HHS Public Access

Author manuscript

Cell. Author manuscript; available in PMC 2015 April 20.

Published in final edited form as:

Cell. 2011 December 23; 147(7): 1589–1600. doi:10.1016/j.cell.2011.11.041.

Interaction between differentiating cell and niche-derived signals in hematopoietic progenitor maintenance

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Summary

Maintenance of a hematopoietic progenitor population requires extensive interaction with cells within a microenvironment or niche. In the *Drosophila* hematopoietic organ, niche-derived Hedgehog signaling maintains the progenitor population. Here we show that the hematopoietic progenitors also require a signal mediated by Adenosine deaminase growth factor A (Adgf-A) arising from differentiating cells that regulates extracellular levels of adenosine. The adenosine signal opposes the effects of Hedgehog signaling within the hematopoietic progenitor cells and the magnitude of the adenosine signal is kept in check by the level of Adgf-A secreted from differentiating cells. Our findings reveal signals arising from differentiating cells that are required for maintaining progenitor cell quiescence, and that function with the niche-derived signal in maintaining the progenitor state. Similar homeostatic mechanisms are likely to be utilized in other systems that maintain relatively large numbers of progenitors that are not all in direct contact with the cells of the niche.

Keywords

VEGF; PDGF; *Drosophila*; hemocyte; niche; Pvr; adenosine signaling; lymph gland; progenitor; quiescence

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Introduction

The mammalian hematopoietic niche displays complex interactions between populations of HSCs and progenitors to maintain their numbers (Garrett and Emerson, 2009). The relative *in vivo* contributions of cues emanating from the microenvironment in regulating stem cell versus progenitor maintenance remains unclear (He et al., 2009; Martinez-Agosto et al., 2007; Orkin and Zon, 2008). Several (Jude et al., 2008; Tumbar et al., 2004), but not all (Li and Clevers, 2010) stem cell and progenitor populations demonstrate slow cell cycling and this property of "quiescence" is critical for maintaining their integrity over a period of time.

In vivo genetic analysis in *Drosophila* allows for the study of stem cell properties in their endogenous microenvironment (Losick et al., 2011). *Drosophila* blood cells, or hemocytes, develop within an organ called the lymph gland, where differentiating hemocytes, their progenitors, and the cells of the signaling microenvironment or niche, are found (Jung et al., 2005). Differentiated blood cells in *Drosophila* are all myeloid in nature and are located along the outer edge of the lymph gland, in a region termed the cortical zone (CZ, (Jung et al., 2005), Figure 1A). These arise from a group of progenitors located within an inner core of cells termed the medullary zone (MZ). The MZ cells are akin to the common myeloid progenitors (CMP) of the vertebrate hematopoietic system. They quiesce, lack differentiation markers, are multipotent, and give rise to all *Drosophila* blood lineages (Jung et al., 2005; Krzemien et al., 2010). MZ progenitors are maintained by a small group of cells, collectively termed the posterior signaling center (PSC), that function as a hematopoietic niche (Crozatier et al., 2004; Krzemien et al., 2007; Mandal et al., 2007). Clonal analysis has suggested the existence of a niche-bound population of hematopoietic stem cells (Minakhina and Steward, 2010), although such cells have not yet been directly identified.

The PSC cells express Hedgehog (Hh), which is required for the maintenance of the MZ progenitors (Mandal et al., 2007). Cubitus interruptus (Ci) is a downstream effector of Hh signaling similar to vertebrate Gli proteins; it is maintained in its active Ci^{155} form in the presence of Hh and degraded to the repressor Ci^{75} form in the absence of Hh (Smelkinson and Kalderon, 2006). PSC-derived Hh signaling causes MZ cells to exhibit high Ci^{155} (Mandal et al., 2007).

Proliferation of circulating larval hemocytes is also regulated by Adenosine Deaminase Growth Factor-A (Adgf-A), which is similar to vertebrate adenosine deaminases (ADAs). Adgf-A is a secreted enzyme that converts extracellular adenosine into inosine by deamination (Dolezal et al., 2003; Maier et al., 2001). Two distinct adenosine deaminases, ADA1 and ADA2/CECR1, are found in humans. CECR1 is secreted by monocytes as they differentiate into macrophages (Zavialov et al., 2010). In *Drosophila*, mutation of *Adgf-A* causes an increased adenosine levels and increase in circulating blood cells (Dolezal et al., 2005; Zurovec et al., 2002). Extracellular adenosine is sensed by the single *Drosophila* adenosine receptor (AdoR) that generates a mitogenic signal through the G-protein/adenylate cyclase/cAMP-dependent Protein Kinase A (PKA) pathway (Dolezelova et al., 2007). A target of PKA is the transcription factor Ci, which also transduces the Hedgehog signal. We were intrigued by the potential link between adenosine and Hedgehog signaling,

both through PKA mediated regulation of Ci, and propose a model that the niche signal and the CZ signal interact to maintain the progenitor population in a quiescent and undifferentiated state within the MZ of the lymph gland.

Results

Signals from differentiating hemocytes regulate hematopoietic progenitor quiescence

During the mid second instar, blood cells initiate differentiation in the larval lymph gland marking the beginning of cortical zone (CZ) formation (Figure 1A). The first cells that express differentiation markers appear stereotypically at the peripheral edge of the lymph gland (Figure 1A). These differentiating cells will eventually populate an entire peripheral compartment that will comprise the CZ. The timing of the first signs of differentiation matches closely with the onset of quiescence among the precursor population, eventually giving rise to the medullary zone (MZ).

The close temporal synchronization of CZ formation and the quiescence of MZ progenitors raised the intriguing possibility that the onset of differentiation might regulate the proliferation profile of the progenitors. To test this hypothesis, we induced cell death by expressing the pro-apoptotic proteins Hid and Reaper in the differentiating hemocytes and assayed for the effect of their loss on the progenitor population. We found that loss of CZ cells induces proliferation of the adjacent progenitor cells, which are normally quiescent at this stage (Figure 1B-C; S1A-B).

We knocked-down candidate ligands in the lymph gland by RNA interference (RNAi) and monitored for a loss of progenitor quiescence. This survey identified Pvf1 as a signaling molecule that is required for the maintenance of quiescence within the lymph gland. Expressing $Pvf1^{RNAi}$ using Gal4 drivers specific to either niche (PSC) cells using *Antp-gal4*, progenitor cells using *dome-gal4*, or differentiating cells using *Hml-gal4* showed that PSC-specific knockdown is sufficient to induce progenitor proliferation (Figure 1D; S1C), whereas *Pvf1* knockdown in progenitors or differentiating cells has no effect on the lymph gland (Figure S1G-I). These results indicate that Pvf1 synthesized in the PSC is required for progenitor quiescence.

To determine the site of Pvf1 function, its receptor Pvr was knocked down in the lymph gland using a similar approach. Interestingly, we found that Pvr^{RNAi} expressed under the control of drivers specific to differentiating cells (*Hml-gal4* and *pxn-gal4*) causes a loss of progenitor quiescence (Figure 1E; S1D). The BrdU incorporating cells do not express differentiation markers (Figure S1A-F). Thus, differentiation follows the proliferative event. Lymph glands are not similarly affected when *Pvr* function is downregulated in the progenitors themselves (Figure S1J). These results indicate that Pvf1 originates in the niche and activates Pvr in maturing hemocytes, and that this signaling system is important for the quiescence of MZ progenitors. These results did not explain, though, how maturing cells might signal back to the progenitors causing them to maintain quiescence.

Given the previously known role of Adgf-A in the control of hemocyte number in circulation (Dolezal et al., 2005), we investigated whether this protein plays a similar role in

the lymph gland. Remarkably, downregulation of the secreted Adgf-A protein in the differentiating hemocytes of the CZ, achieved by expressing *Adgf-A*^{RNAi} under *Hml-gal4* control, induces loss of quiescence of MZ progenitors (Figure 1F; S1E), similar to that seen with loss of Pvr in the CZ. This suggests that Adgf-A may act as a signal originating from differentiating hemocytes that is required for maintaining progenitor quiescence. In support of this idea, while overexpression of *Adgf-A* in differentiating hemocytes alone does not affect normal zonation (Figure S4G), it suppresses the induced progenitor proliferation caused by downregulation of *Pvr* (Figure 1G; S1F). Note that the phenotype in Figure 1C is similar to that seen in Figures 1E and F, but for different reasons. For loss of signaling molecules (Figures 1E-F), it is the break in the signaling network necessary for reducing adenosine that causes continued proliferation and eventual differentiation. For *rpr/hid* (Figure 1C) the signaling cell itself has been removed, thereby causing a lack in a backward signal. Quantitative analysis of the data (from Figure 1B, E, G, see legend) is consistent with a role for Adgf-A downstream of Pvr and leads us to the model shown in Figure 1H, the mechanistic details of which are genetically dissected in this study.

