Regulation of B-cell development and tolerance by different members of the miR-17 ~ 92 family microRNAs

Maoyi Lai¹*, Alicia Gonzalez-Martin¹*, Anthony B. Cooper¹*,†, Hiroyo Oda¹,†, Hyun Yong Jin¹,², Jovan Shepherd¹, Linling He¹, Jiang Zhu¹, David Nemazee¹ & Changchun Xiao¹

The molecular mechanisms that regulate B-cell development and tolerance remain incompletely understood. In this study, we identify a critical role for the miR-17 ~ 92 microRNA cluster in regulating B-cell central tolerance and demonstrate that these miRNAs control early B-cell development in a cell-intrinsic manner. While the cluster member miR-19 suppresses the expression of Pten and plays a key role in regulating B-cell tolerance, miR-17 controls early B-cell development through other molecular pathways. These findings demonstrate differential control of two closely linked B-cell developmental stages by different members of a single microRNA cluster through distinct molecular pathways.
A defining feature of B-cell development is the process of gene rearrangements in the B-cell receptor (BCR) locus, through which B cells acquire the capacity to express a BCR of a given specificity on the cell surface. These rearrangements occur in an orderly manner over time, sequentially involving the immunoglobulin heavy (IgH) and light (IgL) chain genes during the pro-B and the pre-B stages of B-cell development, respectively. This is thought to depend on an orderly accessibility of the corresponding loci to the RAG1 and 2 recombinases, which mediate rearrangements of variable (V), diverse (D), and joining (J) gene segments through the process called V(D)J recombination. At the end of this process, each immature B cell expresses a single IgH and a single IgL chain, with a single and, most times, unique antigen specificity. The combinatorial and stochastic nature of gene rearrangements leads to the generation of immature B cells with self-reactive receptors. At this immature stage, the newborn B cells undergo the first checkpoint for self-reactivity, to eliminate potentially autoreactive cells by central tolerance mechanisms. Once a newborn B cell encounters a self-antigen for which its BCR is specific, it attempts to escape autoreactivity by continuing V(D)J recombination at the IgL locus (receptor editing) or dying by apoptosis (clonal deletion). When the cell has passed this developmental checkpoint, it differentiates into a mature B cell. Self-reactive B cells can be further regulated in the periphery through peripheral tolerance mechanisms, including the induction of energy. Previous studies of human B cells showed that self-reactivity is progressively diminished during normal B-cell development, consistent with the idea that several tolerance mechanisms are at work at different stages of the life of B cells.

Despite intensive study, our understanding of molecular pathways regulating B-cell development and tolerance is still incomplete. Specifically, the function of individual microRNAs (miRNAs) in B-cell development and tolerance remains poorly understood. miRNAs are endogenously encoded single-stranded RNAs of ~22 nucleotides in length. To date, ~2,500 human and ~1,900 mouse miRNAs have been identified and many of them play essential roles in the immune system. They regulate gene expression by pairing with messenger RNAs through imperfect sequence complementarity, resulting in reduced protein output by miRNA cleavage, translational repression or promotion of mRNA decay. It has been estimated that 25–40% miRNA precursors are located in close proximity (~10 kb) of other miRNA precursors, constituting miRNA clusters. The majority of miRNA clusters are first transcribed into single polycistronic primary transcripts (pri-miRNAs) and then cleaved by Drosha into individual hairpins (pre-miRNAs), which are further processed by Dicer to produce mature miRNAs. Gene expression profiling studies have shown that the expression of different miRNAs in a cluster is generally co-regulated, suggesting that they may cooperate with each other to accomplish common functions. Furthermore, comparative genomics show that miRNA clusters are evolutionarily stable and conserved across species, suggesting functional importance of such organization. Some clusters consist of miRNAs with identical seed regions (termed homogeneous miRNA clusters), probably a result of gene duplication. The regulatory effect of homogeneous miRNA clusters may simply be an increase in gene dosage. Other clusters are composed of miRNAs with different seed regions (termed heterogeneous miRNA clusters). It remains unclear how members of heterogeneous miRNA clusters operate together to accomplish common functions.

In this study we dissected the roles of the miRNA-17~92 family miRNAs at different stages of B-cell development. The miR-17~92 family consists of three miRNA clusters: miR-17~92, miR-106a~363 and miR-106b~25. Together, these three clusters contain 15 miRNA stem loops that give rise to 13 distinct mature miRNAs, which fall into four miRNA subfamilies (miR-17, miR-18, miR-19 and miR-92 subfamilies), with members in each subfamily sharing the same seed sequence.

The genomic organization and mature miRNA sequences of this family are conserved in all vertebrates. During lymphocyte development, these miRNAs are highly expressed in progenitor cells, with expression levels decreasing two- to threefold on maturation. Mouse genetic studies showed that miR-17~92-deficient mice were runted and died at birth due to lung and heart hypoplasia. In the haematopoietic system, there was a partial block of early B-cell development at the pro- to pre-B transition. Deletion of miR-106a~363 and miR-106b~25 has no obvious phenotypic consequences, whereas compound mutant embryos lacking both miR-17~92 and miR-106b~25 died at midgestation, suggesting functional redundancy between these miRNA clusters. Conversely, overexpression of miR-17~92 family miRNAs occurs frequently in a broad spectrum of human cancers and in lymphocytes from patients with autoimmune diseases. These observations suggest that miR-17~92 is involved in lymphoma development and autoimmune diseases.

