European Journal of Immunology

Molecular immunology and signaling

Basic

Research Article

MiR221 promotes precursor B-cell retention in the bone marrow by amplifying the PI3K-signaling pathway in mice

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Hematopoietic stem cells and lineage-uncommitted progenitors are able to home to the bone marrow upon transplantation and reconstitute the host with hematopoietic progeny. Expression of miR221 in B-lineage committed preBI-cells induces their capacity to home to the bone marrow. However, the molecular mechanisms underlying miR221-controlled bone marrow homing and retention remain poorly understood. Here, we demonstrate, that miR221 regulates bone marrow retention of such B-cell precursors by targeting PTEN, thus enhancing PI3K signaling in response to the chemokine CXCL12. MiR221-enhanced PI3K signaling leads to increased expression of the anti-apoptotic protein Bcl2 and VLA4 integrin-mediated adhesion to VCAM1 in response to CXCL12 in vitro. Ablation of elevated PI3K activity abolishes the retention of miR221 expressing preBI-cells in the bone marrow. These results suggest that amplification of PI3K signaling by miR221 could be a general mechanism for bone marrow residence, shared by miR221-expressing hematopoietic cells.

Keywords: CXCL12 · CXCR4 · PreBI-cells · Transplantation · VCAM1

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Introduction

B-lymphocytes develop from hematopoietic stem cells (HSC) throughout life in the BM [1]. HSCs can self-renew or differentiate into multipotent progenitors, common lymphoid progenitors (CLP), and common myeloid progenitors, whereby they gradually lose their lineage pluripotency. HSC, multipotent progenitors, common myeloid progenitors, and CLP reside in distinct niches [2–5]. These niches provide hematopoietic progenitors with the major chemokine C-X-C motif chemokine 12 (CXCL12) and with integrin ligands, like VCAM1. Both factors are essential to attract and retain the cells in their environment [6]. Upon transplantation, HSCs and their direct progeny are able to migrate to the BM and reconstitute the host with long and short lasting waves of hematopoiesis.

CLPs differentiate, via the transitional $CD19^-$ pro-preB-cell stage towards committed $CD19^+$ D_HJ_H rearranged preBI-cells [7]. This differentiation is mediated by the cytokine IL-7 and the transcription factors like Early B-cell Factor (EBF) and paired box protein 5 (PAX5). PreBI-cells are dependent on kit-L and IL-7 [8], while they become less responsive to CXCL12 and less adhesive to VCAM1 [9]. This loss of adhesion may allow preBI-cells to leave

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their environment and move away from the IL-7 source. This is necessary to induce V_H- to D_HJ_H- rearrangements, which lead to the expression of pre-B-cell receptor on the surface. Committed B-lineage cells, from the preBI cell stage onwards, have lost their capacity to migrate into the BM upon transplantation and reconstitute the host with B-lineage progeny [10].

Normal fetal liver-derived preBI-cells [10], as well as PAX5-deficient pro-preB-like cells [11] can be expanded by long-term proliferation, remaining stable at their states of differentiation in vitro on stromal cells in presence of IL-7. The capacity to proliferate in vitro under same tissue culture conditions documented their close relationship in B-cell differentiation. However, the two stages of differentiation differ in several capacities. PAX5-deficient pro-preB-cells could be induced in vitro, in the appropriate tissue culture conditions, to differentiate multiple hematopoietic lineages, except to B-cells [12]. Important for the studies presented here is, that PAX5-deficient preB-cells were found to express high levels of miR221, like other hematopoietic progenitor cells including HSCs, while normal preBI-cells expressed very low levels of miR221 [13].

Upon transplantation, normal, PAX5 sufficient preBI-cells cannot home into the BM of a host and consequently do not establish residence there [10]. Nevertheless, B1a-cells develop in the spleen and peritoneum after transplantation of preBI-cells [10, 14]. In contrast, the PAX5-deficient miR221^{high} cells can home to the BM of transplanted hosts and establish a stable pool of approximately 5–10 $\times$ 10^5 cells [11]. Moreover, they give rise to T-lymphoid and myeloid progeny [15].

MiRNAs are small non-coding RNAs, which act as negative regulators of gene expression. MiRNAs have been shown to be important in development and function of the immune system [16, 17]. They are involved in the regulation of cellular processes like apoptosis, proliferation, lineage commitment, and differentiation in the hematopoietic system, including B-cell development [18–21]. We previously investigated the possible role of miR221 expression during the phase of differentiation from pro-preB-cells to preBI-cells by re-expressing miR221 in normally low-expressing preBI-cells [13]. Interestingly, the expression of miR221 in preBI cells renews their capability to home into the BM, reside there, and establish a pool of preBI-cells. However, the detailed molecular mechanism, by which miR221 expression results in transplantability of preBI-cells to BM, remained unclear.

Here, we identify a mechanism of miR221-controlled BM retention of B-cell progenitors. We found, that miR221 enhances PI3K signaling upon stimulation with CXCL12, by directly targeting the Phosphatase and Tensin homolog phosphatase (PTEN). This results in sustained cellular adhesion to VCAM1 and increased anti-apoptotic signaling, enabling preBI-cells to home to BM and reestablish a preBI-cell pool in the BM.

The characterization of the miR221-PI3K signaling axis contributes to a better understanding of how these cells interact with their environment in the BM to establish and continuously maintain normal cell development and pool sizes. We propose that earlier hematopoietic progenitors during their development, and cells that return to BM from the periphery, could share this mechanism.

Results

CXCL12 stimulation increases the adhesion of CLP and pro-preB-cells, but not of preBI-cells to VCAM1

The receptor for the chemokine CXCL12, CXCR4, is expressed throughout hematopoietic and B-cell lineage development. However, the influence of CXCL12/CXCR4-mediated signaling differs during B-cell development. CXCL12 has been shown to cause strong migratory responses in early B-cells, but not in later preB-cells, immature, mature, and peripheral B-cells in vitro [22]. Also, CXCL12 has been found to promote adhesion of early B-cells to VCAM1 in mice and human [9, 23]. These differences in responses to CXCL12 appeared not to be due to the difference in CXCR4 receptor expression, since surface CXCR4 expression had been found to be expressed in all human B-cell progenitors in the BM.

In order to extend this CXCR4 surface expression analysis in B-cell development of mice, we analyzed developing B-cell subpopulations [24, 25] in mouse BM (Fig. 1A). This analysis shows that CXCR4 surface expression in mouse B-cell development is comparable to human [26]. It is interesting to note that pre-BII cells show an enhanced surface expression of CXCR4. Nevertheless the function of CXCR4 during this development remains elusive. Therefore, we tested the CXCR4 function in early B-cell development.

In agreement with previous studies in mice and humans, we found that CXCL12 increased the adhesion on VCAM1-coated plates of ex vivo sorted CLPs and pro-preB-cells. In contrast, ex vivo enriched ($B220^+CD19^+C\text{-}kit^+$) or in vitro cultured CXCL12-stimulated preBI-cells did show only a marginal increase in adhesion (Fig. 1B). This showed that the functional difference in adhesion did not correlate with the CXCR4 surface abundance.

In contrast, the expression pattern of miR221 correlates with the ability of hematopoietic progenitor cells to respond to CXCL12 with enhanced and sustained adhesion (Fig. 1C). Therefore, in subsequent in vitro experiments, we reasoned to test the influence of miR221 re-expression in preBI-cells on CXCL12-stimulated cellular responses.

MiR221 expression induces sustained adhesion of preBI-cells to VCAM-1 upon stimulation with CXCL12

In our previous work, we had studied the actions of miR221 expression during B-cell development by re-expressing miR221 at the first stage of development. These cells no longer express miR221 and do not become resident in BM after transplantation. We transduced preBI-cells with a retrovirus transcribing the mature form of miR221 under the control of a doxycycline-inducible tetO promoter and detected a tenfold increase of miR221 after induction with doxycycline (Fig. 1C). This level of expression was comparable to the endogenous levels of miR221 expression in CLPs and pro-preB-cells. Upon induction of miR221 by addition of doxycycline preBI-cells regain the ability to migrate to BM upon

transplantation in comparable numbers to PAX5$^{-/-}$ pro-preB-cells (Supporting Information Fig. S1A).

Since increased residence of the miR221-expressing preBI-cells in BM could be influenced by cellular responses involving cellular adhesion, we compared preBI-cells expressing miR221 with non-expressing cells for their adhesive potential. When miR221 expression was induced in preBI-cells, adhesion to VCAM1 was increased upon stimulation with CXCL12 (Fig. 1D). This strongly suggested that miR221 re-expression increased CXCL12/CXCR4-stimulated adhesion response of preBI-cells. At the same time, surface expression of CXCR4 or C-KIT was not altered by miR221 expression (Supporting Information Fig. S1D). We conclude, that miR221 re-expression in CXCL12/CXCR4-stimulated preBI-cells increased the adhesion to VCAM1 in vitro. It suggests, that one of the molecular modes, by which miR221 re-expression mediates increased residence of these cells in BM, is the adhesion to ligands of integrins, such as VCAM1.