Step 1: Signaling from the niche to the differentiating cells

Pvf1 is expressed in the same cells as Hedgehog within the PSC in the primary lobe of the second instar lymph gland (Figure 1 I, J). Additionally, punctate dots that label with the anti-Pvf1 antibody can be seen throughout the lymph gland (Figure 1I) in Rab11-positive recycling vesicles (Figure 1K). When *Pvf1* RNAi is targeted to the PSC, the Pvf1-positive punctae are eliminated throughout the lymph gland, suggesting that Pvf1 expression is limited to the PSC and the protein is transported by transcytosis from the PSC to the rest of the cells of the lymph gland. Additionally, clones of cells created within the lymph gland that lack the *Pvr* receptor are greatly reduced in Pvf1-positive punctae (Figure 1L,L′). The simplest explanation is that intercellular Pvf1 transport involves receptor-mediated endocytosis and the recycling of Pvf1 in Rab11 positive vesicles. With regard to Pvr expression, we found that it is absent from the PSC at all developmental stages. Pvr is initially expressed at low levels in early blood progenitors, but is then strongly up-regulated in the first differentiating hemocytes of the forming CZ during the second instar (Figure 1M). By the late third instar, all cells of the lymph gland (except PSC cells) express detectable levels of Pvr, although CZ cells continue to be higher in their expression (Figure 1N).

The loss of the progenitor cell quiescence phenotype upon loss of Pvf1/Pvr signaling apparent in the second instar (Figure 1D, E) is manifested in the third instar as a terminal phenotype in which all progenitor cells of the lymph gland differentiate. Cells occupying the position of the MZ in normal lymph glands all express differentiation markers in the mutants, leaving no progenitors behind. This is a correlation in every genetic background where the precursor population fails to quiesce in the second instar and continues to incorporate BrdU; the third instar phenotype is complete differentiation of the cells that would, in wild type, occupy the MZ and remain undifferentiated. This phenotype is observed when *Pvf1*^{RNAi} is expressed in the PSC (Figure 2B) or when *Pvr*^{RNAi} is expressed in the differentiating hemocytes (Figure 2C). Pvr receptor activation is reduced when Pvf1 is downregulated in the PSC (Figure S3A-B). Importantly, TUNEL staining (Figure S1K-N′)

and *p35* misexpression studies (Figure S1O-P) show that Pvr^{RNAi} does not cause an increase in cell death levels compared to wild type when expressed in the developing CZ. Furthermore, cells lacking Pvr differentiate properly as assessed by retention of their phagocytosis function (Figure 2D). The phenotype caused by loss of Pvf1/Pvr signaling is reminiscent of the fully-differentiated phenotype seen upon loss of *hedgehog* function (Mandal et al., 2007). However, despite the similarity in loss of function phenotypes, Hedgehog expression in the PSC is not altered when Pvr is removed from the CZ (Figure 2C) or Pvf1 is removed from the PSC (Figure 2E). This suggests that the Pvf1-Pvr signaling process operates in parallel to the niche signal from Hh.

Knockdown of the second Pvr ligand Pvf2 by *RNAi* in the PSC does not cause a loss of progenitor quiescence and subsequent differentiation (Figure S2C-D). Rather, Pvf2 controls Shg (E-Cadherin) expression and prevents inappropriate migration of the progenitors into the CZ (Figure S2E-J, H).

Step 2: Signaling within the developing CZ

VEGFR-like receptors including Pvr function in the context of multiple effector molecules including Ras (Learte et al., 2008; Sims et al., 2009), Jun kinase (JNK) (Bond and Foley, 2009; Ishimaru et al., 2004; Mathieu et al., 2007), and STAT (Fu and Zhang, 1993; Li et al., 2002). Loss of Ras function in the CZ, achieved using a dominant negative form, does not phenocopy *Pvr* loss-of-function in these cells (Figure 2F). Also the *bona fide* marker of JNK activation, *puc-lacZ*, is not active in the CZ (not shown). In contrast, similar to *Pvr*, inactivation of *Stat92E* in the differentiating cells of the CZ using either $Stat92E^{RNAi}$ (Figure 2G), $Stat92E^{DN}$ (Figure 2H), or the STAT inhibitor, dPIAS (Figure 2I) causes loss of MZ progenitors. Furthermore, single copy loss of *Pvr* and *Stat92E* in the genetic combination Pvr^{C2195}/+; $Stat92E^{06346}$/+ gives a similar loss of progenitor cell phenotype (Figure 2J; S3J). This second site non-complementation between *Pvr* and *Stat92E* suggests a close genetic relationship between them within a developmental pathway (see for example, (Giansanti et al., 2004; Rogge et al., 1991). In support of STAT functioning downstream of Pvr in differentiating cells, misexpression of $Stat92E^{act}$ is sufficient to suppress the Pvr^{RNAi} phenotype (Figure 2L). Taken together, these data indicate that the developmental role of Stat92E in MZ progenitor cell maintenance is non-autonomous and attributable to a function of Stat92E in the CZ.

Stat92E loss-of-function clones generated outside of the CZ do not cause a differentiation phenotype (Figure 2M). Whole animal mutants in which the entire lymph gland lacks *Stat92E* do show differentiation of the progenitors (Krzemien et al., 2007) likely due to loss of Stat92E function in the CZ. Furthermore, Stat92E canonically functions downstream of the Domeless (Dome) receptor and the JAK homolog Hopscotch. However neither *dome* nor *hopscotch* loss of function causes a loss of progenitor quiescence and ectopic differentiation (Figure 2N-P). Domeless is not expressed in the CZ and as expected, expression of $dome^{DN}$ in the CZ has no effect on progenitor maintenance (Figure S3C-D). Therefore during normal development STAT functions downstream of Pvr in the CZ and the Dome/JAK-STAT pathway does not appear to have a role in this process. As previously proposed, Dome/JAK-

STAT signaling in the MZ progenitors is required for their competency for immune-related responses (Krzemien et al., 2007; Makki et al., 2010).

Within the CZ, expression of a *GFP* reporter of active STAT signaling can be seen in a few scattered cells (Figure 2Q1-9). These cells are negative for MZ markers (Figure 2Q1), a subset actively proliferate (Figure 2Q2), and they also label with an antibody (Janssens et al., 2010) against the activated form of the Pvr receptor (Figure 2Q3-4). These cells express *Hml* (Figure 2Q5) but lack all markers of terminal differentiation (Figure 2Q6-9). The activity of this STAT reporter is reduced in a number of relevant genetic backgrounds involving components of this model (Figure S3E-H″). The reporter-expressing cells are likely "intermediate progenitors" (Krzemien et al., 2010) that receive an active Pvr/STAT signal and it is possible that these cells are the first to initiate the backward signal required for the Domeless positive progenitor quiescence (Figure 2R).