We have generated a conditional miR-17~92 transgenic allele (term miR-17~92 Tg) whose expression can be turned on by Cre recombinase. When miR-17~92 Tg was turned on specifically in B cells using CD19-Cre, transgenic mice exhibited premature death. About 80% of those mice developed lymphomas, demonstrating that miR-17~92 is a powerful cancer driver. Notably, the other 20% miR-17~92 transgenic mice died of autoimmune diseases. We speculate that miR-17~92 might play a critical role in the control of B-cell tolerance. In this study, we use two newly generated in vivo models to investigate the function and mechanism of miR-17~92 family miRNAs in regulating B-cell development and tolerance, and demonstrate functional specificity of different members of this cluster at two closely linked developmental stages of B cells.

**Results**

miR-17~92 regulates B-cell central tolerance. To analyse the effect of transgenic miR-17~92 expression on B-cell central tolerance, we used the recently established IgM<sup>B</sup>-macrolslf mouse model. In this model, mice are engineered to ubiquitously express a superantigen reactive to the heavy chain constant region of IgM, the first BCR expressed on the surface of immature B cells. As receptor editing changes only the light chain, it fails to eliminate superantigen reactivity and all developing B cells undergo cell death by clonal deletion. As a consequence, these mice have almost no mature B cells in the spleen and lymph nodes. We analysed B-cell development in IgM<sup>B</sup>-macrolslf recipients (with surface marker CD45.1<sup>+</sup>) reconstituted with bone marrow from CD19-CremiR-17~92 Tg/Tg (TG) or wild-type (WT) mice (with surface marker CD45.2<sup>+</sup>) (Fig. 1a). The WT→IgM<sup>B</sup>-macrolslf chimeras exhibited a severe B-cell lymphopenic phenotype recapitulating that of the IgM<sup>B</sup>-macrolslf mice, characterized by a complete developmental block at the immature B-cell stage in the bone marrow that results in the absence of mature B cells in the spleen. Strikingly, B-cell development in the TG→IgM<sup>B</sup>-macrolslf chimeras was similar to that in WT→WT chimeras (Fig. 1b,c). Consistent with the restoration of peripheral B cells, serum IgM levels were also substantially restored in the TG→IgM<sup>B</sup>-macrolslf chimeras (Supplementary Fig. 1a). Moreover, these rescued B cells appeared to be functional, as TG→IgM<sup>B</sup>-macrolslf chimeras mounted a close-to-normal antibody response upon NP-CGG immunization (Supplementary Fig. 1b). This is the first time that...
**Figure 1 | miR-17 ~ 92 controls B-cell central tolerance.** (a) Outline of bone marrow reconstitution experiment. In the bone marrow of IgMβ-macroself recipient mice, all nascent immature B cells undergo negative selection as a result of the interaction between the surface IgM and the ubiquitously expressed anti-IgM superantigen. (b) Representative flow cytometry plots showing B-cell development in the bone marrow and spleen of recipient mice. (c) Numbers of donor-derived splenic B cells (CD45.2+CD19+IgM+) in bone marrow-reconstituted mice, with each dot representing a single mouse and the horizontal bar indicating the average cell number for each group. (d) BCR repertoire analysis based on IGHV gene usage in splenic B cells from WT C57BL/6J (WT) and CD19Cre;miR-17 ~ 92 transgenic (TG) mice. B cells were activated in vitro to facilitate the analysis. Results are presented as fold change of TG over WT mice. The dotted line was set at the arbitrary value of 3, to indicate IGHV genes with more than threefold increase in usage in TG mice as compared with WT mice. Data are representative of three (b,c) and two independent experiments (d) (mean ± s.e.m. in d) with n = 5 (WT to WT) or 6 (WT to IgMβ-macroself and TG to IgMβ-macroself) in b,c and n = 3 in d.

We observed such a complete restoration of B-cell development in the IgMβ-macroself central tolerance model by a miRNA gene.

Central tolerance, when functionally intact, protects against autoimmunity by purging a large majority of self-reactive B cells from the B-cell compartment. As B cells with elevated miR-17 ~ 92 expression were able to escape from central tolerance in IgMβ-macroself model, in which all B cells are self-reactive, we reasoned that the BCR repertoire in TG mice might be significantly different from that of WT mice. To assess this, we analysed the BCR repertoire by performing deep sequencing of Ig heavy chain V genes (IGHV) of WT and TG mice. As predicted, TG mice exhibited an IGHV landscape different from that of WT mice, with drastically increased usage in TG mice (>3-fold) of three IGHV genes (IGHV12-3, IGHV4-1 and IGHV11-2) (Fig. 1d). Among these, VH11 and VH12 gene families are predominantly associated with germline-encoded autoantibodies of phosphatidylcholine specificities. A large numbers of VH11+ B cells, which are rarely detected in the spleen of WT mice, were present in the spleen of TG mice, as detected by using an anti-idiotypic monoclonal antibody for VH11 (Supplementary Fig. 1c). Conversely, we found reduced usage of IGHV11-2 and IGHV12-3 in B cells deficient for the miR-17 ~ 92 miRNA family (CD19tKO) (Supplementary Fig. 1d). In addition, we measured the presence of anti-double-stranded DNA autoantibodies in the serum of WT and TG mice at terminal analysis. High titres of autoantibodies were detected in 4 out of 45 TG mice, but were not found in any of the WT mice (Supplementary Fig. 1e).

