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A single microRNA miR-195 rescues the arrested B cell development induced by EBF1 deficiency

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eLife Assessment

This **useful** study reports that the exogenous expression of the microRNA miR-195 can partially compensate in early B cell development for the loss of EBF1, one of the key transcription factors in B cells. While this finding will be of interest to those studying lymphocyte development, the evidence, particularly with regard to the molecular mechanisms that underpin the effect of miR-195, is currently **incomplete**.

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Abstract

Accumulated studies have reported that hematopoietic differentiation was primarily regulated by transcription factors. Early B cell factor 1 (EBF1) is an essential transcription factor for B lymphopoiesis. Contrary to the canonical notion, we found that a single miRNA, miRNA-195 (miR-195) transduction let EBF1 deficient hematopoietic progenitor cells (HPCs) express CD19, carry out V(D)J recombination and class switch recombination, which implied that B cell matured without EBF1. A part of the mechanism was caused by FOXO1 accumulation via inhibition of FOXO1 phosphorylation pathways in which targets of miR-195 are enriched. These results suggested that some miRNA transductions could function as alternatives to transcription factors.

Introduction

Developmental hierarchy in hematopoiesis has been widely researched, and it is well-known that proper stimulation leads hematopoietic stem cells (HPCs) into B cell lineage. Lineage specification is primarily regulated at the transcriptional level, thus lineage-specific transcription factors are considered to indispensable for differentiation (1, 2). B cell development requires multiple transcription factors, especially Early B cell Factor 1 (EBF1), Paired Box 5 (Pax5), and E2A. Pax5 and E2A are critical transcription factors for early B cell development, but they cannot rescue EBF1-deficient HPCs from failure of B cell lineage commitment (3). Conversely, ectopic expression of EBF1 is able to rescue Pax5, E2A, and PU.1 deleted progenitor cells from B lymphopoiesis arrest, and thus EBF1 is considered more potent than the other transcription factors (2, 4, 5). As the most potent transcription factor, EBF1 is essential for pre-pro-B cell to become pro-B cell; namely, *Ebf1*$^{-/-}$ cell express B220 but is disable to express CD19 (6).

MicroRNAs (miRNAs) are small non-coding RNA containing approximately 22 nucleotides that regulate several target protein expressions mediating deadenylation and translation by post-transcriptionally repressing or decaying target messenger RNAs (mRNAs) (7–10). Although similar to transcription factor, miRNAs regulate large numbers of target mRNAs and deeply contribute to various cell events, the regulation is mainly required for negative regulation of leaky gene expression and often called as fine-tuning (11, 12). In hematopoiesis, miRNAs are expressed in lineage specific manner and their profiles greatly influence on cell differentiation (13–15). Focusing on B cell development, it is revealed that Dicer, a key enzyme of miRNA generation, is essential for pre-to pro-B cell transition (16). Individual miRNA is also studied and miR-150 and miR-126 are identified as relational factor to B cell lineage development. miR-150 regulate B cell differentiation by controlling c-Myb expression and miR-126 partially rescues EBF1 deficient B cell lineage commitment by modulating IRS-1 expression (17, 18). Both miRNAs dramatically contributed to B cell development processes, but they were not able to recover B cell development from EBF1 deficiency. Conceived from these vigorous functions of miRNAs on B cell development, in this time, we analyzed ability of miR-195, recently revealed as an important factor for several cell differentiation, on B cell lineage commitment in EBF1 deficient HPCs (19, 20).

Results

miR-195 induces B cell character in EBF1 deficient HPCs

To assess the contribution of miR-195 on B cell development, miR-195 was transduced into mouse fetal liver (FL)-derived Lin^{-} c-kit^{+} HPCs and the cells were differentiated to B220 and CD19 expressing pro-B cells with IL7, Flt ligand and SCF on OP9 stroma cells. After 7 days of culture, certain numbers of the cells gradually expressed CD19, and the positive cells was increased by miR-195 transduction (Fig. 1A). This result suggests that miR-195 has ability to shift the HPCs differentiation toward B cells. Next, we attempted to differentiate *Ebf1*$^{-/-}$ FL HPC to B cell with miR-195 transduction (Fig. 1B). As by a previous study (6), control *Ebf1*$^{-/-}$ FL HPCs expressed B220 but did not express CD19.