Step 3: A signal from differentiating cells to progenitor cells

As alluded to in Figure 1F, the primary candidate for a secreted signaling protein that regulates progenitor quiescence and is derived from differentiating cells is Adgf-A (Novakova and Dolezal, 2011). When *Adgf-A* is eliminated from differentiating cells, the progenitor population fails to quiesce, suggesting a central role for Adgf-A in CZ-derived signaling. Ultimately, the loss of quiescence associated with loss of *Adgf-A* function, either in whole-animal mutants (Figure 3B) or when $Adgf\text{-}A^{RNAi}$ is expressed in differentiating cells (Figure 3C), causes complete differentiation of the hematopoietic progenitor cell population. The loss of progenitors is not due to defects in PSC-derived signaling, as evidenced by intact Antp and Hh expression (Figure S4A-C). Also, the loss of progenitors in $Adgf\text{-}A^{RNAi}$ backgrounds is not associated with changes in reactive oxygen species levels, as they remain unchanged when Adgf-A function is downregulated in the differentiating hemocytes (Figure S4D-E). The phenotypic attributes due to loss of Adgf-A are the same as those associated with loss of Pvr or STAT function in differentiating cells, suggesting that Adgf-A may function in the same signaling pathway. In support of this idea, genetic interaction studies demonstrate that the combination of a single loss-of-function allele of *Adgf-A* with a single loss-of-function allele of *Pvr* (Figure 3D; S3J) or *Stat92E* (Figure 3E; S3J) is sufficient to cause a loss of progenitors in the developing lymph gland. These results are strongly indicative of a close functional relationship between Pvr, STAT, and Adgf-A signaling within the developing CZ. Additionally, the phenotypes caused by loss of *Pvr* or *Stat92E* in the differentiating cells can be suppressed by the simultaneous over-expression of *Adgf-A* in these cells (Figure 3F,G), further demonstrating that Adgf-A functions downstream of Pvr and STAT to regulate progenitor maintenance. Based upon similar mutant phenotypes, close genetic interaction, and the ability of *Adgf-A* to suppress *Pvr* and *Stat92E* phenotypes, we propose the signaling pathway schematized in Figure 3H.

As described previously, the primary function of Adgf-A is to inactivate adenosine, and loss of *Adgf-A* causes an increase in extracellular adenosine levels (Dolezal et al., 2005; Zurovec et al., 2002). Additionally, adenosine levels can also be regulated by intracellular uptake mediated by the nucleoside transporter ENT3 (Baldwin et al., 2005). The adenosine that remains extracellularly can bind the adenosine receptor AdoR to transmit a G protein/

adenylate cyclase/PKA-mediated signal into the cell. Within the developing lymph gland, expression of *ENT3*^{RNAi} in the progenitors causes them to differentiate (Figure 4B), presumably because loss of *ENT3* among progenitors causes a local accumulation of extracellular adenosine that then signals through AdoR to cause them to proliferate and differentiate. This differentiation phenotype does not occur when *ENT3* is downregulated in differentiating cells (Figure 4C). In contrast, expression of *AdoR*^{RNAi} in the progenitor population causes an expansion of these cells at the expense of differentiation (Figure 4D) and over-expression of *AdoR* in progenitors causes a loss of progenitor maintenance (Figure 4E). These results indicate that adenosine signaling through AdoR in progenitor cells negatively impacts their maintenance, and that this signaling is critically controlled during development to maintain progenitors by the expression of Adgf-A in the newly differentiating cells.

AdoR is a seven transmembrane domain receptor that signals through G-proteins to activate adenylate cyclase (Dolezelova et al., 2007). Consistent with this notion, over-expression of an inhibitory G-protein, G_{alphai} (Yu et al., 2005) in progenitors blocks their differentiation (Figure 4F). Similarly, reduction of *Drosophila* adenylate cyclase function in progenitors using *rutabaga*^{RNAi} (Figure 4G) or *rutabaga* mutants (Figure S4P) causes the larger number of cells to be maintained as progenitors when compared with wild type (Figure S4F), suggesting that cAMP signaling normally promotes differentiation. Finally, the phenotype associated with loss of *Pvr* (which in our model will block Adgf-A function and increase extracellular adenosine; Figure 4H-J) or Adgf-A can be suppressed in an *AdoR* mutant background (Figure S4H-L). In both cases, the loss of quiescence correlates with differentiation of cells that normally occupy the MZ compartment. These results further establishes a role for Pvr and Adgf-A in counteracting adenosine signaling through AdoR and adenylate cyclase in progenitor cells leading to their maintenance as quiescent hematopoietic progenitors.

Step 4: A feedback loop that limits adenosine levels

Expressing *AdoR*^{RNAi} (Figure 4K) or *PKA*^{RNAi} (Figure 4L) in the CZ causes non-autonomous differentiation of MZ progenitors. This phenotype is suppressed by over-expression of *Adgf*-A in the CZ (Figure 4 M-N). In addition, although Pvr expression remains unaffected, no staining for activated Pvr is detected in cells that are mutant for *AdoR* (Figure 4P). The simplest model is that AdoR and Pvr collaborate together in a differentiating cell to activate Adgf-A, which is secreted and regulates the level of adenosine (Figure 4Q).

Step 5: Balancing PSC versus CZ signal

PKA occupies a unique position in being associated with both adenosine signaling (Dolezelova et al., 2007) and Hh signaling (Collier et al., 2004). Adenosine activates and Hh inhibits PKA, which promotes the formation of the repressor form of Ci, Ci^{75} (Collier et al., 2004), suggesting perhaps a link between the CZ and the niche-derived signals. PKA function was reduced in the MZ by expressing either the dominant negative *PKAmR** or the dominant negative regulatory subunit *PKA-R2* or *PKA*^{RNAi} (Figure 5A-D). In each case the result is a normal sized lymph gland in which there is a relative increase in the size of the

MZ at the expense of the CZ. The proliferative profile of progenitors with loss of function PKA does not change (Figure S4N-O). Thus loss of PKA activity causes cells to be maintained in a progenitor state but does not cause an overall expansion in the total number of cells in the lymph gland. Similarly, overactivating *PKA* using a mutated mouse PKA catalytic subunit (*PKAmC**) that is resistant to inhibition by its regulatory subunit causes differentiation of hematopoietic progenitors (Figure 5E). Most importantly, a single copy loss of *PKA* in an *Adgf-A* mutant background rescues the progenitor cell phenotype (Figure 5F-H). Adenosine levels are expected to be high in an *Adgf-A* mutant background, thus causing proliferation followed by differentiation. However, simultaneously reducing PKA function downstream of the adenosine receptor has an attenuating effect on this signal and hence suppresses the mutant phenotype. It is important to point out that the nature of the signal that initiates differentiation of the precursors remains unknown and is still operational in all of the above genetic backgrounds.

A direct readout of Hedgehog signaling is the expression level of the active form of Ci (Ci^{155}) and the transcriptional target *ptc*. As expected, over-expression of *PKA-R2*, which inhibits PKA function, causes higher levels of active Ci and Ptc (Figure 5I, I′; S4M, M′), consistent with increased Hedgehog signaling. Additionally, staining for Ci^{act} shows that compared with wild type, the *Adgf-A/Adgf-A* mutant shows lower Ci expression; this is enhanced upon *PKA*/+ heterozygosity (Figure S4S-T). The critical issue is to show whether Ci modulation is AdoR-dependent and we tested this possibility by decreasing the level of *AdoR* in MZ cells. This causes an increase in the level of activated Ci in the progenitor cell population showing that AdoR-mediated cAMP-dependent PKA activation modulates Ci activity (Figure 5J, J′). Ultimately, the Adgf-A-related signal limits the amount of PKA activated by adenosine signaling and therefore the degradation of active Ci. Similarly, active Hedgehog niche signaling also inhibits PKA, limiting degradation of Ci into its repressive form. Together, the CZ and the PSC signals maintain a balance of Ci activity within the MZ thereby controlling quiescence within the hematopoietic progenitor cell population (Figure 5K).

Discussion

The role of a niche signal is well established in many developmental systems that involve stem cell/progenitor populations (Fuchs et al., 2004; Losick et al., 2011; Orkin and Zon, 2008; Scadden, 2006). In the *Drosophila* lymph gland the niche expresses Hh and maintains a group of progenitor cells (Mandal et al., 2007). This current study establishes an additional mechanism, parallel to the niche signal that originates from differentiating cells, which also regulates quiescence of hematopoietic progenitors.

The cells of the lymph gland proliferate at early stages, from embryo to mid second instar. At this stage, cells farthest from the PSC initiate differentiation and the rest enter a quiescent phase defining a MZ. In wild type, the cells of the MZ remain quiescent and in progenitor form throughout the third instar and we show here that this process requires a combination of the PSC and CZ signals. If either signal is removed, the progenitor population will eventually be lost due to differentiation. In many different genetic backgrounds, if quiescence is lost, the progenitor population initially continues to incorporate BrdU during

the second instar without expressing any maturation markers (Figure S1A-F). The differentiation phenotype, characterized by the expression of such markers, follows this abnormal proliferation. The net result is that whenever the progenitors accumulate BrdU (but not express any markers of differentiation) in the second instar, all cells of the lymph gland are differentiated and no MZ remains in the third instar. While we do not know the nature of the signal that triggers hemocyte differentiation, withdrawal of Wingless may play a role in this process (Sinenko et al., 2009).