We next investigated the function of physiological levels of miR-17 ~ 92 family miRNAs in B-cell central tolerance. We analysed receptor editing in WT mice and mice with B-cell-specific deletion of this miRNA family (by either Mb1-Cre or CD19-Cre, termed Mb1tKO and CD19tKO mice, respectively). Deletion of the miR-17 ~ 92 family miRNAs in B cells impaired receptor editing, as indicated by increased k/λ-light chain ratios and reduced percentages of λ-light chain-positive (Igλ+) immature B cells (Supplementary Fig. 2a–d). Conversely, transgenic miR-17 ~ 92 expression promoted receptor editing (Supplementary Fig. 2c–d). These results show that endogenous levels of miR-17 ~ 92 family miRNAs exquisitely regulate receptor editing at the B-cell central tolerance checkpoint. We further analysed the effect of transgenic miR-17 ~ 92 expression on B-cell central tolerance in the IgHEL;mHEL mouse model. In this model, IgHEL mice express a transgenic BCR that is specific for hen egg lysozyme (HEL) in their B cells, whereas mHEL mice express a membrane-bound HEL in a wide range of cells. In IgHEL;mHEL double transgenic mice, developing B cells
are deleted in the bone marrow and few mature B cells appear in the peripheral lymphoid organs\(^3\). Consistent with the previous report, we detected very small numbers of B cells bearing IgHEL (IgD\(^{+}\)) in the spleens of IgHEL mice reconstituted with bone marrow cells from WT IgHEL mice (WT; IgHEL). The number of splenic IgHEL (IgD\(^{+}\)) B cells increased by fivefold when miHEL mice were reconstituted with bone marrow cells from IgHEL mice with B-cell-specific transgenic miR-17~92 expression (TG;IgHEL) (Supplementary Fig. 3). These data, together with previous results from the IgM\(^{+}\)-macrophage model (Fig. 1b,c), demonstrate a general effect of elevated miR-17~92 expression on B-cell selection against self-antigens. Therefore, our data show that miR-17~92 is a critical regulator of B-cell central tolerance at the immature B-cell stage.

**miR-19 plays a key role in B-cell central tolerance.** We next investigated the functional contribution of individual miR-17~92 members in regulating B-cell central tolerance. The six miRNAs of miR-17~92 fall into four miRNA subfamilies (the miR-17, miR-18, miR-92 and miR-92 subfamilies), with members in each subfamily sharing the same seed region and probably similar functions. We generated two groups of lentivectoral vectors expressing either the miR-17~92 cluster with one miRNA subfamily deleted, or four tandem copies of individual miRNAs (Fig. 2a). Northern blot analyses confirmed that these lentivectoral vectors expressed encoded miRNAs at levels comparable to a lentiviral family deleted, or four tandem copies of individual miRNAs numbers of splenic B cells that escaped IgMb-macroself-mediated regulation of B-cell tolerance was determined by quantifying the next investigated the functional contribution of individual miR-17~92 members in regulating B-cell central tolerance. To assess whether miR-17~92 regulates the expression of these target genes in immature B cells, we purified B220\(^{+}\)IgM\(^{+}\)CD93\(^{+}\)B cells from TG mHEL mice reconstituted with B cells co-expressing miR-19 and control (GFP) expressing miR-19 alone (GFP-Ametrine\(^{+}\)) and control (GFP) expressing miR-19 alone (GFP-Ametrine\(^{+}\)). Remarkably, homozygous deletion of the Pten gene (Pten\(^{−/−}\)) completely restored the B-cell compartment, which is comparable to TG→IgM\(^{+}\)-macrophage chimeras (Fig. 3d,e). We next used the lentiviral expression system to co-express miR-19 and Pten in HSCs, as illustrated in Fig. 3f, to test whether restoring Pten expression can prevent the break of B-cell central tolerance by miR-19 in the IgM\(^{+}\)-macrophage recipients. The miR-19-expressing lentivirus encodes GFP, whereas the Pten-expressing lentivirus encodes Ametrine, therefore allowing distinguishing cells transduced by a single lentivirus from those transduced by both. Terminal analysis of the reconstituted mice showed no difference in the total numbers of splenic B cells that escaped the central tolerance checkpoint, whether Pten or its vector control was introduced to the miR-19 overexpression scheme. However, we observed a drastic difference in the compositions of these escaped B cells. In the miR-19 + control group, the number of B cells expressing miR-19 alone (GFP + Ametrine\(^{−}\)) was comparable to that of B cells co-expressing miR-19 and control (GFP + Ametrine\(^{+}\)). In the miR-19 + Pten group, the escaped B cells were predominantly those expressing miR-19 alone (GFP + Ametrine\(^{−}\)), whereas the number of B cells co-expressing miR-19 and Pten (GFP + Ametrine\(^{+}\)) was close to the IgM\(^{+}\)-macrophage background level (Fig. 3g,h). Taken together, these results suggest a miR-19-Pten pathway that regulates B-cell central tolerance.