CXCL12 is known to induce specific cellular responses, such as cell polarization, filopodial and lamellopodial protrusions, as well as formation of focal adhesion contacts for cell migration and adhesion. These processes are associated with integrin activation and remodeling of the actin cytoskeleton [27–29]. Doxycycline-induced re-expression of miR221 in CXCL12/CXCR4-stimulated preBI-cells lead to a quick and strong response on VCAM1-coated plates, with the formation of membrane protrusions and a less round cell shape (Fig. 1E). Cells became polarized and formed F-actin-dense filopodial and lamellopodial structures (Fig. 1F). In contrast, preBI-cells not expressing miR221 failed to show such strong morphological changes.

We conclude that miR221-re-expression induces CXCL12/CXCR4-stimulated preBI-cells to form filopodial and lamellopodial protrusions, enhanced focal adhesion contacts, and increased adhesion via VLA4-integrin/VCAM1-interactions.

MiR221 expression amplifies signaling via PI3K

To investigate the molecular mechanism, by which miR221 increased cell adhesion and polarization, we focused on signal transduction pathways, which could be differentially triggered by CXCL12.

Cultured, proliferating preBI-cells showed activated MAP kinases Erk1/2 after stimulation with CXCL12, which are known to be major mediators of proliferation (Fig. 2A). However, miR221 re-expression did not affect the levels of Erk1/2 phosphorylation after stimulation (Fig. 2A, Supporting Information Fig. S2B and D).

We then tested the influence of miR221 re-expression on signaling pathways, which are known to influence adhesion, migration and survival of hematopoietic cells, notably the PI3K-mediated pathway. Active PI3K-signaling leads to increased levels of PIP_3, which results in the recruitment of AKT to the plasma membrane and subsequent phosphorylation of AKT at Ser^{473} by mTORC2 [30] and PDK1 [31]. This leads to full activation of AKT.

Importantly, in miR221-expressing preBI-cells the PI3K downstream effector AKT showed increased phosphorylation at the Ser^{473} residue after CXCL12 stimulation, when compared with cells not expressing miR221 (Fig. 2A, Supporting Information Fig. S2A and C). Moreover, we found increased levels of FAK autophosphorylation in miR221 expressing preBI-cells in response to CXCL12 (Fig. 2B). Our data suggests that miR221-enhanced PI3K signaling is part of a possible molecular mechanism for BM homing and retention in miR221 expressing cells.

Inhibition of PI3K signaling in vitro blocks integrin-mediated adhesion

Our experiments, described above, show an increased cellular response and adhesion in miR221-expressing cells. Hence, inhibition of PI3K should reduce the CXCL12-induced adhesion to VCAM1. Therefore, we pre-incubated these cells with Wortmannin, a pan-PI3K inhibitor which inhibits PI3K by covalent inactivation [32, 33]. We tested the effect of PI3K inhibition by Wortmannin in the preBI-cell adhesion assay on VCAM1-coated plates. In addition, we also tested the effects of the more selective class I PI3K inhibitor ZSTK-474. Wortmannin and ZSTK-474 both block the positive effect of CXCL12 on adhesion to immobilized VCAM1 (Fig. 2C and D). This suggests, that PI3Kinase Class I molecules have a non-redundant function in the process of cellular adhesion.

Adhesion to VCAM1 has been found to be mediated by Very Late Antigen-4 (VLA4). VLA4 is expressed on the surface of preBI-cells. VLA4 is an integrin $\alpha4/\beta1$ heterodimer, which binds to VCAM1 and fibronectin. Integrins are known to undergo a chemokine-induced conformational change from inactive to activated conformations in order to promote cellular adhesion [34–37].

The monoclonal antibody 9EG7 binds specifically to the activated, open conformation, but not to the closed, inactive conformation of β-1 integrin [35]. When we tested miR221 expressing cells for the expression of this activated form of β-1 integrin, we found higher levels of the activated form, expressed on the surface, compared to the WT pre-BI-cells (Fig. 3A and B). Incubation of miR221 expressing preBI-cells with the activated β-1 integrin-specific antibody (9EG7) blocked adhesion to VCAM-1 coated plates (Fig. 3C). Thus, increased adhesion of miR221-expressing preBI cells appears to be mediated by the activation of β-1 integrin and by the increased binding of this form of integrin to VCAM1 after CXCL12 stimulation (Fig. 3D). Furthermore, the activation of the open, L binding conformation of β-1 integrin by CXCL12 is dependent on PI3K activity (Fig. 3E), since inhibition of PI3K with class I inhibitors inhibits the appearance of the open conformation. Moreover, we find activated integrins to cluster with F-actin-dense regions, detectable after CXCL12-stimulated binding to VCAM1 (Fig. 3F and G). Thus, our data suggest, that miR221-mediated regulation of PI3K signaling could be a central regulatory element of early B-cell adhesion capability.

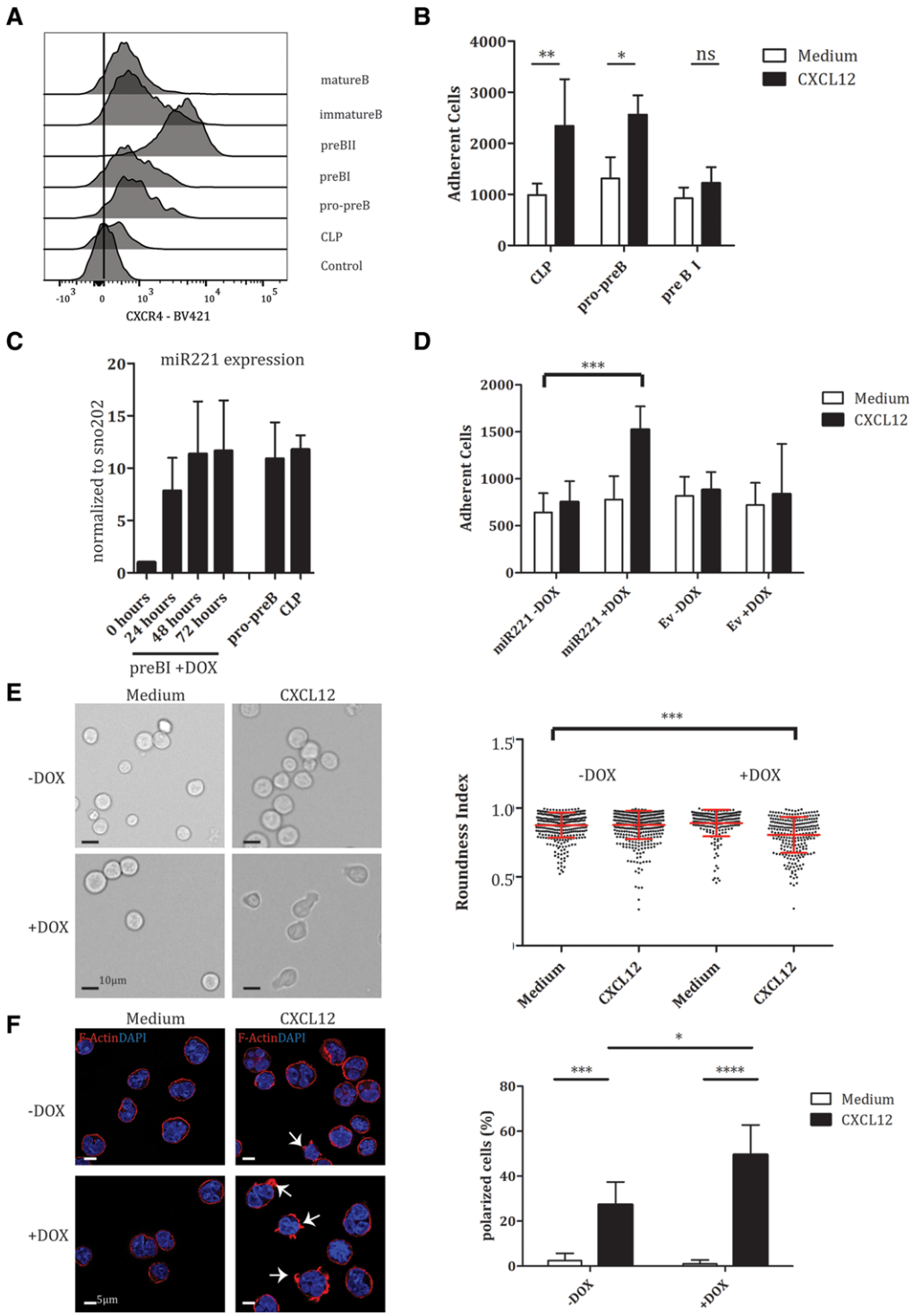

Figure 1. MiR221 promotes sustained adhesion responses to VCAM1 in preBI-cells in response CXCL12. (A) Representative FACS analysis of three independent experiments, CXCR4 expression analysis in CLPs and BM B-cell subsets; for gating strategy see Supporting Information Figure S1B and C. (B) Adhesion assay with ex vivo sorted common lymphoid progenitors, pro-preB-cells and preBI-cells, which were stimulated with medium or CXCL12 on VCAM1 coated wells; pooled data from three independent experiments. (C) Doxycycline inducible expression of miR221 in in vitro culture preBI-cells. Doxycycline induced miR221 expression, measured by RT-PCR in three independent experiments, is shown relative to non-induced preBI-cells and ex vivo sorted pro-preB-cells and CLPs. Time points of measurement after addition of doxycycline are indicated. Samples were measured in triplicates. (D) Adhesion assay with in vitro cultured preBI-cells and empty vector controls, in presence and absence of doxycycline to induce miR221 expression, on VCAM1-coated wells; cells were either stimulated with normal medium or CXCL12; pooled data is shown of five independent experiments; Statistical analysis was performed by Two-way ANOVA; *** < 0.001; only significant differences are indicated; (E) Representative images of the morphological response of miR221 expressing preBI-cells after 3 min stimulation with CXCL12 on VCAM1 coated wells, was assessed by time lapse imaging; Scale bars indicate 10 µm. Membrane protrusions were measured with Image J

