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bantam miRNA is important for *Drosophila* blood cell homeostasis and a regulator of proliferation in the hematopoietic progenitor niche

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ABSTRACT

The *Drosophila* hematopoietic system is utilized in this study to gain novel insights into the process of growth control of the hematopoietic progenitor niche in blood development. The niche microenvironment is an essential component controlling the balance between progenitor populations and differentiated, mature blood cells and has been shown to lead to hematopoietic malignancies in humans when misregulated. MicroRNAs are one class of regulators associated with blood malignancies; however, there remains a relative paucity of information about the role of miRNAs in the niche. Here we demonstrate that *bantam* miRNA is endogenously active in the *Drosophila* hematopoietic progenitor niche, the posterior signaling center (PSC), and functions in the primary hematopoietic organ, the lymph gland, as a positive regulator of growth. Loss of *bantam* leads to a significant reduction in the PSC and overall lymph gland size, as well as a loss of the progenitor population and correlative premature differentiation of mature hemocytes. Interestingly, in addition to being essential for proper lymph gland development, we have determined *bantam* to be a novel upstream component of the insulin signaling cascade in the PSC and have unveiled dMyc as one factor central to *bantam* activity. These important findings identify *bantam* as a new hematopoietic regulator, place it in an evolutionarily conserved signaling pathway, present one way in which it is regulated, and provide a mechanism through which it facilitates cellular proliferation in the hematopoietic niche.

1. Introduction

The cellular organization and molecular signaling that occurs in *Drosophila* hematopoiesis strikingly parallels that which is seen in the hematopoietic stem cell niches of vertebrate animals, including several mammals [1–3]. An elegant example of such similarity is the recent discovery of the *Drosophila* hematopoietic progenitor niche which like its vertebrate counterpart, uses homologous signaling pathways for hematopoietic progenitor maintenance [4–8]. The primary hematopoietic organ, the lymph gland, is comprised of three morphologically and molecularly distinct compartments: (1) the posterior signaling center (PSC), which is the stem cell-like niche, (2) the medullary zone comprised of self-renewing hematopoietic progenitors, that eventually undergo progressive differentiation into the increasingly lineage restricted mature blood cells of (3) the cortical zone which directly surrounds the medullary zone. Cells of the PSC are specified by the homeotic gene *Antennapedia* (*Antp*) and maintained by the *Drosophila* ortholog of vertebrate Early B-cell Factor: Collier (Col) [6,9]. Regulatory signals, such as Hedgehog (Hh), emanate from the PSC in a non-cell-autonomous manner to preserve the progenitor population of the medullary zone [5,6,10]. In lymph glands lacking a PSC or that do not express hh, there is depletion of the progenitor population and concomitant, premature differentiation of mature hemocytes.

This emphasizes the essential role of this cellular domain in controlling the vital balance between proliferation of progenitors and lineage commitment to mature blood cells. It is thus of fundamental importance to elucidate the signals that govern maintenance of the PSC [4–6,11–14].

Several reports have detailed key contributions by miRNAs in regulating the development of specific hematopoietic lineages [15–20]. Furthermore there is evidence of their causative role in mediating malignant diseases in the hematopoietic system [21–24]. Owing to their important developmental roles and ability to target known oncogenes and tumor suppressors, miRNAs are a

Abbreviations: PSC, posterior signaling center; Antp, Antennapedia; Col, Collier; Hh, hedgehog; HSC, hematopoietic stem cell; InR, insulin-like receptor; col-Gal4, P85col-Gal4; Foxo, forkhead-related transcription factor; Bam, bag of marbles; Rbf, retinoblastoma-family protein.

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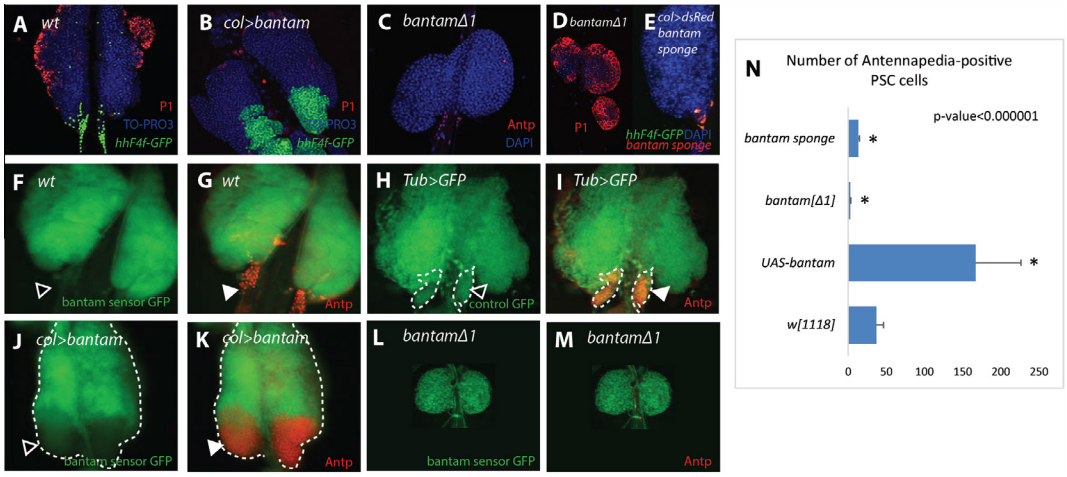