**Impaired B-cell development in the absence of miR-17~92.** A previous study showed that germine deletion of miR-17~92 led to a partial block of early B-cell development at the pro- to pre-B transition and Bim was suggested to be a key functional
Figure 2 | miR-19 plays a critical role in regulating B-cell central tolerance. (a) Lentiviral vectors expressing the FL miR-17~92, its deletion mutants and individual members. miRNAs sharing the same seed region belong to the same subfamily and are depicted in the same colour. (b) Northern blot analysis of miRNA expression in HeLa cells transduced with indicated lentiviruses. (c) Outline of experimental strategy. HSCs were enriched from WT donors to facilitate the transduction by lentiviruses encoding various combinations of miR-17~92 miRNAs (LV-miRNAs). (d) Representative flow cytometry plots of lymphocytes from the spleen of recipient mice. It is noteworthy that lentiviruses encoding miR-17~92 (FL) fully rescued the B-cell compartment, and that virus transduced cells (CD45.2<sup>+</sup> GFP<sup>+</sup>) outcompeted WT helper-derived cells (CD45.2<sup>+</sup>/GFP<sup>+</sup>/CD45.1<sup>+</sup>/TCR<sub>β</sub>+) and non-transduced cells (CD45.2<sup>+</sup>/GFP<sup>+</sup>/CD45.1<sup>+</sup>/TCR<sub>β</sub>−). (e) Numbers of splenic B cells in IgM<sup>+</sup>-macrophage recipient mice. B-cell number above the dash line indicates break of tolerance. Data are representative of 3 (b) or 8 (d) or pooled from 8 independent experiments (e) (mean ± s.e.m. in e) with n = 5 (Vector) or 14 (FL) in d, and n = 5 (Vector and del18), 14 (FL), 10 (del17 and 4 × 19), 4 (del19 and del92) or 6 (4 × 17, 4 × 18 and 4 × 92) in e.
target of miR-17~92 in this process\textsuperscript{19}. However, another study reported that transgenic mice with ubiquitous overexpression of miR-17, a member of the miR-17~92 cluster, exhibited overall growth retardation and severely reduced numbers of B lineage cells in both the bone marrow and the spleen\textsuperscript{34}. Therefore, the cell autonomous function of the miR-17~92 family, as well as the functional contribution of individual miRNAs of this family, in B-cell development remains to be elucidated. To investigate the cell-intrinsic role of miR-17~92 family miRNAs in B-cell development, we generated Mb1-Cre;miR-17\textsuperscript{92}\textsuperscript{fl/fl};miR-106a\textsuperscript{B}\textsuperscript{363}/C0\textsuperscript{0}/C0;miR-106b\textsuperscript{B}\textsuperscript{25}\textsuperscript{0}/C0\textsuperscript{0}/C0 mice (termed Mb1tKO). In these mice, miR-17~92 is deleted specifically in the B-cell lineage by Mb1-Cre, a Cre allele expressed from the earliest stage of B-cell development\textsuperscript{35}, whereas the two paralogue clusters, miR-106a\textsuperscript{B}\textsuperscript{363} and miR-106b\textsuperscript{B}\textsuperscript{25}, are deleted in the germline, resulting in a complete absence of this family in the B-cell lineage.
Analysis of Mb1tKO mice revealed a twofold reduction in B-cell numbers in the bone marrow (Fig. 4a). Consistent with the previous study of miR-17~92 germline knockout mice, Mb1tKO mice showed an accumulation of pro-B cells (B220<sup>hi</sup> cKit<sup>+</sup>) and a reduction of pre-B cells (B220<sup>hi</sup>CD25<sup>+</sup>) (Fig. 4b,c). The splenic B-cell number in Mb1tKO mice was also reduced by threefold (Fig. 4d). Apoptosis of pro-B and pre-B cells of Mb1tKO mice was determined by flow cytometry analysis of Annexin V and active caspase 3. We observed an increased apoptotic rate in pre-B cells of Mb1tKO mice when compared with their counterparts in WT mice (Fig. 4e). In conclusion, the miR-17~92 family miRNAs control the pro- to pre-B transition during B-cell development in a cell-autonomous manner and the complete absence of this miRNA family leads to increased apoptosis of pro-B cells.

