H)]. Deletion of the Su(H)-binding domain in a transgenic construct denoted H-C2 results in loss of H activity. Overexpression of H-C2 (Class 1 to 5[4]) by varying the copy number and heat shock (hs) induction levels of the hs promoter of the H-C2 transgene[4] generated the phenotypes of increasing ectopic venation.

Table 1 Changes in mRNA expression of selected genes in space-returned 3rd instar larvae that are involved in wing development.

Gene Name	Fold change	*p*-value	Biological processes
Vrille (CG14029)	-1.30	0.00	Imaginal disc-derived wing hair organization and biogenesis
Absent, small or homeotic discs 2 (CG6677, *ash2*)	+0.60	0.00	Imaginal disc-derived wing morphogenesis; Imaginal disc derived wing vein specification; Phenotypes of alleles manifest in wing vein L3, wing margin
CTP:phosphocholine cytidylyl transferase 1 (CG1049)	+0.60	0.00	Imaginal disc-derived wing morphogenesis
Pox neuro (CG8246)	-0.60	0.00	Imaginal disc-derived wing morphogenesis; Phenotypes of alleles manifest in ventral wing blade
Bx42 (CG8264)	+0.50	0.00	Notch signaling pathway; phenotypes of alleles manifest in anterior cross vein

Table 3 Overexpression of Hairless (H-C2) induces an ectopic vein phenotype in the adult *Drosophila* wing, but does not significantly affect stereotyped venation patterning. Stereotyped and ectopic wing venation resulting from overexpression of H-C2 (Johannes and Preiss[1]) was quantified by VESGEN in vascular maps (Figure 1) to obtain densities of vessel length (L_v, px px^{-2}), vessel area (A_v, px^2 px^{-2}) and vessel number (N_v, px^{-2}). Results for the wildtype and Class 5 wing are reproduced here.

phenotype	wing veins		ectopic veins		
	L_v	A_v	L_v	A_v	N_v
wild type	0.0250	0.0789	0.0004	0.0006	1
Class 5 ectopic veins, Compared to wildtype	0.0257 1.03×	0.0892 1.13×	0.0095 24×	0.0254 42×	18 18×

Table 2 Changes in mRNA expression of various space-returned genes across the wing disc and vein development signaling pathways in adult female flies.

Gene Name	Fold change	*p*-value	Biological processes
Epidermal growth factor receptor (EGFR) signaling pathway			
Stem cell tumor (CG33166)	-1.50	0.00	EGFR signaling pathway; Wing vein morphogenesis; Phenotypes of alleles manifest in wing vein
Aveugle (CG30476)	-0.80	0.00	EGFR signaling pathway; Phenotypes of alleles manifest in wing vein and wing disc
Rhomboid-4 (CG1697)	+0.70	0.00	EGFR signaling pathway; Phenotypes of alleles manifest in wing and wing vein
Rhomboid-7 (CG8972)	-0.70	0.00	EGFR signaling pathway; Expressed in developing wing veins
Pointed (CG17077)	+0.80	0.00	EGFR signaling pathway; Imaginal disc derived wing morphogenesis
Notch signaling pathway			
Sp1070 (CG9138)	+2.20	0.00	Negative regulation of notch signaling pathway; Notch binding
Bunched (CG5461)	+1.90	0.00	Negative regulation of notch signaling pathway; Phenotypes of alleles manifest in wing discs
Brainiac (CG4934)	-0.90	0.00	Notch signaling pathway
Shibire (CG18102)	+2.10	0.00	Positive regulation of notch signaling pathway; Wing vein extension; Veined wing generated song production
Hedgehog receptor activity			
Smoothened (CG11561)	-0.80	0.00	Negative regulation of notch signaling pathway; Wing disc anterior/posterior pattern formation; Smoothened signaling pathway
Other			
Discs overgrown (CG2048)	+0.80	0.00	Establishment of imaginal disc derived wing hair orientation
Piopio (CG2079)	+1.50	0.00	Apposition of dorsal and ventral imaginal disc derive wing surfaces; Imaginal disc derived wing morphogenesis
Held out wings (CG10293)	+0.90	0.00	Apposition of dorsal and ventral imaginal disc derive wing surfaces
Penguin (CG1685)	-0.80	0.00	Apposition of dorsal and ventral imaginal disc derive wing surfaces
Guftagu (CG11861)	+0.70	0.00	Imaginal disc derived wing morphogenesis