Fig. 1. Characterization of *bantam* miRNA expression and function in the PSC of the lymph gland. Anti-Antp staining and *hhF4f-GFP* transgene expression mark the PSC. Mature plasmatocytes are labeled with P1 staining and cell nuclei are stained with DAPI. (A) Wild type (wt) control lymph gland w^{1118} showing normal PSC size. (B) PSC specific overexpression of *bantam*, through *col-GAL4*. (C and D) lymph glands from $bantam^{\Delta 1}$ homozygotes. (E) *col*-driven expression of a *bantam sponge-dsRed* transgene. (F) Region of endogenous *bantam* activity indicated by loss of GFP expression in *bantam* sensor GFP background (empty arrowhead). (G) Anti-Antp staining indicates that the observed region of endogenous *bantam* activity occurs in the cells of the PSC (arrowhead). (H) Lymph gland expressing control sensor transgene express GFP due to insensitivity to *bantam* activity (empty arrowhead) (I) Region verified as PSC with anti-Antp staining (arrowhead) (J and K) *col*-driven *bantam* in *bantam sensor* GFP background. (J) Region of *bantam* activity (empty arrowhead) verified as (K) expanded PSC by anti-Antp staining (arrowhead). (L and M) Loss of *bantam*, in $bantam^{\Delta 1}$ homozygous null mutants. (N) Statistical analysis of PSC cells in loss of function and gain of function of *bantam*.

key part of the intricate molecular networks that drive or suppress cancer development and progression [16]. However, despite their clear relationship with hematopoietic development and cancer, there remains a paucity of knowledge regarding the expression patterns, the identity of their target genes, and in vivo function of miRNAs in hematopoietic stem cell (HSC) populations and their niches [25–27]. Since both uncontrolled HSC expansion as well as loss of HSC is deleterious for humans, an understanding of the molecular mechanisms of HSC fate decision is thus of considerable importance.

In this study, we investigate the role of *bantam* miRNA, in hematopoiesis and elucidate its relationship with the Insulin-like receptor (InR) pathway and dMyc in positively regulating growth [28–30]. The InR pathway is a critical, conserved pathway responsible for regulating cell size and number in *Drosophila* [31–33]. Previous studies have shown that InR pathway signaling impacts PSC size and maintenance of the blood progenitor population [34–38]. Similarly dMyc, the *Drosophila* transcriptional activator orthologous to the mammalian proto-oncoprotein c-Myc, has also been shown to be an important positive regulator of growth in the PSC [35,39–41]. Our studies present a novel role for this crucial miRNA specifically in regulating the hematopoietic niche through interacting with these signaling factors established to be essential in controlling growth and proliferation.

2. Materials and methods

2.1. Fly stocks

We used the following lines in this study: *P85col-Gal4* (*col-Gal4*) (M. Crozatier) [5,9]; *hhF4f-GFP* [14]; *bantam sensor GFP*, *control sensor*, *UAS-bantam-A*, $bantam^{\Delta 1}$ and *UAS-dsRed bantam sponge* (S. Cohen) [28,42]; UAS-myr::tdTomato, UAS-InR, UAS-dMyc, UAS-dMyc RNAi (TRiP JF1761), UAS-foxo, and w^{1118} obtained from the Bloomington Stock Center and UAS-Akt1 RNAi (GD1361) obtained from the Vienna Drosophila RNAi Center. Flies were reared on a conventional cornmeal-based medium. Egg-lays were done at 25 °C and the lymph glands of 5-days old (unless otherwise indicated) 3rd instar larvae were dissected. As a consequence of the delayed developmental timeframe of $bantam^{\Delta 1}$ homozygous null mutants, due in part to reduced growth rates, all larvae analyzed were 7-days old and kept at 29 °C for purposes of keeping comparative analysis consistent [29].

2.2. Immunostaining

Immunostaining was performed as described previously [14]. The following primary antibodies were used to determine protein expression in lymph glands dissected from control or mutant animals: mouse anti-plasmatocyte (P1) antibody (1:100) [43,44] and mouse anti-Antp antibody (1:100; 4C3, Developmental Studies Hybridoma Bank) [6]. As secondary antibodies, we used Alexa 488 or 555-conjugated mouse IgG antibodies (Invitrogen). Cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Samples were analyzed and imaged with a Zeiss Axioplan 2 fluorescence microscope or a Nikon A1R-NP laser-scanning confocal microscope.

2.3. Counting PSC cell number

A minimum of twenty, individual lymph glands were analyzed for each genotype. Statistical analyses of Antp-positive cell counts were performed as previously described [45] and analyzed with a *t*-test.

3. Results and discussion

3.1. bantam miRNA is an essential regulator of growth in the PSC and blood cell homeostasis in the lymph gland

Extensive studies have been conducted to determine the functions and interactions of signaling pathways in regulating the larval hematopoietic organ. However, it is not currently known whether miRNAs are involved in PSC development. We were specifically interested in investigating the impact of *bantam* miRNA, a known positive regulator of growth in *Drosophila*, on the hematopoietic system [28,30,46–48].