Our experimental analysis has demonstrated a novel role for Pvr in maturing hemocytes and its ligand, Pvf1, in the cells of the PSC. Pvf1 expression increases at a stage when the lymph gland is highly proliferative. At this critical window in development, Pvf1 originating from the PSC is transported to the differentiating hemocytes, binds to its receptor Pvr, and activates a STAT-dependent signaling cascade. At this stage, Pvf1 is sensed by all cells but it is only in the differentiating hemocytes that it activates Adgf-A in an AdoR/Pvr-dependent manner. This secreted factor Adgf-A is required for regulating extracellular adenosine levels. High adenosine would signal through AdoR and PKA to inactivate Ci and reduce the effects of the niche-derived Hedgehog signal leading to differentiation of the progenitor cells. The function of the Adgf-A signal is to reduce this adenosine signal and therefore reinforce the maintenance of progenitors by the Hedgehog signal. Thus, the Adgf-A and Hh signals work in the same direction but Adgf-A does so by negating a proliferative signal due to adenosine. In wild type, equilibrium is reached through a signal not originating from the niche that opposes this proliferative process. The attractive step in this model is that the CZ and niche (in this case Hh-dependent) signals both impinge on common downstream elements allowing for control of the progenitor population relative to the niche and the differentiated cells. Most importantly, this is a mechanism for maintaining quiescence within a moderately large population of cells that is not in direct contact with a niche. By the time the three zone PSC/MZ/CZ system is set up in the late second instar all the cells of the MZ express high levels of E-cadherin, become quiescent and are maintained as progenitors and are capable of giving rise to all blood cell lineages (Jung et al., 2005; Krzemien et al., 2010). Under such circumstances, the interaction between a niche-derived signal and an equilibrium signal originating from differentiating cells can maintain homeostatic control of the progenitor population. Several vertebrate stem cell/progenitor scenarios such as during bone morphogenesis (Mendez-Ferrer et al., 2010) and hematopoiesis (He et al., 2009; Orkin and Zon, 2008) or in the *Drosophila* intestine (Mathur et al., 2010) have progenitors and differentiating cells in close proximity that could pose an opportunity for a similar niche and differentiating cell-derived signal interaction. In fact, evidence for such interactions have recently been provided for vertebrate skin cells (Hsu et al., 2011).

The role of small molecules such as adenosine has not yet been adequately addressed in vertebrate progenitor maintenance. A small molecule such as extracellular adenosine is unlikely to form a gradient over the population of cells and maintain such a gradient over a developmental time scale. It is much more likely that this system operates similar to the “quorum sensing” mechanisms described for prokaryotes (Ng and Bassler, 2009). A critical level of adenosine is required for proliferation and by expressing the Adgf-A signal this threshold amount is lowered, causing quiescence in the entire population.

We describe a developmental mechanism that is relevant to the generation of an optimal number of blood cells in the absence of any overt injury or infection. However, a system that utilizes such a mechanism to maintain a progenitor population could potentially sense a disruption upon induction of various metabolic stresses to cause differentiation of myeloid cells. Various mitochondrial and cellular stresses can cause an increase in extracellular adenosine (Fredholm, 2007), but whether they are relevant to this system remains to be studied. In the past, we have observed dual use of reactive oxygen species (ROS) as well as Hypoxia Inducible Factor-a (HIF-α) in both development and stress response of the *Drosophila* hematopoietic system (Mukherjee et al., 2011; Owusu-Ansah and Banerjee, 2009). Responses to injury have been described in the *Drosophila* intestine (Amcheslavsky et al., 2009; Jiang et al., 2009), and in satellite cells (Ten Broek et al., 2010) that respond during injury, a stress related signal could be the initiating factor that overrides a maintenance signal. Thus the equilibrium generated through developmental interactions is disrupted to promote a cellular response to stress signals.

Experimental Procedures

For sources of all stocks and antibodies please see Supplementary information. Progenitor and differentiated cell types for all genotypes are assessed by combining 3-5 middle optical sections of the lymph gland lobe. Flp-out clones within MZ were generated using a lineage tracing system controlled by *hand-gal4* and Hml^{Δ}*-gal4* as described (Evans et al., 2009). Genotypes for, *hs-flp* derived clones: [*hs-flp Ay-gal4 UAS-GFP UAS-dome*^{DN}]. FRT clones: [*hs-flp FRT40A Pvr*^{C2195} *FRT40A Ubi-GFP*] and [*hs-flp FRT82B Stat92E*^{06346} *FRT82B Ubi-GFP*].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Banerjee lab for helpful discussions. We acknowledge VDRC, NIG, DHSB, BDSC, E. Bach, J. Calderon, J. Darnell, T. Dolezal, J. Hooper, D. Montell, P. Rorth, A. Sehgal, B. Shilo, M.Yoo, and M. Zeidler for reagents. Supported by NHLBI R01 HL067395 to UB and NHLBI K08 HL087026 to JM.

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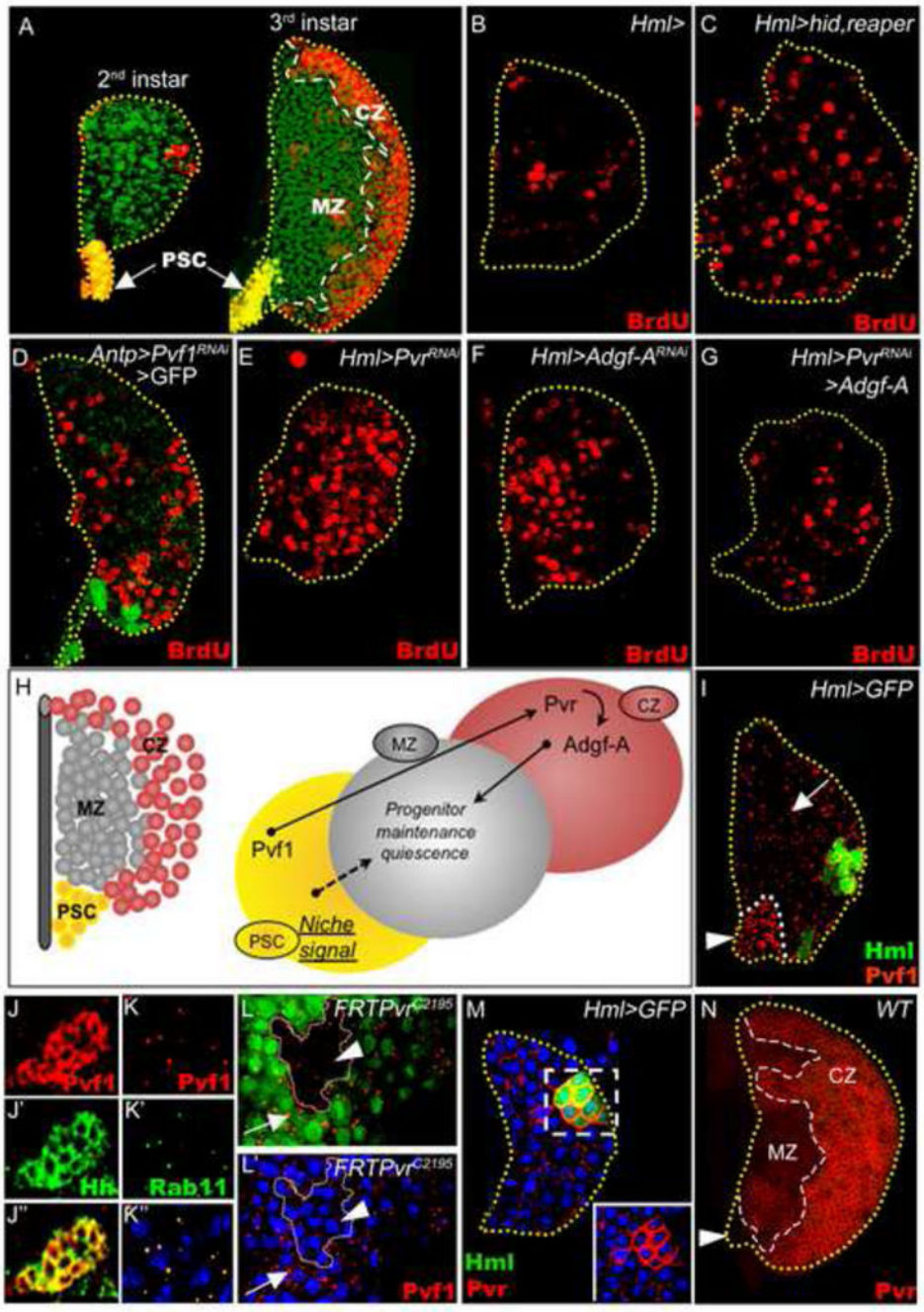