RESULTS

Microarray data from larvae (Table 1) and adult flies (Table 2) returned from space measured significant changes in genes important for wing development and vein patterning compared to ground controls. For instance, the hedgehog pathway regulates the positioning of longitudinal veins such as L3 and L4. Expression of the gene *Smoothened* with hedgehog receptor activity was significantly down regulated (-0.8 fold; *p*-value-0.00) in space-returned adult flies. Similarly, expression of *Rhomboid 7* (-0.7 fold; *p*-value-0.00) and *Aveugle* (-0.8 fold; *p*-value-0.00) were significantly down regulated in space-returned adult flies compared to ground controls. Expression of *Rhomboid* and *Aveugle* is critical in EGF-regulated stereotyped patterning of veins. In the case of space-returned larvae, expression of *ash2* was significantly up-regulated (+0.6 fold; *p*-value-0.00), suggesting possible changes in intervein cell fate that determines intervein patterning.

By confirming vascular parameters generated with VESGEN (Table 3, Figure 1), the eight stereotyped wing veins remained quite constant in genetically perturbed phenotypes compared to wildtype, including the most perturbed phenotype, Class 5. For example, A_v and L_v for stereotyped Class 5 vessels are 1.03× and 1.13× that of the wildtype. In Class 5, only the stereotyped PCV is incomplete. However, ectopic veins increased in number by N_v from 1 in the wildtype to 18 in Class 5; for the ectopic vessels, L_v increased from 0.0004 to 0.0095 px-px^2. A_v, L_v, and N_v for ectopic vessels are 24×, 42× and 18× greater compared to wildtype.

CONCLUSIONS AND DISCUSSION

Major regulators of wing disc development include genes important for the epidermal growth factor receptor (EGF), Notch, Hedgehog (Hh), Wingless (Wg), and Dpp signaling pathways. Most of these genes also play a vital role in wing vein morphogenesis. We measured significant changes in expression for a number of such genes that include *Smoothened*, *Rhomboid 7, Aveugle*, and *ash2*. Altered wing venation of *Drosophila* resulting from a series of increasingly perturbed gene expression was successfully mapped by NASA's VESGEN software to reveal that normal stereotyped vascular patterning was not significantly changed, despite the presence of increasingly abnormal ectopic vascularization. In the future, space-dependent changes in vascular patterning may be mapped by VESGEN to offer useful phenotypic read-outs of changes in genetic and other molecular signaling during *Drosophila* development and vascular adaptations of other important experimental model tissues such as *Arabidopsis* leaves and the rodent GI and retina (Figure 2).

REFERENCES

1. Johannes B, Preiss A: Wing vein formation in *Drosophila melanogaster*: hairless is involved in the cross-talk between Notch and EGF signaling pathways. Mechanisms of Development 2002, 115:3-14.
2. Vickerman MB, Keith PA, McKay TL, Gedeon DJ, Watanabe M, Montano M, Karunamuni G, Kaiser PK, Sears JE, Ebrahem Q, Ribita D, Hylton AG, Parsons-Wingerter P: VESGEN 2D: Automated, user-interactive software for quantification and mapping of angiogenic and lymphangiogenic trees and networks. Anat Rec A 2009, 292:320-32.
3. Marcu O, Lera MP, Sanchez ME, Levic E, Higgins LA, Shmygelska A, Fahlen FA, Nichol H, Bhattacharya S: Innate Immune Responses of *Drosophila melanogaster* Are Altered by Spaceflight. PLOS ONE 2011, 6:1-8.

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