MiR221 downregulates the protein expression of PTEN

The PI3K signaling pathway is controlled by negative regulators, including the Phosphatase and Tensin homologue PTEN, Src homology-2 (SH2) domain-containing inositol phosphatase (SHIP1), PH domain and leucin-rich repeat protein phosphatase (PHLPP), and phosphatases protein phosphatase 2A (PP2A) phosphatases. PHLPP and PP2A directly dephosphorylate AKT at the Ser^{473} residue, leading to an inactivation of AKT [38, 39]. PHLPP harbors miR221 binding sites in its 3‘ UTR. PTEN and SHIP are phosphatases, which dephosphorylate phosphatidylinositol-3,4,5-trisphosphatePIP_3 to PIP_2 ($PI_{4,5}P_2$ and $PI_{3,4}P_2$, respectively) and thus regulate the PI3K-Akt pathway at a different level [40, 41]. PTEN also harbors miR221-binding sites (Supporting Information Fig. S3A and B). Therefore, we next analyzed the differential expression of the major negative regulators of the PI3K-AKT signaling pathways. We hypothesized, that a specific downregulation of negative PI3K regulators by miR221 would be a potential mechanism for a direct miR221-mediated activation of PI3K.

Re-expression of miR221 in preBI-cells did not alter the expression of PP2Ac on protein level. PHLPP expression appeared to be mildly affected after 48 h of miR221 expression, but this repression had no lasting effect (Fig. 4A). However, miR221 expression stably reduced PTEN protein expression (Fig. 3B), while PTEN RNA levels were not significantly reduced (Fig. 3C). These results suggest, that miR221-re-expression in preBI-cells reduces the levels of translated PTEN protein, which results in increased PI3K signaling.

The miR221 target sequences in the PTEN 3’UTR suggest a direct regulation of PTEN expression by miR221. In human, PTEN has been shown to be a direct target of miR221, where miR221 binds a site of PTEN-3’UTR, which is conserved between mice and humans [42]. In mice, we have identified an additional binding site in the mouse 3’UTR of PTEN, which is also responsive to miR221 in LUC assays (Supporting Information Fig. S3A–D).

If expression of miR221 decreases PTEN expression by specific targeting of the PTEN 3’-UTR, expression of a PTEN gene devoid of miR221-binding sites (3’-UTR), should not be influenced by miR221 expression. Therefore, ectopic PTEN expression should complement and abolish the PI3K activation effect, caused by miR221 downregulation of endogenous and miR221-sensitive PTEN.

We overexpressed the full length PTEN-mRNA, devoid of a 3’-UTR, in miR221 expressing preBI-cells, using Venus (YFP) as a marker for the expression construct (Fig. 3D). Venus (YFP) positive clones, tested for PTEN expression, showed an increased expression of PTEN (Fig. 3E). Induction of miR221 in these cells reduced PTEN protein expression to initial levels (-DOX), suggesting that only the endogenous PTEN is being targeted by miR221 (Fig. 3F and G), and the ectopic PTEN is insensitive to miR221. Taken together, we conclude from these experiments, that miR221 expression leads to direct downregulation of PTEN protein by interaction with its 3’-UTR.

MiR221 expression leads to CXCR4-PI3K mediated Bcl2 upregulation

PI3K-Akt mediated signaling has been shown to promote cell survival in many normal and malignant cells. PI3K integrates survival pathways via Akt and the subsequent inhibition of pro-apoptotic proteins and activation of anti-apoptotic proteins, as well as integrin activation signals [43, 44]. We hypothesized, that PI3K signaling could also contribute miR221-mediated increased preBI-cell survival in BM. We found here, that preBI-cells, which express miR221, have elevated levels of the anti-apoptotic protein Bcl2, when compared to empty vector (EV) or cells not induced by doxycycline (Fig. 5A). Stimulation of miR221-expressing cells with CXCL12 results in a strong activation of the PI3K-Akt pathway. Therefore, we tested, whether elevated protein expression of Bcl2 is induced by CXCL12, which is provided by OP9 stromal cells. Indeed, blockade of the receptor for CXCL12, CXCR4, by AMD3100 reduces the Bcl2 expression to levels of preBI-cells not expressing miR221 (Fig. 5B and C). To test, whether the increased Bcl2 expression is further dependent on the enhanced activity of PI3K, we treated the miR221-expressing preBI-cell with the PI3Kδ class I-specific inhibitor IC87114, to inhibit PI3Kδ, which is specifically expressed in hematopoietic cells [45, 46]. Treatment with IC87114 further reduced Bcl2 expression in miR221-expressing preBI-cells, resembling the effect of CXCL12 signaling inhibition (Fig. 5B and C). IL-7 has been shown to be important for early B-cell survival and differentiation, and is mainly involved in signaling via STAT5 and PI3K [47]. Culturing preBI-cells on OP9 stromal cells at suboptimal IL-7 (10–100 pg/mL) concentrations yielded higher cell numbers of preBI-cells expressing miR221 than controls (Fig. 5D), indicating that these cells are more sensitive to low IL-7 concentrations. We also wanted to test, whether these increased levels of Bcl2 confer protection against stress-induced apoptosis in preBI-cells. Therefore, we cultured preBI-cells in the presence of miR221 and treated these cells with low concentrations of H_2O_2 to induce ROS-mediated cell stress and apoptosis [48]. We assessed

(20× magnification), as an index of cellular roundness, with value 1 representing a perfect circle; Statistical analysis was performed by One-way ANOVA with Dunn’s test for multiple comparisons, *** < 0.001; pooled data from three independent experiments with approximately 100–150 cells analyzed per experiment, is shown; only significant differences are indicated. (F) Representative confocal images (63× magnifications) of preBI-cells on VCAM-1 coated wells 1 min after CXCL12 stimulation. Cells were stained with DAPI (blue) and Phalloidin-A647 (red) to detect F actin dense structures. White arrows indicate lamella/filopodia formation with high density of F-actin. Scale bars indicate 5 μm. Statistical analysis of polarized cells is shown in the right panel. Pooled data shows percentage of polarized cells per image and was assessed in five independent experiments with 10–20 cells analyzed per experiment; Statistical analysis was performed by unpaired *t*-test; **** < 0.0001, *** < 0.001, * < 0.05; in all experiments error bars indicate SD of mean.