However, miR-195 transduced *Ebf1*$^{-/-}$ FL HPCs highly expressed CD19 (Fig. 1C). In normal B cell development, CD19 expression follows B220 expression and hence CD19 positive cells show B220 expression as well. Thus, miR-195-transduced *Ebf1*$^{-/-}$ FL HPCs which including B220 negative CD19 positive population may simply reflect up-regulation of CD19 expression, but not B cell development. To exclude this possibility, gene expressions of miR-195-transduced *Ebf1*$^{-/-}$ FL HPCs by cDNA microarray assay were investigated then indicated that miR-195-transduced cells more expressed B cell lineage related genes, e.g., *Pax5*, *Aicda*, *Rag1*, *Rag2*, *CD79b* and *Runx2*, whereas less expressed T cell and NK cell lineage related genes, including *Gata3*, *Id2*, *Lck*, *CD3e* and *Il2rb* and also myeloid lineage related genes for example, *Cebpe*, *Ly6g*, *Fcgr1*, *Fcgr2b* and *fcgr3* (Fig. 1D). (1–6) Among the B-lineage transcription factors, *Pax5* and *Erg* were modestly but significantly upregulated ($\log_2$FC ~1.2 and ~0.9, respectively) in miR-195-transduced *Ebf1*$^{-/-}$ cells compared to controls. While these changes were moderate, they were consistent across replicates