**A central role of miR-17 in early B-cell development.** We next analysed which individual miRNA subfamily of the miR-17~92 cluster plays an essential role in the pro- to pre-B-cell transition. To evaluate the functional contribution of each miRNA subfamily of the miR-17~92 cluster to the regulation of early B cell development, we restored the expression of miR-17~92 miRNAs, either individually or in combination (Fig. 2a), in Mb1tKO HSCs by lentiviral transduction and determined their ability to undergo the pro- to pre-B-cell transition. A mixed bone marrow reconstitution approach, in which Mb1tKO B cells were competing with their WT counterparts in WT mice (Fig. 4e). In conclusion, the miR-17~92 family miRNAs control the pro- to pre-B transition during B-cell development in a cell-autonomous manner and the complete absence of this miRNA family leads to increased apoptosis of pro-B cells.

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**PTEN, Phlpp2 and Bim do not control early B-cell development.** The PI3K pathway has been shown to control early B-cell development. Our results showed that the miR-17~92 family miRNAs suppress the expression of PTEN and Phlpp2 in immature B cells (Fig. 3a) and mature B cells. Flow cytometry analysis of pAkt in developing B cells of Mb1tKO mice showed decreased Akt phosphorylation in pro-B cells when compared with their WT counterparts (Fig. 6a). Previous studies have also suggested Bim as a key functional target mediating miR-17~92 regulation of B-cell development. We found slightly increased protein levels of PTEN and Bim. For those reasons, we speculated that miR-17~92 family miRNAs regulate early B-cell development by suppressing the expression of PTEN, Phlpp2 and Bim.

To test whether decreased PTEN and Phlpp2 expression rescues the B-cell development defect of Mb1tKO mice, we generated a panel of mouse strains by introducing Pten and/or Phlpp2 deletion to Mb1tKO mice. As shown in Fig. 6b,c and Supplementary Fig. 6a,b, heterozygous and homozygous deletions of Pten and Phlpp2, either individually or in combination, were not able to rescue the pro- to pre-B transition block in Mb1tKO mice. Interestingly, homozygous deletion of Pten was detrimental to pro-B cells on the Mb1tKO background, as shown by the complete loss of B220<sup>hi</sup> cKit<sup>+</sup> pro-B cells when both Pten alleles were deleted.

Previous studies showed that Mb1-Cre-mediated deletion of Dicer led to a severe block of early B-cell development at the pro- to pre-B transition, which is similar to but stronger than
that caused by germline deletion of miR-17~92 (ref. 19), or by the complete deletion of the miR-17~92 family miRNAs specifically in the B-cell lineage (that is, Mb1tKO mice in this study). An Emi1-Bcl-2 transgene (Bcl2Tg) was able to partially rescue the pro- to pre-B transition block caused by Dicer deletion37,38. We therefore tested whether the same Bcl2Tg was able to restore B-cell development in Mb1tKO mice. As shown in Supplementary Fig. 6c,d, although Bcl2Tg did restore the splenic B-cell numbers, probably by prolonging the survival of mature B cells in the periphery39, it did not have any measurable effect on the pro- to pre-B transition block in Mb1tKO mice. Taken together, these results exclude PTEN, Phlpp2 and Bim as major mediators of the regulation of early B-cell development by the miR-17~92 family miRNAs. Thus, other target genes and molecular pathways must play more important roles in this process.

Discussion
The present study revealed critical roles of the miR-17~92 family miRNAs in two sequential events in the development of B cells:
the pro- to pre-B cell transition and the establishment of B-cell central tolerance. The use of two novel in vivo models, Mb1tKO and the IgM<sup>+</sup>-macrolf mice, enables us to dissect the dynamic functional contribution of individual members of a miRNA cluster in two closely linked developmental stages of B cells. As miR-17~92 plays essential roles in both events, functional analysis of individual miRNAs encoded by this cluster in each event provides new insights into the question of specificity versus redundancy of individual members of heterogeneous miRNA clusters. Our findings demonstrate that different members of the miR-17~92 cluster play central roles in controlling B-cell development and tolerance through different molecular pathways.

To our knowledge, miR-17~92 is the first miRNA cluster that has been discovered to control B-cell tolerance. During early B-cell development, miR-17~92 miRNAs are relatively abundant in pro- and pre-B cells, but their expression goes down drastically when developing B cells transit from pre-B to immature B cells<sup>13,18</sup>. As miR-17~92 is generally thought to play pro-survival and pro-proliferation roles, keeping its expression level low renders immature B cells sensitive to self antigen-induced apoptosis, when the opportunity of receptor editing is exhausted. Among the six miRNAs encoded by miR-17~92, miR-19 plays a key role in controlling B-cell central tolerance, at least partly through suppressing the expression of PTEN, a negative regulator...
of the PI3K-Akt pathway. This PI3K-Akt pathway was previously shown to be essential for BCR tonic signalling, which is essential for the survival of mature B cells. Our results demonstrate that this pathway also plays critical roles in supporting the survival of immature B cells.