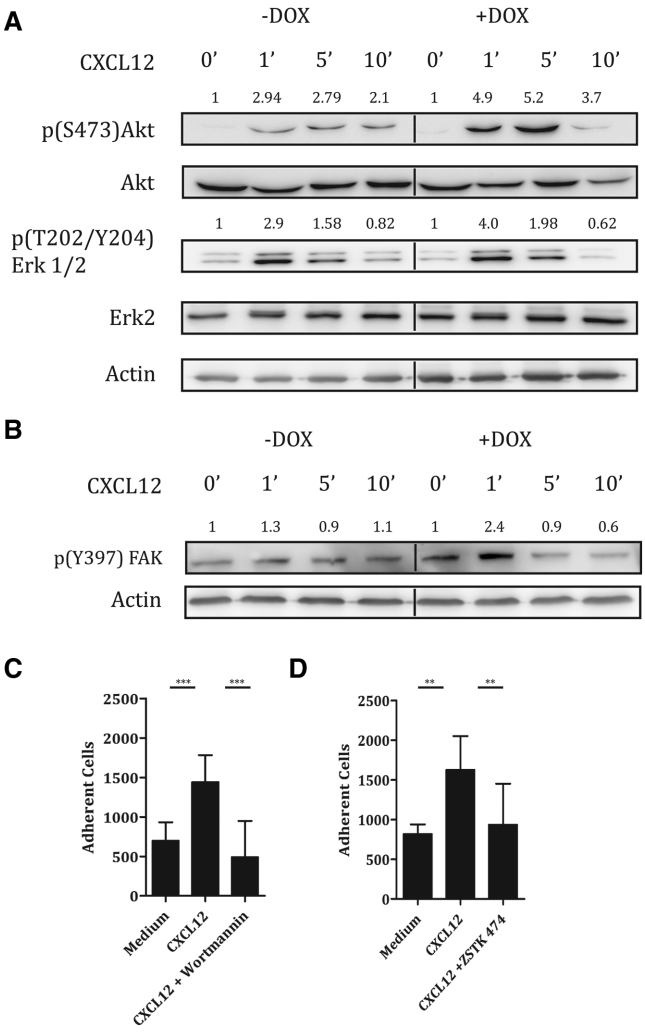

Figure 2. MiR221 expression in preBI-cells leads to stronger activation of PI3K-Akt pathway in response to CXCL12. (A) Western blot analysis of phosphorylated Erk1/2 and Akt proteins in miR221 expressing preBI-cells (+DOX) compared to normal preBI-cells (-DOX). Cells were stimulated with CXCL12 for indicated times. Total Erk2 and total Akt were used as controls and actin was used as loading control. Values indicate relative pAkt and pErk phosphorylation normalized to Actin loading control and total Akt and Erk respectively. Representative blots of two independent experiments are shown. (B) Western blot analysis of phosphorylated FAK proteins in miR221 expressing preBI-cells (+DOX) compared to normal preBI-cells (-DOX). Cells were stimulated with CXCL12 for the indicated times. Values indicate relative pFAK phosphorylation normalized to Actin loading control (for quantification procedure see materials and methods). Representative blots of two independent experiments are shown. (C) Adhesion assay with miR221 expressing preBI-cells on VCAM1- coated wells after stimulation with CXCL12, in the presence or absence of Class I PI3K inhibitor ZSTK474 (pooled data of three independent experiments is shown) and in the presence or absence of Wortmannin as a pan PI3K inhibitor (D) (pooled data from four independent experiments is shown); statistical analysis was performed by unpaired t-test *** < 0.001, ** < 0.01. All error bars indicate SD of mean.

cell viability 22 h after treatment. MiR221-expressing preBI-cells were more resistant to cytotoxic stress, caused by H_2O_2 than their counterparts (Fig. 5E). These results show that miR221-enhanced PI3K activity enables preBI-cells to increase anti-apoptotic Bcl2 expression in response to CXCL12 and IL-7 provided by stromal cells, which is important for early B-cell survival [49].

Inhibition of PI3K and ectopic expression of PTEN reduce miR221 mediated transplantability

Next, we wanted to test our model, that miR221-mediated amplification of PI3K signaling is responsible for BM retention of miR221-expressing preBI-cells in vivo. We administered Wortmannin to CD45.2$^+$Rag$^{-/-}$ mice, which were transplanted with 5 $\times$ 10^5 mir221-expressing CD45.1$^+$ preBI-cells. Wortmannin treatment significantly reduced the amount of GFP$^+$CD45.1$^+$CD19$^+$ donor cells in the BM. In contrast, cell numbers of host CD19$^+$ cells, which do not express miR221 were not affected (Fig. 6A–C), suggesting a lower sensitivity and dependence of miR221-non-expressing cells to PI3K inhibition. Most of the transplanted cells remained IgM-negative after Wortmannin treatment. These experiments confirm our findings, that miR221-enhanced PI3Kinase functions mediate in vivo retention of preBI-cells in BM. Similar to termination of miR221-expression [13] the inhibition of PI3Kinase terminates preBI-cell retention in BM in vivo, thereby not enhancing differentiation. Ectopic PTEN expression, devoid of miR221 binding sites, should complement and abolish the PI3K activation effect, caused by miR221 downregulation of endogenous and miR221-sensitive PTEN. This should reduce retention of preBI-cells in the BM and, thus, complement the miR221 expression effect. We transplanted 1 $\times$ 10^6 miR221-expressing preBI-cells into RAG1$^{-/-}$ mice, and compared the numbers of transplanted cells, with the numbers of miR221-expressing preBI-cells, which overexpress miR221-insensitive PTEN. The numbers of GFP$^+$ (miR221-expressing) preBI-cells, retained in the BM, were reduced upon overexpression of miR221-insensitive PTEN 1 wk after transplantation (Fig. 6D). These results are in line with our observation, where inhibition of PI3K by Wortmannin also results in reduced preBI-cell numbers in BM.

Taken together, our results show that miR221 posttranscriptionally suppresses PTEN, a negative regulator of PI3K signaling. This regulation leads to enhanced PI3K signaling and subsequent phosphorylation of Akt, upregulation of Bcl2 expression, and FAK auto-phosphorylation. MiR221-enhanced PI3K activity leads to sustained binding to the integrin L VCAM1 and increased survival. Hence miR221-expressing B-cell precursors are able to strongly respond to niche factors like CXCL12 and reside in their respective BM niche (Fig. 6E).

Discussion

The BM provides an environment for the development of many hematopoietic cell lineages. Different microenvironments are proposed to give rise to different cell types. It has been suggested that each cell type occupies a specialized microenvironment, which provides essential adhesion molecules, chemokines, and cytokines like VCAM1, CXCL12, and IL7.