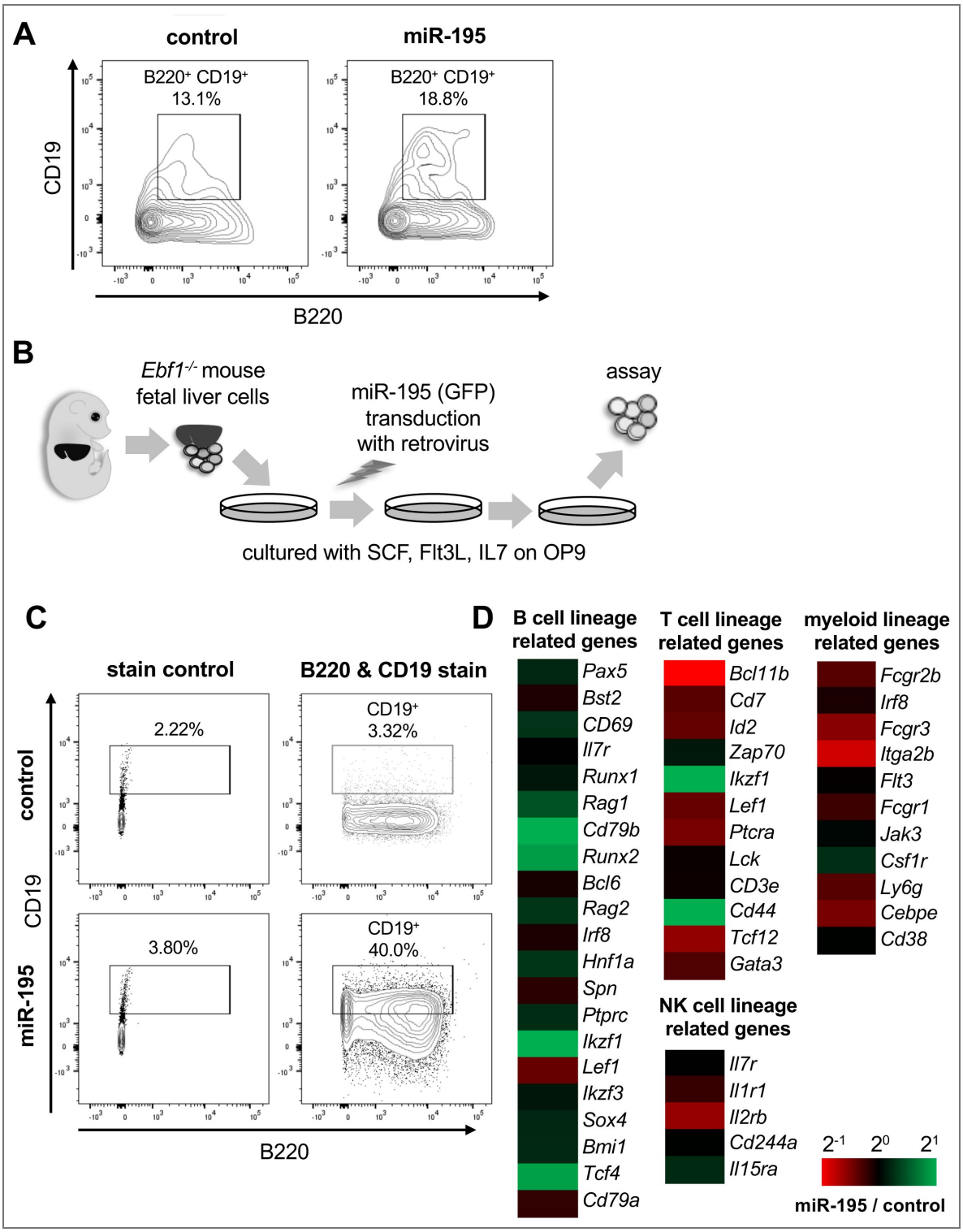

Fig. 1. miR-195 promotes HPCs to differentiate into the pro-B cell stage without EBF1.

(A) Flow cytometry analysis of control and miR-195-expressing Lin⁻ cells. HPCs from fetal livers of wild-type mice were cultured for 7 days on OP9 with SCF, Flt3-ligand, and IL-7, after infection with control or miR-195 retrovirus. Representative result of control (upper panel) and miR-195 (lower panel) viral infections is shown (n=3). (B) Outline of the *in vitro* culture system of *Ebf1*$^{-/-}$ HPCs. (C) Flow cytometry analysis of control and miR-195-expressing *Ebf1*$^{-/-}$HPCs. Shown data is representative of n=3. (D) Microarray analysis of miR-195-expressing *Ebf1*$^{-/-}$ HPCs. Log_2 fold-changes in the expression levels of genes related to B (left panel), T (middle-upper panel), NK (middle-lower panel), and myeloid (right panel) cell lineages were classified and are shown as colored columns. The analysis was carried out in duplicates.

and suggest partial restoration of the B cell transcriptional program. These results suggested that not only CD19 expression but also up-regulation of several B cell developmental factors and down-regulation of other lineage related genes were involved in the promotion of B cell lineage commitment by miR-195.

EBF1 deficient HPCs were able to commit B cell lineage by transduction of miR-195 with bone marrow niche modification

The ectopic miR-195 expression led *Ebf1*$^{-/-}$ HPCs induced differentiation toward B cell. However, a large part of the miR-195-transduced HPCs expressed CD19 but not B220, which implied that they strayed from the canonical B cell differentiation steps (Fig. 1C). In addition to the inner state, the microenvironment known as niche was also critically involved in hematopoiesis (21). Especially in early B cell development, bone marrow niches precisely control the maintenance and differentiation of lineage precursors by cytokines and chemokines (22). To explore the development of miR-195-transduced *Ebf1*$^{-/-}$ FL HPCs under bone marrow niches, we engrafted miR-195-transduced *Ebf1*$^{-/-}$ early B cells into NOG and B6RG mice, in which absence of B cell makes the engrafted B cell visible (Fig. 2A). After 7 days, the engrafted cells successfully adapted in the bone