Several previous studies suggested that the miR-17~92 family miRNAs are essential for the pro- to pre-B transition during early B-cell development, but discrepancy exists among those reports. In a study employing Mb1-Cre-mediated deletion of Dicer, which abolished the expression of all mature miRNAs, B-cell development was severely blocked at the pro- to pre-B transition. miR-17~92-mediated suppression of Bim expression was proposed to be the major underlying mechanism. Indeed, germline deletion of miR-17~92 caused a partial B-cell development block at the pro- to pre-B transition and the Bim protein level was higher in miR-17~92-deficient pre-B cells. The pro- to pre-B transition block caused by Dicer deletion was partly rescued by a Eμ-Be Il2 transgene, further supporting an important role of Bim in mediating miR-17~92 control of early B-cell development. However, another study reported that transgenic mice with ubiquitous overexpression of miR-17, a member of the miR-17~92 cluster, exhibited a severe reduction in the number of B lineage cells in both the bone marrow and the spleen. The latter study casted doubt on the cell autonomous function of the miR-17~92 family miRNAs in controlling early B-cell development. In the present study, we generated and analysed mutant mice harbouring B-cell-specific deletion of the miR-17~92 family miRNAs (Mb1KO mice). These mutant mice exhibited a pro- to pre-B transition block that is similar to that caused by Mb1-Cre-mediated deletion of Dicer and germline deletion of miR-17~92 (refs 19,37). Therefore, the miR-17~92 family miRNAs do play a cell-intrinsic role in controlling early B-cell development. Our functional dissection experiments revealed that, among the six miRNAs encoded by the miR-17~92 miRNA family, (a) Phospho-Akt T308 (p-AKT) and Bim protein levels were measured in pro-B and pre-B cells of control and Mb1KO mice by flow cytometry and presented as overlay histograms. (b) Representative flow cytometry plots and cell numbers of splenic IgM+ B cells (B220+IgM+), pro-B cells (B220+IgM−) and pre-B cells (B220+IgM−CD25+) in the bone marrow of mice of indicated genotypes. (c) Representative flow cytometry plots and cell numbers of splenic IgM+ B cells (B220+IgM+) in mice of indicated genotypes. Data are representative of 2 (a) or 8 (b,c), or pooled from 8 (b,c) independent experiments (mean ± s.e.m. in b,c) with n = 4 (a: Phospho-Akt (T308)) or 2 (a: Bim) in a, n = 5 (Control, Mb1KO, Mb1KO;Pten+/−, Phlpp2−/− and Mb1KO;Pten+/−, Phlpp2−/−), 7 (Mb1KO;Pten+/−), 9 (Mb1KO;Pten+/−, 8 (Mb1KO;Phlpp2−/−) and Mb1KO;Pten+/−, Phlpp2−/−, Phlpp2−/−) in b (BM Ig+ and preB) and c, and n = 4 (Control and Mb1KO), 5 (Mb1KO;Pten+/−, Mb1KO;Pten+/−, Phlpp2−/−, Mb1KO;Pten+/−, Phlpp2−/− and Mb1KO;Pten+/−, Phlpp2−/−), 9 (Mb1KO;Pten+/−), 6 (Mb1KO;Phlpp2−/− and Mb1KO;Phlpp2−/−) or 3 (Mb1KO;Pten+/−, Phlpp2−/−) in b (proB).
synergize with miR-17 to exert this function. Surprisingly, although PTEN is an important mediator of the miR-17~92 function in immature B (shown in this study) and mature B cells32,33, it does not seem to play critical roles in miR-17~92 regulation of early B-cell development. In addition, our results excluded an important role of miR-17~92 regulation of Bim in controlling early B-lymphocyte development, which was suggested by previous studies19,37. Thus, other unknown molecular pathways must mediate miR-17~92 family miRNA control of the pro- to pre-B-cell transition. The elucidation of these pathways warrants further investigation.

Our experimental approach provided a unique opportunity to investigate how individual members of a heterogeneous miRNA cluster work together to exert their functions in a dynamic manner. Previous studies of miR-17 cluster work together to exert their functions in a dynamic fashion,56,64 and it may be possible that at different developmental stages of a single cell lineage, or in different cellular contexts, the functional relevance of these molecular pathways may differ to a large degree. That would explain the differential contribution of individual members of a miRNA cluster to its roles in different cellular contexts. Thus, miR-17 plays a critical role in regulating B-cell central tolerance through suppressed expression of Pten, whereas miR-17 controls the pro- to pre-B transition through other unknown molecular pathways.

In summary, our study identified critical roles of miR-17~92 in two closely linked developmental stages of the B-cell lineage, pro- to pre-B transition and immature B cells. Interestingly, different members of this cluster play central roles in these two processes by regulating different molecular pathways. These findings illustrate dynamic functional specificity of individual members of a heterogeneous miRNA cluster in B-cell development.