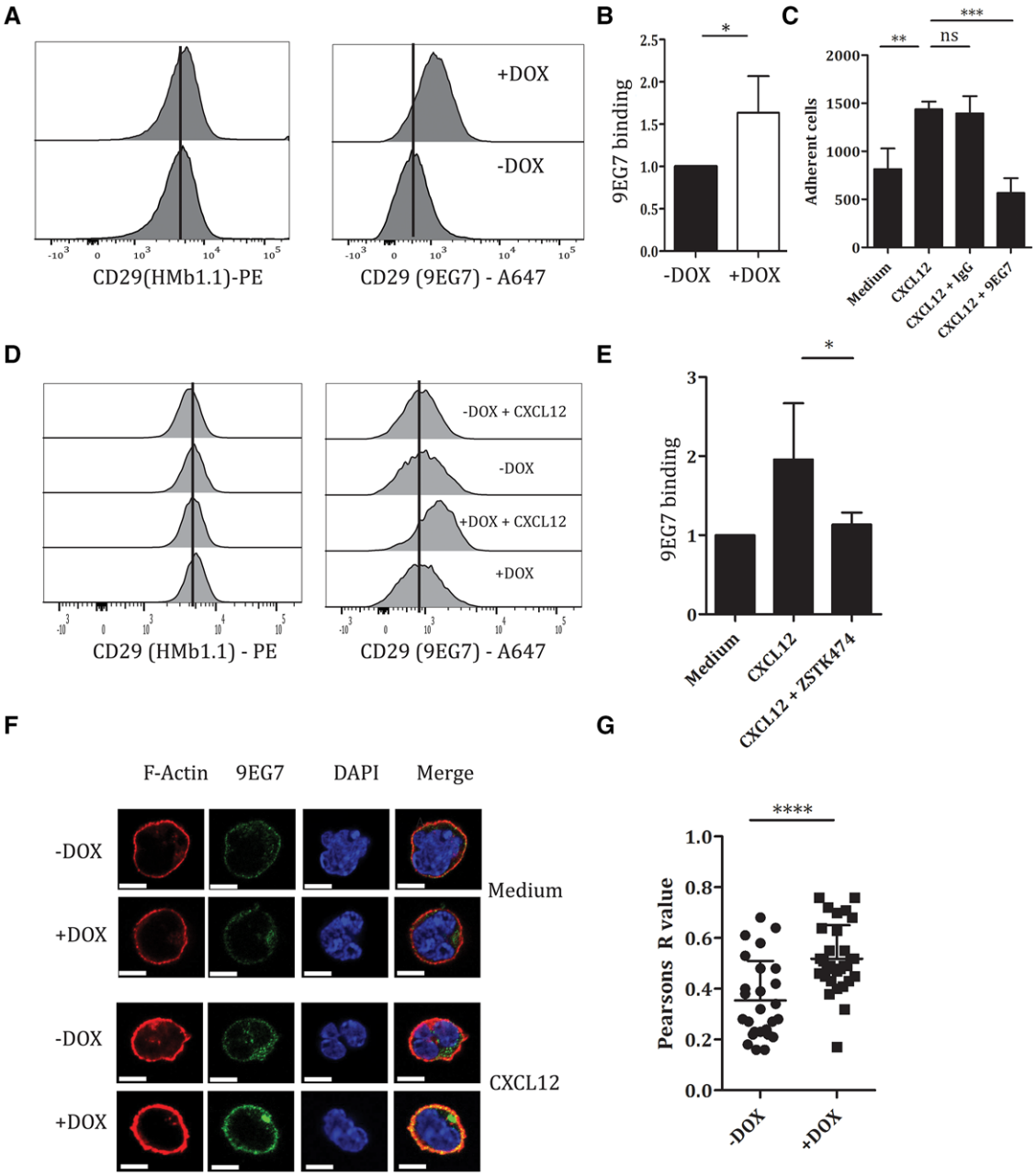

Figure 3. MiR221 leads to integrin activation and co-localization with F-actin in preBI-cells. (A) FACS analysis of CD29 and activated CD29 (9EG7) in preBI-cells cultured on OP9 cells in the presence and absence of doxycycline, e.g., miR221. (B) Bar diagram shows relative geometric mean expression of 9EG7 normalized to CD29 ($n = 4$); * < 0.05 unpaired t test. (C) Adhesion assay with in vitro cultured preBI-cells in the presence of doxycycline and CXLC12, on VCAM1-coated wells. Cells were incubated with anti CD29 (9EG7) antibodies or IgG controls prior to adhesion assay; pooled data of three independent experiments is shown; Statistical analysis was performed by One way ANOVA with Tukey test for multiple comparisons; * < 0.05, ** < 0.01, *** < 0.001. All error bars indicate SD with indicated mean. (D) Expression of the 9EG7 epitope was assessed in the presence and absence of doxycycline (e.g., miR221), when stimulated with CXCL12 or Medium. Expression of the 9EG7 epitope and total CD29 amount was measured by FACS. Representative histograms of three independent experiments are shown. (E) FACS quantification (geo mean) of the 9EG7 activated integrin after stimulation with CXCL12 and in the presence or absence of Class I PI3K inhibitor ZSTK474; pooled data of 4 independent experiments with three samples per experiment. (F) PreBI-cells, in the presence and absence of doxycycline (miR221), were stimulated with CXCL12 for 5 min on VCAM1-coated wells. PreBI-cells were stained with anti CD29 (9EG7) (green), Phalloidin (red) and DAPI (blue); representative images of four independent experiments are shown (G) Colocalization analysis of F-actin (red) with activated beta 1 integrin (green) in preBI-cells from. Pearson correlation was calculated to assess colocalization by Image J for at least 30 cells per condition from four independent experiments. Statistical analysis was performed by unpaired t test; * < 0.05, **** < 0.0001. All error bars indicate SD of mean.

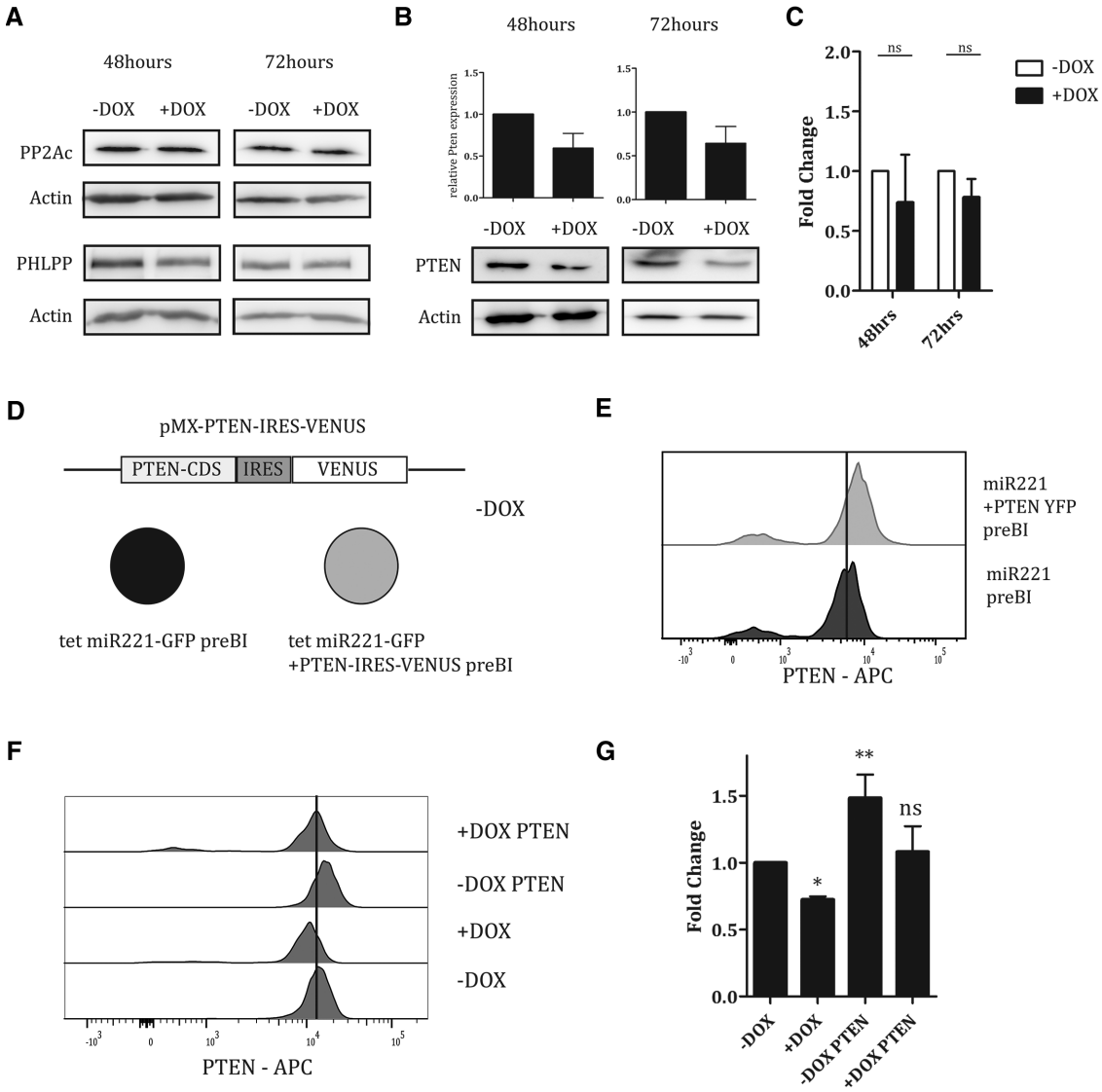

Figure 4. MiR221 expression reduces PTEN protein expression. (A and B) Western blot analysis of PTEN, PP2Ac and PHLPP protein expression in miR221 overexpressing preBI-cells compared with preBI-controls (48, 72 h after miR221 induction). Actin was used as loading control. Quantification is indicated as fold change compared to miR221 non-induced preBI cells, in bar diagrams on top of blots (for quantification procedure see materials and methods). Representative western blots of three independent experiments are shown. (C) RT-PCR analysis of PTEN RNA expression (48, 72 h after miR221 induction); GAPDH was used as a housekeeping gene; fold change of PTEN expression relative to miR221 non induced controls is indicated. Data was assessed in triplicates in four independent experiments. (D) Overexpression schematic of the complete PTEN CDS sequence in miR221 expressing preBI-cells. (E) Flow cytometric analysis of PTEN protein expression in PTEN overexpressing preBI-cells, with and without induction of miR221 by the addition of doxycycline. (F) Representative FACS analysis of differential PTEN expression in the presence and absence of miR221 and miR221 insensitive PTEN. (G) Analysis of the geometric mean of PTEN fluorescence intensity is shown, relative to preBI cells in the absence of miR221 (-DOX); pooled data of three independent experiments is shown with fold change of expression indicated; statistical analysis was performed by unpaired *t*-test; * < 0.05, ** < 0.01, ns = not significant. All error bars indicate SD of mean.

CXCL12 has been shown to be an essential BM retention factor, which guides hematopoietic cells to, and maintains them in their niche. Although hematopoietic progenitors and committed B-lineage cells express the CXCL12-receptor CXCR4, their migratory response to CXCL12 and their CXCL12 stimulation-dependent ability to adhere to VCAM1, differ significantly [9, 22, 23, 50]. We find the surface expression of CXCR4 to be different in B-cell progenitor subsets in the BM, highest on $CD25^+$ preBII cells and lower on CLPs, preBI cells, immature, and mature B-cells. Discrepancies in function and expression of CXCR4 receptor, however, have also been described for human B-cell progenitors [26]. Thus, the surface expression of CXCR4 does not predict the physiology of its function. Similarly, freshly isolated $Lin^-Sca1^+Kit^+$ progenitor cells have been reported to display low surface expression of CXCR4, when isolated from the BM [51]. Enhanced surface expression of CXCR4 has been observed on preBI cells in serum free cultures on immobilized kit L in the absence of CXCL12. This enhanced expression could be downregulated by the addition of