Figure 1. Regulation of progenitor quiescence by differentiating hemocytes mediated by Pvf1/Pvr and Adgf-A signaling

(**A**) A representative lymph gland primary lobe from a mid-second instar larva (left image) consisting of a posterior signaling center (PSC, yellow), progenitors (green), and a few differentiating cells (red). A primary lobe from a later-staged wandering third instar larva (right image) shows three distinct zones: the PSC (yellow), which functions as the niche for the maintenance of progenitors (green) within the medullary zone (MZ), and the peripheral cortical zone (CZ) region, comprised of differentiating blood cells (red).

(**B-G**) Cell proliferation profile in lymph glands from mid-second instar larvae analyzed by BrdU (red) incorporation. (**B**) Control lymph glands (genotype: *Hml-gal4*) at this stage have only a few proliferating cells (9 +/- 6 cells, N=6). (**C**) Induction of cell death in the differentiating cells by expression of Hid and Reaper (*Hml-gal4 UAS-hid UAS-rpr*) causes an increase in the number of BrdU positive cells (red), indicative of the loss of quiescence among the progenitors (compare with **B**). A similar loss of quiescence occurs upon (**D**) down-regulation of Pvf1 in the PSC using $Pvf1^{RNAi}$ (*Antp-gal4 UAS-2xEGFP UAS-*$Pvf1^{RNAi}$), (**E**) down-regulation of Pvr in the differentiating cells using Pvr^{RNAi} (*Hml-gal4 UAS-*Pvr^{RNAi}; 39 +/- 7 cells, N=5, $p<0.004$ when compared to **B**), or (**F**) down-regulation of Adgf-A in the differentiating cells using $Adgf\text{-}A^{RNAi}$ (*Hml-gal4 UAS-*$Adgf\text{-}A^{RNAi}$). (**G**) Over-expression of *Adgf-A* can suppress the proliferation phenotype due to loss of Pvr^{RNAi} (*Hml-gal4 UAS-*Pvr^{RNAi} *UAS-Adgf-A*; 17 +/- 7 cells, N=8, $p<0.03$ when compared with **E**).
(**H**) The color scheme of the model (right) corresponds to the zones represented in the schematic of the lymph gland (left). Maintenance of progenitor quiescence within the MZ requires Pvf1 from the PSC, Pvr function in the CZ, and Adgf-A from the CZ functioning downstream of Pvr.
(**I-N**) Expression of Pvf1 and Pvr in the developing lymph gland. TOPRO-3 (in **K-M**, blue) marks nuclei. (**I** and **M**) Mid-second instar lymph glands from control animals (*Hml-gal4 UAS-2xEGFP*); (**J-L′** and **N**) Third instar lymph glands. **(I)** Pvf1 expression is seen as small punctae present at high levels in the PSC (arrowhead) and dispersed in the rest of the lymph gland (arrow). (**J**) Pvf1 (red, J) expression in the PSC co-localizes with Hedgehog (green, **J′**). The overlap is shown in yellow (**J″**). (**K**) Within the lymph gland proper, Pvf1 (red punctae, **K**) co-localizes with Rab11 (green punctae, **K′**), a marker for transcytosis vesicles. The overlap is shown in yellow (**K″**). (**L**) *Pvr* mutant clones (non-green) were generated within wild-type tissue (green) and stained for Pvf1 (red punctae). Pvf1 is present in the Pvr^{+} tissue (arrow) but is strongly reduced in the clones lacking *Pvr* (arrowhead). (**M, N**) Temporal analysis of Pvr expression. (**M**) The first differentiating cells (green, *Hml-gal4 UAS-2xEGFP*) show strong upregulation of Pvr expression (red, inset). (**N**) At later stages, Pvr expression is relatively high in the CZ as compared to the MZ, and is lacking in the PSC (arrowhead).

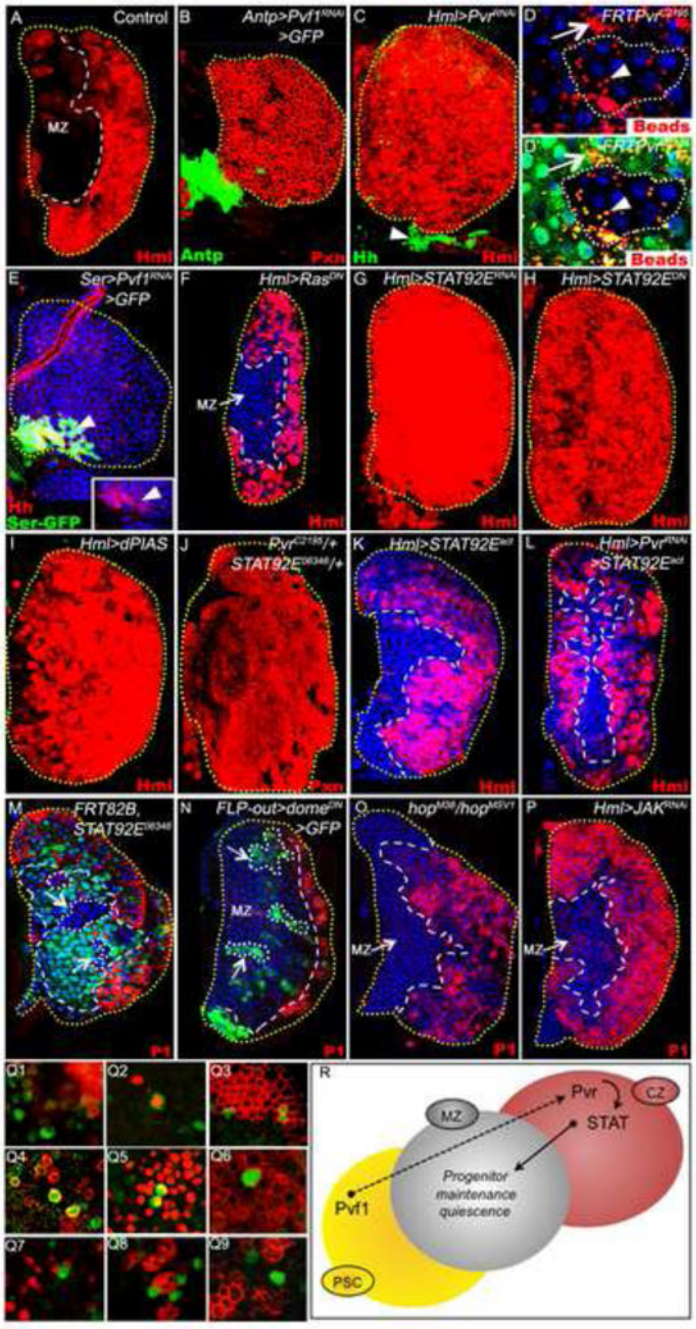

Figure 2. Role of STAT downstream of Pvr

For uniformity, differentiating hemocytes are shown in red even if they are marked with EGFP (*Hml-gal4 UAS-2xEGFP*). Lymph glands shown are from wandering third-instar larvae. TOPRO3 marks nuclei (blue).

(**A**) Control. Normal *Hml-gal4* expression pattern (Hml, red).