marrow. While there was no remarkable change in control cell population, notably, instead of B220 negative CD19 positive cells, the double positive cells were markedly increased in miR-195-transduced *Ebf1*$^{-/-}$ FL early B cells, suggesting that the normal stepwise B cell development occurred (Fig. 2B). In B cell development, most prominent steps after CD19 expression are VDJ recombination and subsequential IgM expression on cell surface. In addition to CD19 expression, EBF1 is also known as essential gene for VDJ recombination, especially V_H to DJ_H recombination (23). To determine whether miR-195-transduced *Ebf1*$^{-/-}$ cells rearranged the VDJ region, we attempt to detect V_H-J_H assembled gene segments in the engrafted mouse bone marrow cells by droplet digital PCR (ddPCR). The data revealed that there were certain number of V_H-J_H segments in the bone marrow of mice engrafted miR-195-transduced *Ebf1*$^{-/-}$ cells (Fig. 2C, Fig. S1). Subsequently, to expect the EBF1 independent reconstitution enabled B cell receptor to express as IgM, we analyzed B cell populations in the engrafted mouse bone marrow. Not much but some miR-195-transduced cells expressed IgM on cell surface likely as normal immature B cell in bone marrow 10 days after engraftment (Fig. 2D). Moreover, these IgM positive cells were also detected in splenocytes. These data suggested that engrafted cells had differentiated into IgM positive immature or mature B cells, and they have been recruited to spleen. The critical function of B cell is changing B cell receptor from IgM to IgG following class switch DNA recombination, which is accompanied by stimuli-induced cell proliferation. To clarify whether miR-195-transduced *Ebf1*$^{-/-}$ B cells have the function, whole splenocyte of the engrafted mice were stimulated with IL-4 and LPS, which causes class switch recombination to IgG1 (24). While control transduced GFP positive cells did not expand by the stimuli, miR-195-transduced GFP positive cells expanded enough to be surely detected and importantly a part of them expressed IgG1 (Fig. 2E). These data suggested that miR-195 has a potential to induce B cell differentiation from HPCs to mature B cells, resulting in class switch recombination even when critical regulator EBF1 is absent.