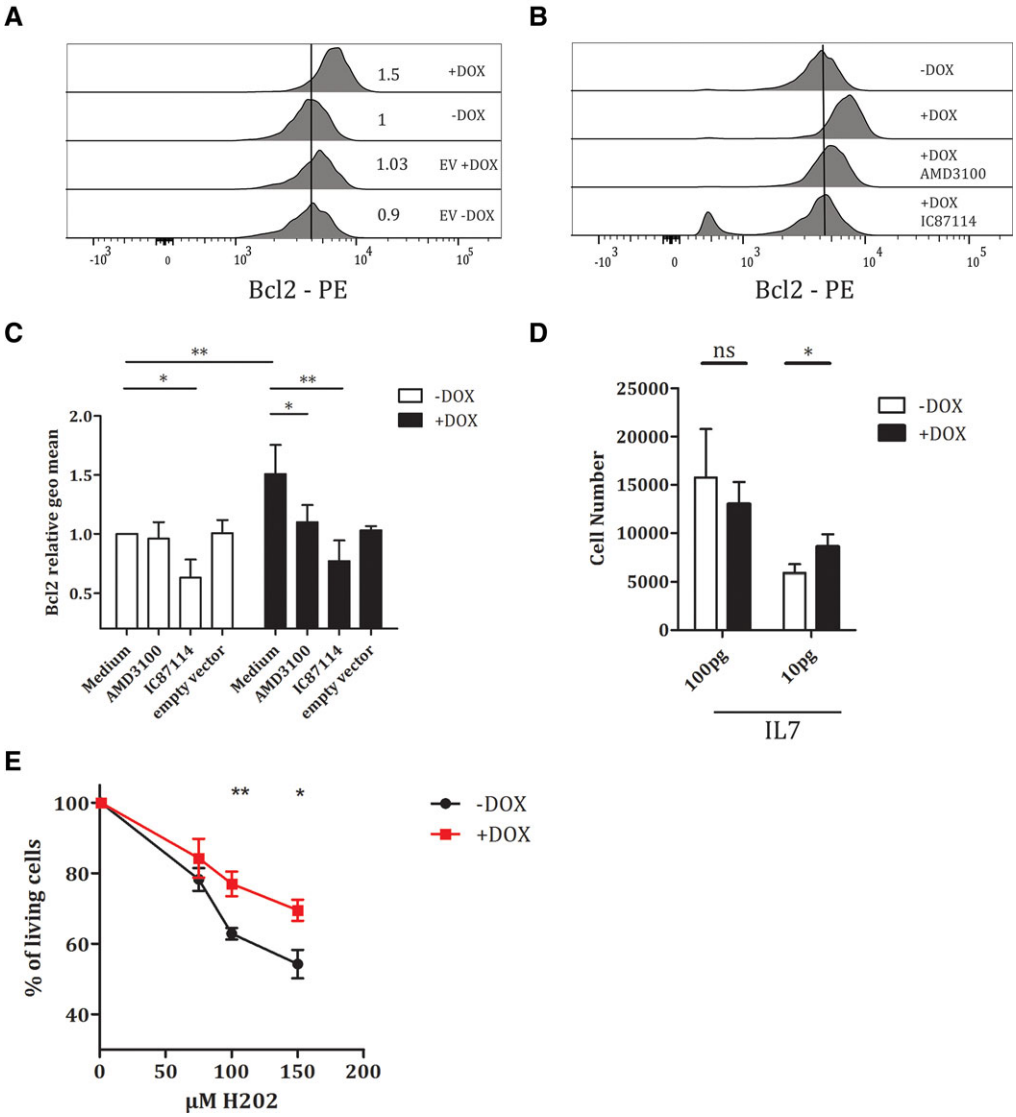

Figure 5. Enhanced PI3K activity leads to CXCL12 mediated upregulation of Bcl2. (A) PreBI-cells were cultured in the presence and absence of doxycycline (e.g., + and – miR221), for 3 days on OP9 stromal feeder cells. Expression of Bcl2 was assessed by intracellular flow cytometric analysis in miR221 expressing (+DOX) versus non-expressing (-DOX) preBI-cells ($n = 7$) and empty vector controls in the presence or absence of doxycycline ($n = 3$). Representative histograms are shown. (B and C) Addition of CXCR4 inhibitor AMD3100 ($n = 7$) and PI3Kδ inhibitor IC87114 ($n = 4$), was performed 24 h before analysis of Bcl2 expression in preBI-cells, cultured for 3 days in OP9 in the presence or absence of doxycycline; fold change expression of Bcl2 geometric mean is shown relative to normal preBI-cells. Pooled data of indicated number of experiments is shown. (D) Cell numbers of PreBI-cells (5000 plated) which were cultured for 3 days on OP9 cells in suboptimal concentrations of IL-7 (as indicated); pooled data of five experiments are shown. (E) PreBI cells were cultured on OP9 stromal cells in the presence and absence of doxycycline. Cells were treated with H_2O_2 with indicated concentrations and percentage of living cells was acquired 22 h after treatment by PI staining. One representative of 4 independent experiments with three replicates per experiment is shown. Statistical analysis was performed by unpaired *t*-test; ns = not significant, * < 0.05, ** < 0.01. All error bars indicate SD of the mean.

CXCL12 [8]. Thus the levels of surface expression of the CXCR4 receptor may reflect the presence or absence of the L CXCL12 and thus may be the result receptor internalization upon receptor–L interaction [8, 26].

Nevertheless the surface expression of CXCR4 cannot explain the change of CXCL12-mediated cellular response activity of B-lineage cells. Thus, B-lineage cells at more mature stages of development, which no longer respond to CXCL12-CXCR4-signaling by increased adhesion, might use altered signaling of the CXCR4 receptor for other functions at these later stages of development. These other potential functions of CXCL12-CXCR4 signaling in other, e.g., more mature B-cells, remain to be investigated.

Our experiments with miR221-overexpressing preBI-cells suggest, but do not yet prove, that miR221-targeted downregulation of PTEN, and subsequent amplification of the Integrin-Pi3K-Akt pathway could be the underlying molecular mechanisms that lead to CXCL12-CXCR4-dependent BM retention and residence, possibly also of other earlier hematopoietic progenitors. In line with this hypothesis, we found miR221 to be expressed in all hematopoietic