(**B**) $Pvf1^{RNAi}$ expression in the PSC (green; *Antp-gal4 UAS-2xEGFP UAS-*$Pvf1^{RNAi}$). All non-PSC cells of the lymph gland express Peroxidasin (Pxn, red).

(**C**) *Pvr*^{RNAi} expression in the CZ (*Hml-gal4 UAS-2xEGFP UAS-PvrR*^{NAi}) causes MZ progenitors to differentiate (Hml, red), although Hedgehog (green) expression in the PSC (arrowhead) remains normal.

(**D** and **D′**) *Pvr*-mutant clones (*Pvr*^{C2195}/*Pvr*^{C2195}, non-green cells) differentiate normally as judged by their ability to phagocytose FluoSphere beads (red), similar to neighboring wild type tissue (green).

(**E**) Hedgehog expression (red, inset) is unaffected when *Pvf1*^{RNAi} is expressed in the PSC (green; *Ser-gal4 UAS-2xEGFP UAS-Pvf1*^{RNAi}); yellow represents co-localization of Hedgehog and *Ser-gal4* expression.

(**F**) Expression of *Ras*^{DN} in differentiating hemocytes (*Hml-gal4 UAS-2xEGFP UAS-Ras*^{DN}) does not affect progenitor fate.

(**G-I**) Loss of *STAT* function in differentiating cells causes progenitor differentiation (Hml, red). This phenotype can be induced by expressing either (**G**) *Stat92E*^{RNAi} (*Hml-gal4 UAS-2xEGFP UAS-Dcr-2 UAS-Stat92E*^{RNAi}), (**H**) *Stat92E*^{DN} (*Hml-gal4 UAS-2xEGFP UAS-Stat92E*^{DN}), or (**I**) *dPIAS (Hml-gal4 UAS-2xEGFP UAS-dPIAS)*.

(**J**) Combined single-copy-loss of *Pvr* and *Stat92E* (*Pvr*^{C2195}/+; *Stat92E0*^{6346}/+). All progenitors express Pxn (red).

(**K**) As a control, expression of *Stat92E*^{act} (Ekas *et al.*, 2010) in differentiating cells has no effect on progenitor fate (*Hml-gal4 UAS-2xEGFP UAS-Stat92*$^{\Delta N\Delta C}$) (**L**) Co-expression of *Stat92E*^{act} and *Pvr*^{RNAi} (*Hml-gal4 UAS-2xEGFP UAS-Stat92Er UAS-PvrR*^{NAi}) suppresses the progenitor differentiation phenotype caused by *Pvr*^{RNAi} alone (compare with **C**).

(**M**) *Stat92E* mutant clones (*Stat92E*^{06346}/*Stat92E*^{06346}) within the MZ (lacking green, demarcated by white dots) do not cause ectopic differentiation (lack of red within the clones).

(**N**) Blocking Domeless function in MZ cells (*FLP-out gal4 UAS-dome*^{DN} clones, green, see methods for details, demarcated by white dots), does not cause them to differentiate (P1, red).

(**O**) JAK mutant animals (*hop*^{M38}/*hop*^{MSV1}) do not exhibit ectopic differentiation (P1, red) of progenitors.

(**P**) Expression of *JAK*^{RNAi} specifically in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-hop*^{RNAi}) does not cause MZ progenitor differentiation (P1, red).

(**Q1-9**) In the third instar, lymph glands exhibit a small fraction of cells that express a reporter of STAT activity (green, *10X Stat92E-GFP*). These cells are negative for *domeless* (*dome-MESO-lacZ*; **Q1**), proliferate (PH3; **Q2**), and express Pvr^{act} (**Q3-4**) and Hml (*Hml-ga4, UAS-lacZ*; **Q5**), but lack differentiation markers: Pxn (**Q6**), Lz (**Q7**), ProPO (**Q8**) and P1 (**Q9**). Cells appear yellow due to co-localization of the STAT reporter and Hml (anti-|3-gal), Pvr^{act}, or PH3.

(**R**) Schematic representation of STAT function in progenitor maintenance. Pvf1 from the PSC is transported to the differentiating cells in the CZ to activate its receptor Pvr, leading to the activation of STAT that, in turn, generates the CZ signal necessary for the maintenance of the progenitors in the MZ. Thus, loss of either Pvf1 from the PSC (in **B**), or loss of Pvr (in **C**) or STAT (in **G-I**) from the CZ results in proliferation and differentiation of MZ progenitors.

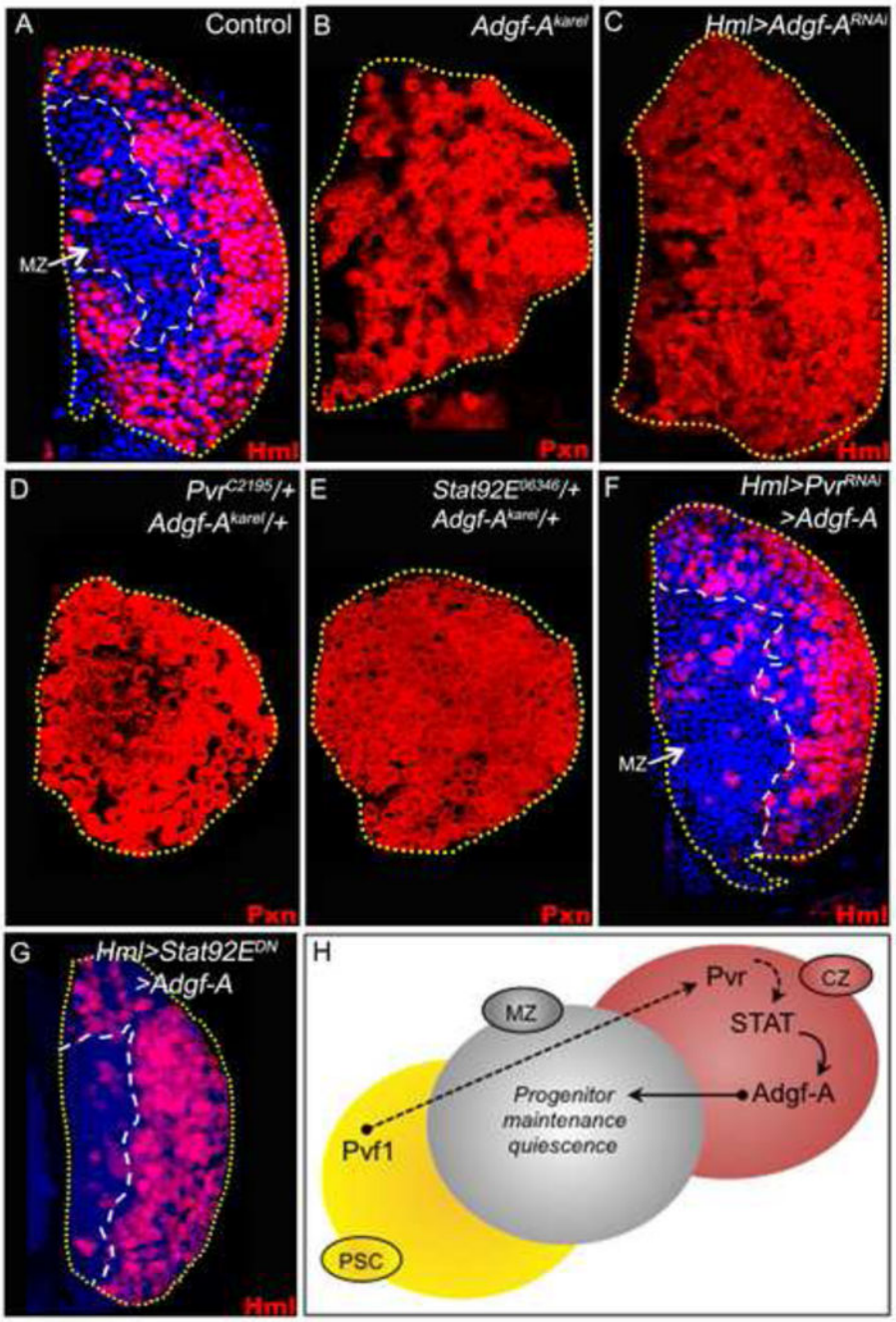

Figure 3. Adgf-A functions downstream of STAT in hematopoietic progenitor maintenance

Third instar lymph glands are shown. Differentiating hemocytes are marked in red, with nuclei marked with TOPRO3 (blue). For uniformity, hemocytes in **A**, **C**, **F** and **G** are shown in red although they are genotypically *Hml-gal4 UAS-2xEGFP*.