Methods

**Mice.** The generation of miR-17~92 Tg (Jax stock 008317), miR-17~92fl/fl (Jax stock 008458), miR-106a~363+/− (Jax stock 008461), and miR-106b~25 (Jax stock 008460), CDF1-Cre (Jax stock 006785), B6.Cr, IgμΔ-macro zelf, Ptenfl/fl (Jax stock 006400) and Eμ-Bcl-2 transgenic mice was previously reported19,24,26,35,38,45,46. Phlpπpfl/fl mice were generated in the Xiao lab using EUCOMM ES clone HEPD00619_1_C08. All these strains are in the C57BL/6j genetic background. Both male and female mice of 8–12 weeks of age were used for all experiments. For early B-cell development characterization, mice were analysed at 6–8 weeks of age. All mice were bred and housed under specific pathogen free (SPF) conditions. All animal experiments were approved by the Animal Care and Use Committee of The Scripps Research Institute.

**Lentiviral vector generation and virus production.** Lentiviral plasmids pWPXlkd, pMD2.G and psPAX2 were gifts from Dr Didier Trono laboratory (Addgene plasmid numbers #12258, 12259 and 12360). The expression vectors for miR-17~92, miRNAsubfamily deletion mutants and individual miRNA components were constructed by stepwise cloning of various combinations of individual miRNAs (precursor sequences with 30–50 nucleotide flanking regions) into pWPXlkd. The Ametrine expression vectors were constructed by replacing the CAG promoter in pWPXlkd with the hCMV promoter. A 1.1-Kb Ametrine-expressing construct was subcloned into an empty lentivirus plasmid backbone to form the pWPXlkd-ΔAmetrine plasmid. The pWPXlkd-ΔAmetrine plasmid was linearized with NotI and in vitro transcribed to produce Ametrine-expressing pWPXlkd lentiviral particles. The T7 promoter in the pWPXlkd plasmid was replaced by a T7 promoter that included a T7 terminator signal to prevent transcription read-through from the T7 promoter.

**Bone marrow transplantation and reconstitution experiments.** HSCs were purified by enrichment of Sca-1+ cells using magnetic cell separation following isolation of bone marrow side population cells using FACs based on the capacity of these cells to actively exclude the vital dye Hoechst 33342 (ref. 47). Briefly, bone marrow cells were prepared from femurs and tibiae. Single cell suspensions of bone marrow cells were re-suspended in HBSS containing 2% fetal bovine serum (FBS), penicillin-streptomycin and 10 mM Hepes buffer, and stained with 8.8 μg ml−1 Hoechst 33342 (Invitrogen) at 5 × 10^6 cells ml−1. After incubation at 37°C for 90 min, the Hoechst 33342-negative side population cells were isolated using a FACSAria cell sorter (BD Biosciences). Purified HSCs were re-injected in StemSpan medium (StemCell Technologies) supplemented with 100 μg ml−1 stem cell factor (SCF), 50 μg ml−1 thrombopoietin (TPO), 100 μg ml−1 Fms-related tyrosine kinase 3 ligand (FLT3-L), 10 μg ml−1 interleukin-6 (all from PeproTech) and 2 μg ml−1 Polybrene (Sigma-Aldrich). Lentiviral transduction (multiplicity of infection = 10) was performed in round bottom 96-well plates, using 10–15 × 10^4 cells per 25 μl reaction volume, at 37°C for 24 h.

Recipient mice were irradiated with two doses of 5 Gy, 3 h apart and subjected to bone marrow transplantation 2 h later by tail vein injection: (i) 5 × 10^6 bone marrow cells from the indicated donors or (ii) 10–15 × 10^6 lentivirally transduced HSCs mixed with 1 × 10^6 congenic, unfractonated bone marrow cells. Recipient mice were maintained with antibiotics-containing food or water for 30 days before switching to normal food and analysed at indicated time points.

**Antibodies, western blot and flow cytomtery analysis.** For western blotting, cell lysates were resolved on 4–20% SDS–PAGE. Antibodies used for western blotting were anti-PTEN (Cell Signaling, 9559; dilution 1/1,000), anti-Phlpπp2 (Bethyl, A300-564A; dilution 1/1,000), anti-IgM (RML-42, 407306; dilution 1/10), anti-IgG (A3-527; dilution 1/10) was performed in 10% SDS–PAGE. Antibodies and reagents with the following specificities were used for western blotting: anti-Caspase 3 (550480; dilution 1/50), anti-CD19 (1D3, 550992; dilution 1/400), anti-CD45.2 (104, 109820; dilution 1/200), anti-CD19 (6D5, 28-1682; dilution 1/200), anti-CD45.1 (A20, 110730; dilution 1/200), anti-B220 (RA3-6B2, 103236; dilution 1/200), anti-CD45.1 (103236; dilution 1/200), anti-CD25 (PC61, 102008; dilution 1/200), anti-TCR b (H57, 160311; dilution 1/100), anti-IgD (11-26, 12-5993-82; dilution 1/500), anti-CD93 (AA4.1, 17-5892-83; dilution 1/200) and anti-β-actin (Sigma-Aldrich, AC-74; dilution 1/10,000). Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 7.