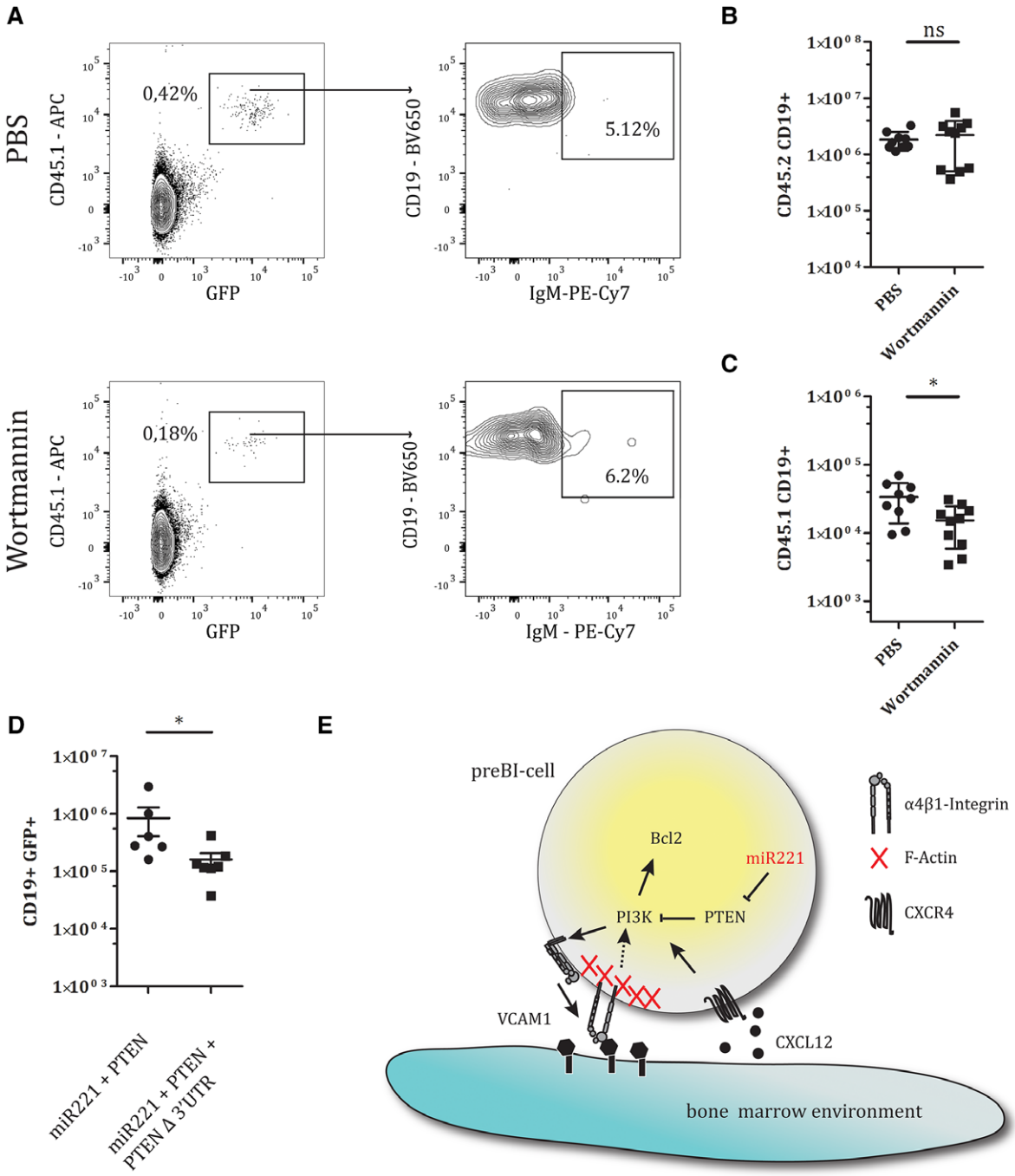

Figure 6. *In vivo*, depletion of miR221 expressing preBI-cells from the BM, by ablation of the PI3K signaling pathway. (A) CD45.2 Rag1$^{-/-}$ mice were sub-lethally irradiated and CD45.1^{+}GFP^{+} miR221 expressing preBI-cells were transplanted. Five days after transplantation, mice were treated with Wortmannin and the BM was analyzed for the presence of transplanted cells. Representative FACS blots of mice treated with PBS and mice treated with Wortmannin are shown (gated on living cells). Percentages indicate the relative cell numbers to total cell numbers of previous gate. Absolute cell numbers CD45.2^{+}CD19^{+} host preBI-cells (B) and transplanted CD45.2^{+}CD19^{+} preBI-cells (C) after treatment with Wortmannin (black squares) or PBS (black circles) in two femur and two tibiae of treated mice. Pooled data of three independent experiments with three mice per experimental group is shown. Statistical analysis was performed by unpaired *t*-test; $^{*}p < 0.05$, ns = not significant; error bars indicate SD of the mean (D) 1 wk after transplantation of PTEN overexpressing preBI-cells, the BM was analyzed by FACS for GFP^{+} cells. Black circles represent total number of cells in two femurs and two tibiae of mice, which were transplanted with miR221-GFP expressing cells and black squares represent PTEN-IRES-VENUS overexpressing miR221-GFP cells. Statistical analysis was performed by Mann–Whitney–*U*-test; $^{*}p < 0.05$, ns = not significant, error bars indicate SEM. Pooled data of two independent experiments with 3–4 mice per experimental group is shown. (E) Schematic overview of miR221 action on enhancing PI3K signaling pathway, which results in increased adhesion and survival signaling in preBI-cells in BM.

progenitor cells, which migrate to and are retained in the BM upon transplantation, while preBI-cells are the first B-lineage cells, which downregulate miR221 and fail to become resident in the BM. Future studies will have to investigate, whether the regulation of this endogenous expression of miR221 controls adhesion and retention in BM also in these earlier hematopoietic progenitors. Furthermore, these studies should address whether these progenitors use the same CXCL12-CXCR4-stimulated molecular pathway of adhesion and retention as the miR221-overexpressing preBI-cells used in our present studies.

Here, we show that CXCL12/CXCR4-mediated retention and residence can be restored in previously non-transplantable preBI-cells by expression of miR221 to normal levels found in normal CLPs. Interestingly, we find that miR221-expressing preBI-cells are established in subosteal regions of the BM after transplantation, similar to hematopoietic precursors, like HSCs after transplantation [52](Supporting Information Fig. 4A–E).

Hematopoietic cells regulate many of their surface molecules including cell adhesion molecules, cytokine, and chemokine receptors, dynamically throughout development. This regulation enables the cells to specifically respond to niche factors and disregard other stimuli. Here, we describe the posttranscriptional regulation of PTEN by miR221 enables preBI-cells to strongly respond to CXCL12 and interact with their environment. We propose that this specific amplification of signal transduction via the PI3K pathway by miR221, might also lead to a strong adhesive and pro-survival response of other miR221-expressing hematopoietic progenitors in the BM, which may favor their transplantability.

We found that class I PI3Kinases is essential for integrin activation and adhesion. PI3K activity has been found to play essential roles in integrin activation and cellular adhesion in T cells and platelets [53, 54]. This activity promotes anti-apoptotic and pro-adhesive functions and miR221 expression enhances this PI3K activity. Our results have been obtained by studying miR221 re-expression in preBI-cells which, during normal B cell differentiation, have just lost miR221 expression. We propose that CXCL21-stimulated and PI3K-mediated survival and adhesion could be common miR221-regulated functions in earlier hematopoietic progenitors expressing miR221. In line with this, ex vivo sorted miR221 expressing CLPs not only showed enhanced adhesion to VCAM1, but also showed increased levels of pAkt after isolation and stimulation with CXCL12 in comparison to ex vivo sorted preBI cells which do not express miR221 (Supporting Information Fig. S2E–G).

L bound integrins transduce signals into the cell via integrin associated signaling complexes, including FAK. FAK is an essential mediator of integrin function and cellular adhesion relaying signals from L bound integrins to PI3K. Additionally, FAK is a direct target of PTEN phosphatase activity, which is reduced in miR221 expressing cells [55, 56]. FAK activation by CXCL12 has been shown to promote adhesion of hematopoietic precursor cells to VCAM1 [57, 58]. Integrin-FAK signaling has been also shown to provide important survival signals via PI3K [44].

Signaling for increased survival is one of the many other effects of PI3K function. PI3K activity can lead to the inhibition of apoptosis through inhibition of pro-apoptotic caspase-9 and the Bcl2-antagonist of cell death [59], which increases the activity of the anti-apoptotic factor Bcl2. In fact, we found in miR221-expressing preBI-cells, cultured on BM-derived OP9 stroma-cells, a CXCL12-PI3K dependent increase of Bcl2 protein expression. Ectopic Bcl2 overexpression has been found to rescue survival of STAT5-deficient B-cells [49], which usually depend on IL-7-STAT5-signaling for survival [47]. Hence, miR221-mediated activation of PI3K could result in increased survival by upregulation of Bcl2 in response to environmental factors like CXCL12 in vivo. This could favor survival of miR221-expressing cells in BM environments with low IL-7 concentration. Therefore, enhanced adhesion to a CXCL12-rich microenvironment could also trigger increased survival signaling in miR221-expressing cells via enhanced PI3K signaling.

The PI3K inhibitor Wortmannin has been used in vivo to study PI3K functions in normal hematopoietic cells and in cancer [60–62]. Depletion of miR221 expressing preBI cells from the BM by Wortmannin suggests that activation of PI3K plays an essential role in the retention of miR221-expressing cells in BM. This is in contrast to host preBI-cells, which do not express miR221. Furthermore, when PTEN lacking miR221 binding sites is overexpressed, BM retention of miR221-expressing cells is reduced. Thus, PTEN is a primary target of miR221 to enhance PI3K activity. The inhibition of PI3K by treatment with Wortmannin and overexpression of PTEN did not alter the amount of differentiated IgM^+ cells in BM. This suggests that the reduction of miR221-expressing preBI-cells in BM is not due to increased differentiation. However, the ablation of transplantation is not complete. This could be due to the incomplete block of PI3K signaling by Wortmannin, in vivo. Another explanation could be that additional putative miR221 targets contribute to the in vivo phenotype. One of such candidates is the phosphatase PHLPP, which could be stably repressed by miR221 in vivo, in contrast to our in vitro experiments and, thus, also contribute to a downstream increase of PI3K signaling. It should be noted, that overexpression of miR222, in preBI cells did not allow these cells to become resident in the BM. Moreover expression of miR222, the paralogue of miR221 with an identical seed sequence, did not result in the repression of PTEN. This differential activity of miR221 and miR222 is in contrast to previous reports in humans, where both miR221 and miR222 have been shown to target PTEN [42]. A possible explanation for this difference in miRNA action might be, that the 3‘UTR of PTEN is different in mice and human, and that miR222 might be less efficiently binding to the mouse 3’UTR of PTEN. The levels of miR222 overexpression might thus be insufficient to repress PTEN protein abundance in mouse preBI cells. Future studies will have to identify the potential difference in actions of miR221 and miR222 in preBI-cells and other hematopoietic progenitors.

Aberrant expression of miRNAs and the loss of PTEN are often associated with increased survival, cell cycle progression, and have been shown to be associated with the development of cancer. We found, that re-expression of miR221 does not enhance normal proliferation of preBI-cells in vitro, nor that our transplanted

preBI-cells expand beyond normal preBI-cell numbers in BM, in vivo. Therefore, we think, that the main functions in preBI-cells affected by high expression of miR221 in actively proliferating cells are not cell cycle and proliferation, but adhesion, migration, and survival. In mast cells, miR221/222 has been shown to even inhibit cell proliferation in contrast to cell cycle enhancing functions of miR221/222 in cancer cells [63]. In fact, these diverging effects of miR221 expression on different types of hematopoietic cells suggest, that physiological expression of miR221 at different cellular stages of hematopoiesis, could affect different cellular functions. This is likely due to the diverse expression and accessibility of target genes at each specific cellular stage.

Finally, we would like to emphasize again, that miR221 re-expression in preBI-cells confers an aberrant property to the cells, which they have naturally lost during commitment to the B-cell lineage, and which is necessary in earlier stages of development. The property to become resident or to be able to move away from a certain BM environment for further development might be essential steps in lymphoid differentiation, which can be regulated by miR221.

Materials and methods

Mice

C57BL/6, RAG1$^{-/-}$(CD45.2, CD45.1) mice were bred and kept in the Max Planck for Infection Biology animal facility under SPF conditions. For cell sorting and analysis of different hematopoietic subpopulations, 6–12 wks old C57BL/6 mice were used. For all transplantation experiments approximately 0.5–1 $\times$ 10^6 miR221 expressing preBI-cells (CD45.1) were transplanted in sub lethally irradiated RAG1$^{-/-}$ mice, with 1 g/L doxycycline and 50 g/L sucrose at pH 3.0 in drinking water. Mice were analyzed 1–2 wks after transplantation. For PI3K inhibition in vivo, Wortmannin (Sigma) was administered three times (in 5 days) intraperitoneally with 1.2 mg/kg to RAG1$^{-/-}$ mice, 1 wk after transplantation of miR221 expressing preBI [62]. All of the experimental procedures complied with the "National Regulations for the Care and Use of Laboratory Animals", approved by the Landesamt for Gesundheit and Soziales, Berlin (T0334/13, G0050-17, G0306-13, G0140-11).