(**A**) Normal expression pattern of *Hml-gal4*, showing differentiating cells (Hml, red). The MZ cells lack markers of differentiation and are marked by TOPRO3 (blue).

(**B**) *Adgf-A*^{karel}/*Adgf-A*^{karel} mutant lymph gland. All progenitors differentiate and express Pxn (red, compare pattern with **A**).

(**C**) Expression of *Adgf-A*^{RNAi} in differentiating hemocytes (*Hml-gal4 UAS-2xEGFP UAS-Adgf-A*^{RNAi}) causes differentiation of progenitors (Hml, red; compare with **A**).
(**D**) Combined single-copy-loss of *Pvr* and *Adgf-A* (*Pvr*^{C2195}/+; *Adgf-A*^{karel}/+). All progenitors differentiate and express Pxn.
(**E**) Combined single-copy-loss of *STAT* and *Adgf-A* (*Stat92E*^{06346}/+ *Adgf-A*^{karel}/+). All progenitors differentiate and express Pxn.
(**F**) Co-expression of *Adgf-A* and *Pvr*^{RNAi} (*Hml-gal4 UAS-2xEGFP UAS-Pvr*^{RNAi} *UAS-Adgf-A*) suppresses the progenitor differentiation phenotype caused by *Pvr*^{RNAi} (compare with Fig. 2C).
(**G**) Co-expression of *Adgf-A* and *STAT*^{DN} (*Hml-gal4 UAS-2xEGFP UAS-STAT*^{DN} *UAS-Adgf-A*) suppresses the progenitor differentiation phenotype caused by *STAT*^{DN} (compare with Fig. 2H).
(**H**) Schematic representation of *Adgf-A* function in progenitor maintenance. Expression of *Adgf-A* from the differentiating cells, mediated by Pvf1/Pvr and STAT signaling, functions as the CZ signal. Therefore, loss of Adgf-A in the CZ (as in **C**) causes progenitor differentiation, and over-expression of Adgf-A in the CZ (in **F** and **G**) suppresses mutant effects of Pvr and STAT. The earlier steps are as described in Figure 2S.

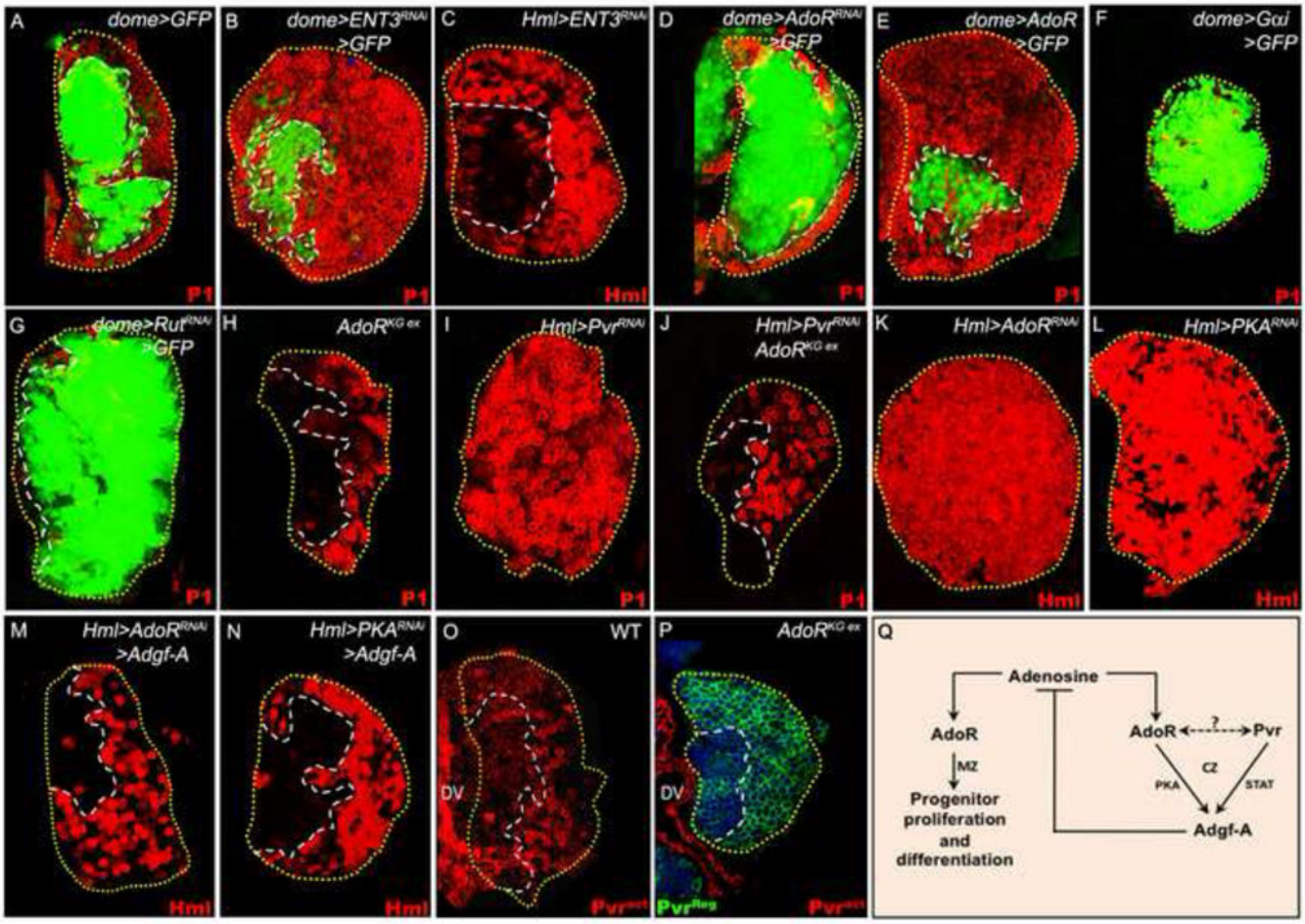

Figure 4. Adenosine signaling regulates hematopoietic progenitor quiescence

In **A-B** and **D-G**, the progenitor cell population is marked with *dome-gal4, UAS-2xEGFP* (green). In **A-N**, differentiating hemocytes are shown in red. All lymph glands shown are from wandering third instar larvae.

(**A-G**) Role of the adenosine transporter ENT3, the receptor AdoR, downstream signalling component G_{α}, and the adenylate cyclase Rutabaga.

(**A**) Control lymph gland showing the normal expression pattern of *dome-gal4 UAS-2xEGFP* (green) in progenitors and P1 (red) in differentiating cells.

(**B**) $ENT3^{RNAi}$ expression in progenitors (*dome-gal4 UAS-2xEGFP UAS-*$ENT3^{RNAi}$) causes a reduction in MZ size (compare with **A**) and a corresponding increase in the CZ.

(**C**) $ENT3^{RNAi}$ expression in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-*$ENT3^{RNAi}$) does not cause progenitor differentiation.

(**D**) $AdoR^{RNAi}$ expression in progenitors (*dome-gal4 UAS-2xEGFP UAS-*$AdoR^{RNAi}$) causes their expansion and reduction of differentiating cells (compare with **A**).

(**E**) *AdoR* overexpression in progenitors (*dome-gal4 UAS-2xEGFP UAS-AdoR*) causes their differentiation (compare with **A**).

(**F**) $G_{\alpha i}$ overexpression in progenitor cells (*dome-gal4 UAS-2xEGFP UAS-*$G_{\alpha i}$) blocks their differentiation.