miR-195 physiologically maintains several B cell populations

As ectopic miR-195 expression revealed its potential in B cell development. Next, to investigate contribution of endogenous miR-195 for B cell lineage populations, miR-195 deficient mice in which the genome around miR-195-5p was eliminated by CRISPR/Cas9 system were established. The analysis of HPC lineage populations in the bone marrow revealed that several B cell-related progenitors were relatively reduced in miR-195$^{-/-}$ mice. Sca-1^{-} c-kit^{+} common myeloid progenitor cell population was increased but controversially, Sca-1^{+} c-kit^{-} (LSK^{-}) cells was decreased in miR-195$^{-/-}$ mice (Fig. 3A). As LSK^{-} cells mainly includes early lymphoid precursor, these results suggested that miR-195 is involved in hematopoiesis including differentiation of stem cells toward lymphoid and early B cells (25). While analysis of each early B cell populations did not show significant difference, whole B220^{+} IgM^{-} pre B cell populations was slightly increased in the BM of miR-195$^{-/-}$ mice (Fig. 3B). In the splenic B cells, marginal zone B (MZB) cells were reduced in

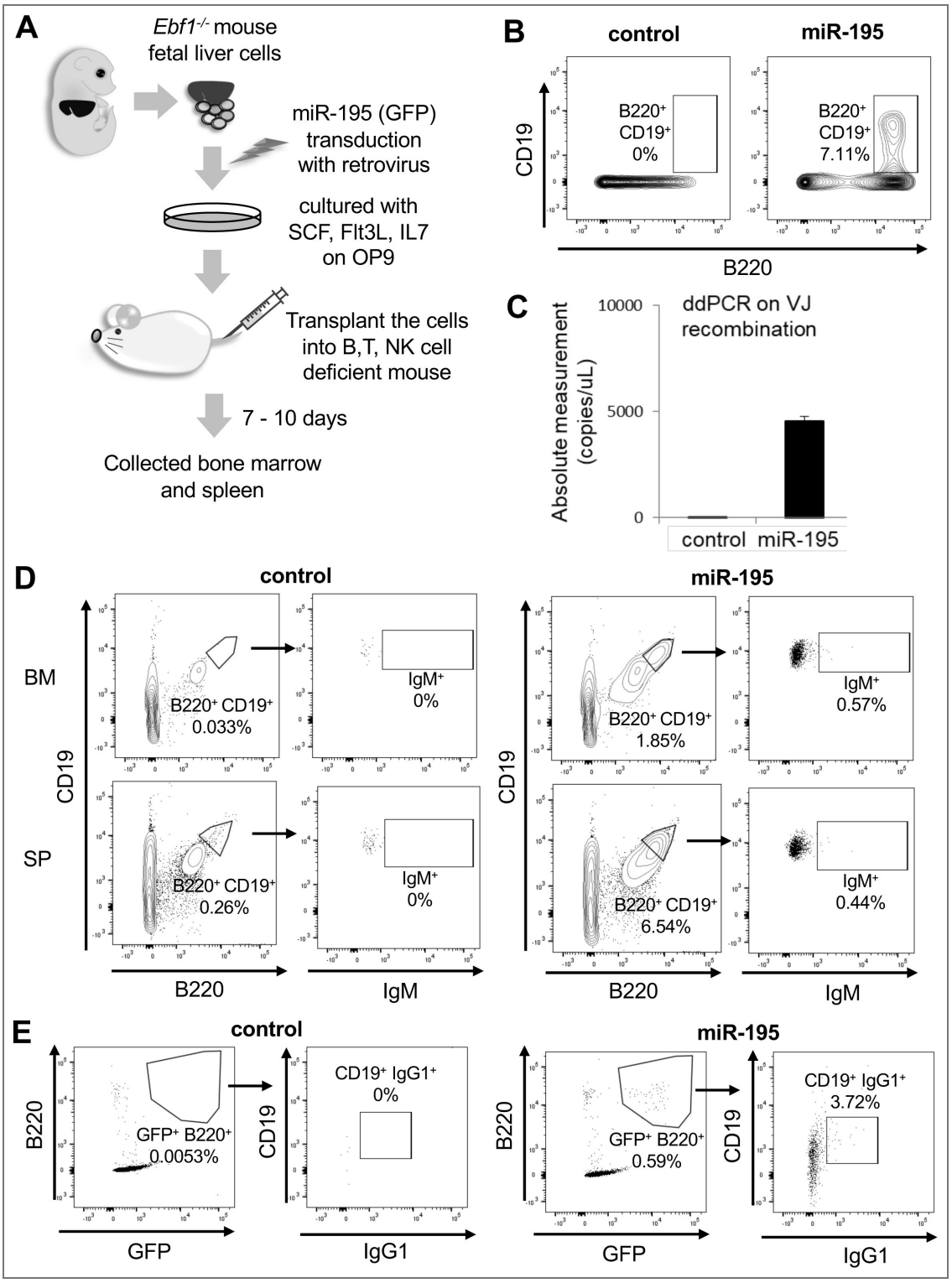

Fig. 2. miR-195 leads *Ebf1*-deficient HPCs to mature into B cells with bone marrow niche assistance.

(A) *In vivo* analysis of B cell development of *Ebf1*$^{-/-}$ HPCs. (B) Flow cytometry analysis of control and miR-195-expressing *Ebf1*$^{-/-}$ HPCs in the bone marrow collected at 7 days after transplantation. (C) Using droplet digital PCR, VJ region fragments were amplified from the genomic DNA of B220^{+} cells in the bone marrow of mice transplanted with control and miR-195-expressing *Ebf1*$^{-/-}$ HPCs. (D) Flow cytometry analysis of control and miR-195-expressing *Ebf1*$^{-/-}$HPCs in the bone marrow (BM) and spleen (SP), at 10 days after transplantation. (E) Flow cytometry analysis of class-switch recombination. Splenocytes of mice transplanted with control and miR-195-expressing *Ebf1*$^{-/-}$ HPCs were cultured for 72 hrs with IgG1 class-switch stimuli, LPS, and IL-4. Each flow cytometric data is representative of *n*=3.

miR-195$^{-/-}$ mice (Fig. 3C). MZB cells was previously reported to be highly dependent on EBF1 activity and disappear in absence of EBF1. B-1 cells was likewise crucially regulated EBF1 as well(26). In the peritoneal cavity of miR-195$^{-/-}$ mice, B-1 cells were significantly decreased (Fig. 3D). These results suggested that miR-195 contributed to maintain several EBF1-dependent mature B cell populations at least in a part. Taken together, these results were consistent with those obtained from ectopic expression of miR-195.

FOXO1 phosphorylation pathway targeted by miR-195 was responsible for B cell lineage commitment