Cell surface staining and flow cytometry analysis were performed following established protocols. Intracellular staining was performed following fixation and permeabilization using BD Phosflow Perm Buff II. Stained cells were analysed on FACS Calibur or LSR II (BD Biosciences). Data were analysed with FlowJo software (Tree Star). Antibodies and reagents with the following specificities were used for staining: anti-B220 (RA3-6B2, 103236; dilution 1/200), anti-CD45.1 (A20, 110730; dilution 1/200), anti-CD45.2 (104, 109820; dilution 1/200), anti-CD19 (6D5, 28-1682; dilution 1/100), anti-TCR b (H57, 160311; dilution 1/100), anti-IgD (11-26, 12-5993-82; dilution 1/500) and anti-CD93 (AA4.1, 17-5892-83; dilution 1/100) were purchased from eBioscience; anti-active Caspase 3 (550480; dilution 1/150), anti-CD19 (1D3, 550992; dilution 1/400), anti-CD45.1 (103236; dilution 1/200), anti-CD45.2 (104, 109820; dilution 1/200), anti-light chain κ (187.1, 561354; dilution 1/50) from BD Bioscience; anti- IgM (115-097-020 and 115-175-075; dilution 1/500) from Jackson ImmunoResearch; and anti-Bum (C345C, 2933; dilution 1/200) and anti-phospho-Akt-Thr308 (24419, 4056; dilution 1/100) from Cell Signaling.

**In vitro immature B-cell culture and enrichment.** Total bone marrow cells were isolated from the femur and tibia. B lineage cells were enriched by magnetic cell sorting using anti-CD19 MACS MicroBeads (Miltenyi Biotech). CD19+ cells were cultured in Advanced DMEM-reduced serum medium (Gibco) supplemented with 10% FCS, 100 μg ml−1 penicillin-streptomycin and 2 μg ml−1 Polybrene in a 37°C, 5% CO2 incubator. Stimulation was performed using anti-IgM (115-097-020 and 115-175-075; dilution 1/500) from Jackson ImmunoResearch; and anti-Bum (C345C, 2933; dilution 1/200) and anti-phospho-Akt-Thr308 (24419, 4056; dilution 1/100) from Cell Signaling.
Bioinformatics analysis of antibody sequence data followed the human antibodiyomics pipeline,53–55. Specifically, the mouse H, κ and λ germine genes from IMGT (http://www.imgt.org) including the V, D and J segments were incorporated into the pipeline where such information is required for given assignment (step 2), error correction (step 3) and determination of H/LCDR3 and v DNA sequence boundaries (step 5). For heavy chains, 313 VH genes along with 39 DH and 9 JH genes were compiled into three libraries, whereas for light chains, 151 VK genes along with 5 JK genes and 19 VL genes were used in library construction, respectively. The mouse antibodyomics pipeline consists of five consecutive steps. Given a data set of NGS-derived mouse antibody sequences, each sequence was (1) reformatted and labelled with a unique index number; (2) aligned to V, D (for heavy chain only) and J families using the current mouse VDJ germline gene database and an in-house implementation of IgBLAST, and sequences with E-value $>10^{-3}$ for V gene assignment were removed from the data set; (3) subjected to a template-based error-correction procedure, in which insertion and deletion (indel) errors in the V gene segment were detected based on the alignment to their respective germline gene sequences. It is noteworthy that only indels of less than three nucleotides were corrected; (4) compared with the template antibody sequences at both the nucleotide level and the amino acid level using a global alignment module in CLUSTALW2 (ref. 56); (5) subjected to a multiple sequence alignment-based procedure to determine the complementarity determining region 3 (CDR3), which was further compared with the template CDR3 sequences at the nucleotide level and to determine the sequence boundary of the V(D)J coding region. After VL variable region sequences were obtained, a bioinformatics filter was applied to detect and remove erroneous sequences that may contain swapped gene segments due to PCR errors. Specifically, a FL read would be removed if the V gene alignment was $<220$ bp. The processed and annotated antibody chain sequences were then subjected to germline gene frequency analysis.

**Statistical analysis.** Data were analysed using unpaired two-tailed Student’s t-test ($^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$). Results are shown as mean with error bars indicating t s.e.m.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files, or from the authors upon a reasonable request. The IGHV deep-sequencing data sets are available in SRA database with the accession code SRP075987.

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C. X. and S. H. performed the initial observation that transgenic miR-17-92 expression breaks B-cell tolerance in the IgM$^+$-macrophage model. M.I. and H.O. performed experiments investigating miR-17-92 regulation of B-cell central tolerance. A.G.M. performed experiments investigating miR-17-92 family regulation of early B-cell development and B-cell central tolerance. M.I. performed functional dissection of individual members of miR-17-92 in B-cell central tolerance and early B-cell development. M.I., A.G.M., H.Y.J., I.H. and J.Z. performed deep-sequencing analysis of BCR repertoire in TG and tKO mice. J.S. managed the mouse colony and provided technical assistance; TSRI Flow Cytometry Core Facility for expert support; Beatriz Gascon for the cover art. C.X. is a Pew Scholar in Biomedical Sciences. This study is supported by the PEW Charitable Trusts, Cancer Research Institute, Lups Research Institute and National Institute of Health (R01AI087634, R01AI089854, R56AI110403 and R56AI12155 to C.X.).

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