B cell cultures

PreBI-cells were propagated on a semi confluent layer of gamma irradiated (30Gy) OP9 stromal cells in IMDM (Gibco), supplemented with 2% FCS, 0.03% Primatone-RL, 100 units penicillin, 100 μg streptomycin, and 1% mouse IL-7 (equivalent to 10 ng/mL recombinant IL-7) at 37°C. For expression of miR221, miR221 preBI-cells were retrovirally transduced with a vector (pSR-LP-TRE), carrying a tet responsive CMVmin promoter, downstream of which was inserted the mature miR221-3p sequence integrated in a miR30 backbone and eGFP [13]. Empty vector controls carry the same vector but without the miR221-3p sequence. For induction of miR221, 1 μg/mL doxycycline was added to the culture for 24–72 h. For PTEN overexpression, miR221 inducible preBI cell lines were retrovirally transduced with a pMX-IRES (CP) Venus construct, carrying the full length CDS of mouse PTEN. To inhibit signaling via CXCR4 and PI3Kδ preBI cells were cultured in the presence of AM3100 (1 μg/mL; Sigma) and IC87114 (5 μg/mL; Sellekchem) respectively for at least 24 h at indicated concentrations.

Flow cytometry and antibodies

Antibodies for flow cytometry used were: CD25-PeCy7, ckit-APC, CD45.1-Alexa700, CD45.1-APC, CD45.2-APC eFluor780, CD4-FITC, Nk1.1-FITC, CD8-FITC, IgM-FITC (eBioscience); CD11c-FITC, CD3-FITC, B220-FITC, CD11b-FITC, TER-119-FITC, CD19-FITC, IL-7R-PeCy7, Flk2-PE, IgM-PeCy7, CD19-APC-Cy7, CD25-APC-Cy7, B220-BV510, CXCR4-BV421, CD19-BV650, CD29-PE (HMβ1-1) (BioLegend), CD29 (9EG7) (BD Pharmingen). Live/dead staining was done with Propidiumiodide. For intracellular staining cells were fixed with 4% PFA for 15 minutes at room temperature and permeabilized with ice cold methanol for 20 min. Antibodies for intracellular flow cytometric analysis used were: anti Bcl2-PE (Miltenyi), anti-PTEN-APC (Miltenyi), anti-pAkt-APC, anti-pERK-PE (eBioscience). For all stainings, cells were blocked with PBS containing 0.5% BSA, 2% FCS and anti-mouse-Fcγ receptor antibody (2.4G2 a kind gift from the DRFZ). Flow cytometry data was collected on a LSR II, LSR-Fortessa and CantoII cytometers (BD Pharmingen) and analyzed with FlowJo10 (Treestar). Cell sorting and part of the data acquisition was performed at the DRFZ facilities. We have adhered to the guidelines for flow cytometry and cell sorting [64].

Adhesion assays

For adhesion assays, flat bottom 96-well plates (Corning 3599) were coated with 1 μg/mL (50 ng per well) recombinant VCAM1 human FcChimera (R&D Systems), overnight in PBS at 4°C. Plates were blocked for 1–2 h with 1% BSA in PBS. Cells were starved for 1 h in IMDM + 0.5% BSA at 37°C in the presence of Wortmannin (1 μg/mL; Sigma), ZSTK474 (1 μg/mL; Sellekchem), anti-CD29 (9EG7) (10 μg/mL) or IgG2a isotype controls (BioLegend), or medium as control for inhibition experiments. Afterward, cells were incubated for 30 min with 100 ng/mL recombinant CXCL12 (PeproTech) for 30 min, before transfer to VCAM1 coated wells with approximately 8000 cells per well, where cells were let to adhere for 30 min in the presence of CXCL12 or medium as control. Cells were washed three to four times with IMDM + 0.5% BSA before remaining cells were collected by addition of ice cold PBS including 0.5% BSA and EDTA. Cell numbers were determined by FACS.

Western blot

For cell signaling experiments, cells were starved for 2–3 h in IMDM medium containing 0.5% BSA. Cells were stimulated with 100 ng/mL CXCL12 for indicated time points. Reaction was stopped on ice and cells were re-suspended in 2% Laemmli Buffer before loading on a 7–10% Acrylamide SDS-PAGE. Proteins were transferred onto a polyvinylidene flouride membrane, blocked with TBS containing 0.05% Tween20 and 3% BSA, and incubated with primary antibodies at 4°C, overnight. The following primary and secondary antibodies were used: phospho–p44/42 (Erk1/2) (Thr202/Tyr204) (D13.14.4E, Cell Signaling), Erk2 (C14, Santa Cruz), phospho-FAK (Y397) (#3283, Cell Signaling), phospho-Akt (Ser473) (#9271, Cell Signaling), Akt (#9272, Cell Signaling), PTEN (217702, R&D Systems), PP2A C-subunit (Millipore), PHLPP (Biomol), rabbit-HRP (#7074, Cell Signaling), mouse-HRP (#7076, Cell Signaling), and actin-HRP (Sigma). Quantification of Western blots: Western blots were quantified with the rectangular selection tool of ImageJ software. Data were normalized to the loading control. The fold induction was calculated by relating normalized expression values to that of the WT preBI cells and unstimulated controls.

Microscopy and image analysis

Femurs and Tibiae from adult mice were obtained from RAG1 deficient mice 1 month after transplantation of preBI-cells. Bones were fixed with 4% PFA for 4 h at 8°C. Bones were frozen in OCT (Sakura Finetek, Torrance, CA), longitudinally sectioned at 7 μm thickness. Sections were stained with anti-GFP-Alexa488, anti-CD105-PE, and anti VCAM1-A647 antibodies overnight at 4°C. If not stated other-wise, confocal images were generated using a 20×/0.8 numerical aperture (NA) air objective lens or a 100×/1.4 numerical aperture oil objective (DIC M27) on a Zeiss LSM710, equipped with Zen 2010 Version 6.0 software. Overview images of BM sections were generated by 2D tile scanning with a 20×/0.8 NA air objective lens. Tiles were recorded with an overlap of 10% and projections. Used resolution set at x: 2048, y: 2048, channels: 3, 12-bit, and images were analyzed using Zen 2012 software (Carl Zeiss MicroImaging).

PreBI cells adherent to 1 μg/mL immobilized VCAM1 were stained with Phalloidin-A647 (Cell Signaling), anti-CD29 (9EG7) (BD Pharmingen), and DAPI. Cells were imaged using a using a 20×/0.8 NA air objective lens or a 100×/1.4 numerical aperture oil objective (DIC M27).

PreBI-cell shape after stimulation with CXCL12 on VCAM1-coated well plates was analyzed by Keyence BZ9000 microscope using a CFI PlanAPO lambda 20×/0.75 NA objective. Images were analyzed by ImageJ. To asses cellular protrusions, the roundness of cell shape was measured; Roundness: $4 \times [Area]/(\pi \times [Major\ axis]^2)$. Colocalization analysis was performed by ImageJ using the Coloc2 Plugin.

Real time PCR

PTEN and miR221 expression was quantified using TaqMan real time PCR assays (ThermoFisher) by the $^{\Delta/\Delta}$CT method. GAPDH and Sno202 were used as housekeeping genes.

Luciferase assays

Luciferase assays have been performed with the DualGlo Luciferase Kit by Promega, according to manufacturer instructions. Briefly: HEK293T cells were transfected with 0.5 μg of Psi-Check2 vector carrying ca. 500 bp of respective 3'UTR sequences of PTEN, containing a miR221 binding site. Together with the vector, containing the WT or mutated PTEN-UTR sequences, 50 nM (final concentration) miR221 mimics were cotransfected. Mutated binding sites were devoid of miR221 sequence. Target sequences were synthetically generated by Gene Strings (Thermo Fisher). Twenty-four hours after transfection Renilla LUC luminescence was measured and normalized to Firefly LUC luminescence.

Acknowledgments: We are also indebted to Dr. Andreas Schmidt and Dr. Lena Lampe for critically reading the manuscript. This study was in parts supported by a Reinhard Koselleck-Grant of the Deutsche Forschungsgemeinschaft (D.F.G.) ME 2764/1-1 to F.M., and the Transregio SFB, TRR130 of the D.F.G.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **CLP**: common lymphoid progenitor · **CXCL12**: C-X-C motif chemokine 12 · **HSC**: hematopoietic stem cells · **PTEN**: Tensin homolog phosphatase · **VLA4**: Very Late Antigen-4

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Received: 3/10/2017
Revised: 22/1/2018
Accepted: 9/2/2018
Accepted article online: 5/3/2018