To elucidate how miR-195 promote B cell development in EBF1 deficient HPCs, we analyzed regulatory networks of predicted miR-195 target genes by using starBase_v2.0 and David Bioinformatics Resources 6.8 in KEGG pathway database (27–33). Several gene regulation networks were detected as candidates of responsible pathways on the miR-195 function (Table S1 and S2). Remarkably, MAPK signaling pathway and PI3K-Akt signaling pathway included various targets of miR-195. Both MAPK and Akt were known to phosphorylate and degradate FOXO1, which was a critical factor in several stages of B cell development (34). Thereby, we focused on the predicted miR-195 targets: *Pik3r1*, *Pdpk1*, *Akt3*, *Raf1*, *Sos2,* and *Mapk3*, which were involved in and activate MAPK and PI3K-Akt pathways. First, to confirm that the predicted targets are actually regulated by miR-195, we picked up 3'UTR of *Mapk3* and *Akt3,* which were especially important in the pathways, and inserted in a luciferase reporter assay plasmid. As expected, the luciferase activity was down-regulated by miR-195 transduction, but it was not impaired by transduction of miR-195 mutant of mature miRNA region (Fig. 4A). Furthermore, to determine whether the predicted targets were actually regulated by miR-195, we measured the expression levels in miR-195-transduced *Ebf1*$^{-/-}$ HPCs, and qPCR analysis showed that miR-195 transduction certainly decreased the mRNA levels (Fig. 4B).

Because of sequence similarity among miR-15/16 family members, the baseline levels detected in control samples may include signal from endogenous miRNAs such as miR-497 or miR-16. Thus, the observed increase ($\log_2$FC ~2.5) may underestimate the actual level of miR-195 overexpression. In line with these findings, *Mapk3* expression was also downregulated in our microarray analysis of miR-195-transduced *Ebf1*$^{-/-}$ cells. However, for *Akt3*, the microarray results were inconsistent across different probes, suggesting probe-dependent variability. Therefore, while qPCR and reporter assays support *Akt3* as a potential target of miR-195, its regulation remains to be further validated. Next, to evaluate inhibition of FOXO1 phosphorylation and degradation by miR-195, we compared protein levels of FOXO1 and phosphorylated FOXO1 (pFOXO1) in miR-195-transduced *Ebf1*$^{-/-}$ HPCs. The western blotting results revealed that miR-195 transduction decreased pFOXO1 levels and increased relative FOXO1 protein levels (Fig. 4C, Fig. S2).

We also performed western blotting for PAX5 and ERG using the same samples. The results showed no significant change in these protein levels between miR-195-transduced and control Ebf1$^{-/-}$ cells (Fig. S3), consistent with the modest upregulation observed in our microarray data. Finally, to determine whether FOXO1 accumulation is sufficient for *Ebf1*$^{-/-}$ HPCs to differentiate into pro-B cells, *Ebf1*$^{-/-}$ HPCs were transduced with *Foxo1* and cultured under the B cell differentiating condition. Similar to miR-195 transduction, Foxo1 transduction arose B220 and CD19 double positive *Ebf1*$^{-/-}$ cells, which was accompanied with CD19 positive but B220 negative population (Fig. 4D). These data indicated that FOXO1 accumulation by inhibition of phosphorylating pathways was responsible for *Ebf1*$^{-/-}$ HPCs to differentiate into B cell lineage.

Epigenetically activated genes in pro-B cells by miR-195 is fewer than by EBF1

In B cell development, epigenetic changes of transcription factors and differentiation molecules are crucial for proper development, which are mainly regulated by EBF1 (35, 36). We investigated transposase-accessible chromatin using deposited sequencing data (ATAC-seq) of *Ebf1*$^{-/-}$ pro-B cells and wild type pro-B cells from GSE92434 and cells in early B cell lineages from GSE100738. While