(**G**) *Rut*^{RNAi} expression in progenitors (*dome-gal4 UAS-2xEGFP UAS-Rut*^{RNAi}) causes their expansion and reduces differentiating cells.
(**H-J**) AdoR function downstream of Pvr.
(**H**) Control *AdoR* mutants (*AdoR*^{KGex}/*AdoR*^{KGex}) develop a small lymph gland but with normal zonation.
(**I**) *Pvr*^{RNAi} expression in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-Pvr*^{RNAi}) causes loss of progenitors.
(**J**) Mutation in *AdoR* (*AdoR*^{KGex}/*AdoR*^{KGex}) partially suppresses the loss of progenitors caused by *Pvr*^{RNAi} in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-Pvr*^{RNAi}, compare with Fig 2**C**).
(**K-N**) AdoR and Pvr together control expression of Adgf-A in differentiating cells.
(**K**) *AdoR*^{RNAi} expression in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-AdoR*^{RNAi}) causes progenitors to differentiate (red).
(**L**) *PKA*^{RNAi} expression in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-PKA*^{RNAi}) causes progenitors to differentiate (red).
(**M**) Co-expression of *Adgf-A* with *AdoR*^{RNAi} in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-AdoR*^{RNAi} *UAS-Adgf-A*) suppresses the progenitor differentiation phenotype caused by *AdoR*^{RNAi} alone (compare with **K**).
(**N**) Co-expression of *Adgf-A* with *PKA*^{RNAi} in differentiating cells (*Hml-gal4 UAS-2xEGFP UAS-PKA*^{RNAi} *UAS-Adgf-A*) suppresses the progenitor differentiation phenotype caused by *PKA*^{RNAi} alone (compare with **L**).
(**O-P**) Activation of Pvr is dependent upon the presence of AdoR.
(**O**) A wild-type (*w*^{1118}) lymph gland showing the expression pattern of activated (phosphorylated) Pvr (Pvr^{act}, red). The red staining on the left is in the heart (dorsal vessel, DV).
(**P**) In *AdoR*^{KG03964ex}/*AdoR*^{KG03964ex} lymph glands expression of Pvr (total protein, Pvr^{reg}, green) is normal. However, Pvr^{act} expression (red) is entirely missing from the lymph gland (compare with red staining in **O**). The red staining on the left is in the dorsal vessel (DV).
(**Q**) Adenosine signaling in the lymph gland. Adenosine signaling through AdoR in progenitors promotes their proliferation and differentiation. This process is kept in check by reduction of available extracellular adenosine. Therefore, in the progenitors, reduction of the adenosine transporter (in **B**) or increase in the adenosine receptor (in **E**) causes differentiation. Also in the progenitors, loss of the adenosine receptor (in **O**) or its downstream components (in **F** and **G**) causes expansion of this compartment at the cost of the CZ. In differentiating cells, Pvr collaborates with AdoR to maintain Adgf-A expression. Therefore, loss of adenosine receptor or its downstream components in the CZ (in **K** and **L**) causes progenitor differentiation and this phenotype is suppressed by overexpression of Adgf-A.

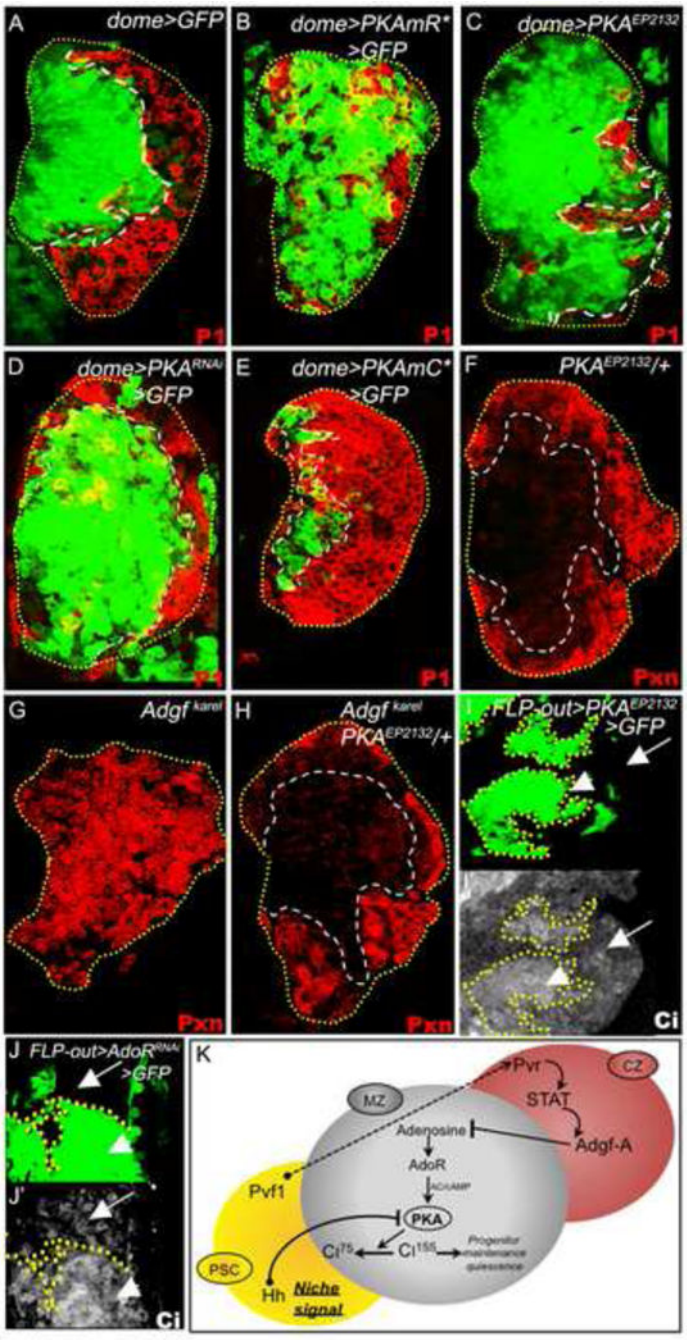

Figure 5. Interaction between PSC and CZ signals

In **A-E**, progenitors are marked with *dome-gal4 UAS-2xEGFP* (green). In **A-H**, the differentiating cells are shown in red. All lymph glands shown are from wandering third instar larvae.

(**A**) Control lymph gland showing the normal expression pattern of *dome-gal4 UAS-GFP* (green) in progenitors and P1 (red) in differentiating cells.

(**B-E**) Role of PKA in progenitor maintenance. Loss of PKA function in progenitors causes their expansion and a corresponding reduction of differentiating cells, as shown by

expressing (**B**) the regulatory domain of PKA (*dome-gal4 UAS-2xEGFP UAS-PK^{mR*}*), which functions as a sink for cAMP, (**C**) *PKA ^{EP2132}* (*dome-gal4 UAS-2xEGFP UAS-PKA^{EP2132}*) which functions as a dominant-negative, and (**D**) *PKA^{RNAi}* (*dome-gal4 UAS-2xEGFP UAS-PKA^{RNAi}*). (**E**) Gain of PKA function in progenitors through the expression of the constitutively active PKA^{mC*} (*dome-gal4 UAS-2xEGFP UAS-PKA^{mC*}*) causes their differentiation. Compare with **A**.

(**F-H**) PKA opposes the function of Adgf-A in progenitor maintenance. (**F**) Normal control *PKA^{EP2132}/+*. (**G**) Control *Adgf-A^{karel}/Adgf-A^{karel}*. Hematopoietic progenitors differentiate. (**H**) *PKA^{EP2132}/+; Adgf-A^{karel}/Adgf-A^{karel}*. The progenitor differentiation phenotype due to loss of *Adgf-A* is suppressed by single copy loss of *PKA* (compare with **G**).

(**I-J**) Regulation of *Ci^{activated}* by PKA and AdoR. (**I-I′**) FLP-out cell clones (green in **I**, see materials and methods) that reduce PKA function (by expressing *PKA^{EP2132}*) exhibit high levels of *Ci^{activated}* (*Ci^{act}*) expression (shown in grayscale, **I′**). (**J**) FLP-out cell clones (green) that reduce AdoR function (by expressing *AdoR^{RNAi}*) also exhibit elevated levels of *Ci^{act}* (shown in grayscale) expression (**J′**).

(**K**) The niche and CZ signals function together to regulate the levels of *Ci^{act}* necessary for progenitor maintenance in the MZ. Note that Ci is activated not only upon loss of PKA (in **I**) but also upon loss of the adenosine receptor (in **J**) thus creating a link between Hedgehog and adenosine signaling.