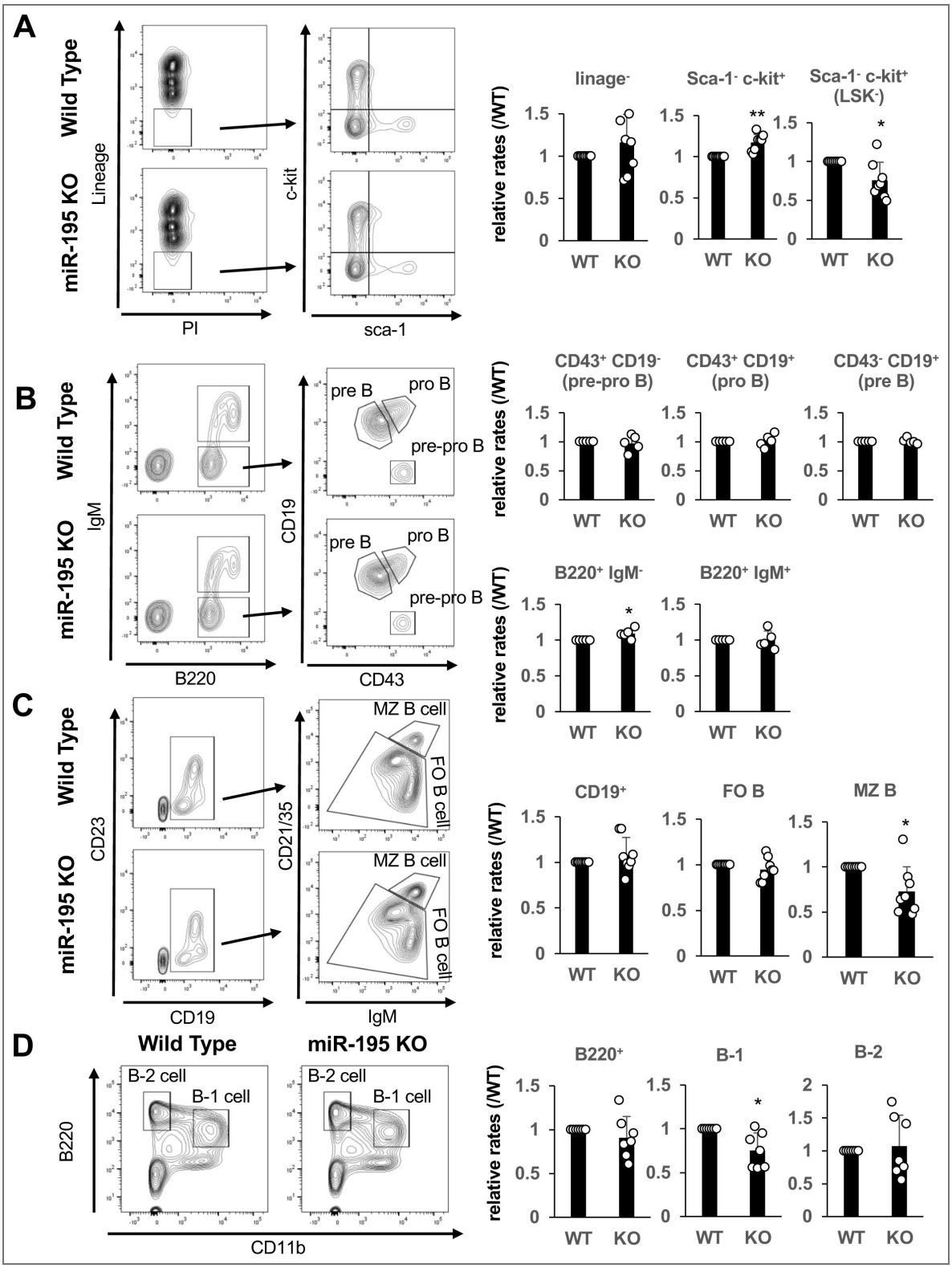

Fig. 3. Several B cell populations are disturbed in the miR-195-deficient mice.

Flow cytometry data of B cell lineage populations in miR-195$^{-/-}$ and littermate WT mice. Representative plots (left side) and mean ± S.D. of relative population rates in each littermate WT mice (right side) are shown. (A) Analysis of early B cell populations in the bone marrow. Pre-pro-B (B220^{+} IgM^{-} CD43^{+} CD19^{-}); pro-B (B220^{+} IgM^{-} CD43^{+} CD19^{+}); pre-B (B220^{+} IgM^{-} CD43^{-} CD19^{+}); *n*=5. (B) Analysis of hematopoietic progenitor populations in the bone marrow; *n*=5. (C) Analysis of B cell populations in the spleen. FO B (CD19^{+} IgM^{+} CD21/35$^{low\text{-}middle}$); MZ B (CD19^{+} IgM^{+} CD21/35^{high}); *n*=8. (D) Analysis of B cell populations in the peritoneal cavity: B-1 (B220^{+} CD11b^{+}); B-2 (B220^{+} CD11b^{-}); *n*=7. Statistical significance was tested using one-sample *t* test. *p<0.05; **p<0.01. WT, wild-type.

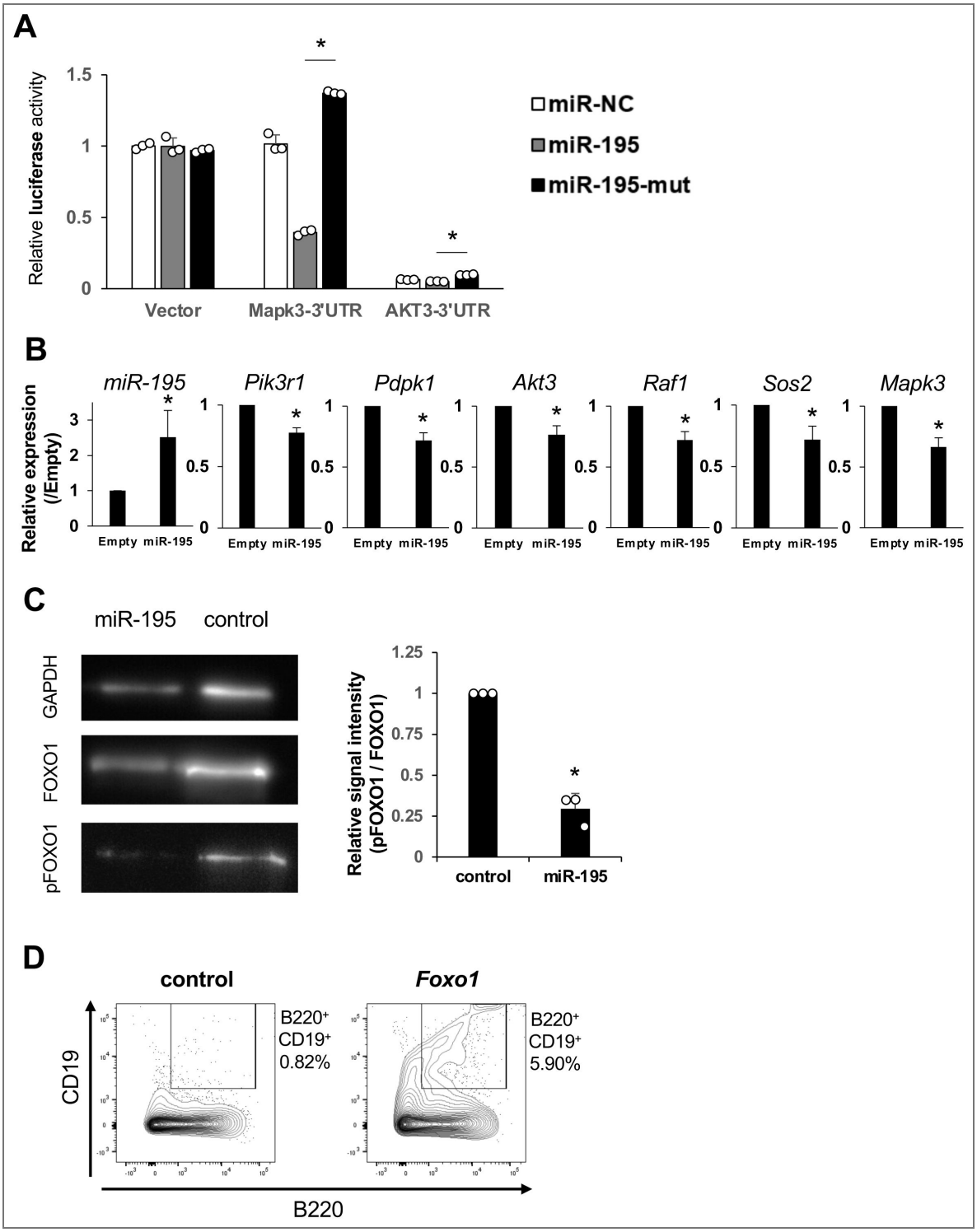

Fig. 4. FOXO1 phosphorylation pathways are key targets of miR-195 for promotion of B cell development.

(A) Relative expression rate of miR-195 and predicted target genes were compared between control (EMPTY) and miR-195-expressing *Ebf1*$^{-/-}$ HPCs. (B) Relative luciferase inhibitory rates of miR-195 onto predicted target 3'-UTR were analyzed using Dual-Luciferase® reporter assay. (C) Western blot of FOXO1 and phosphorylated FOXO1 (pFOXO1) in control and miR-195-expressing *Ebf1*$^{-/-}$ HPCs. Quantification of FOXO1 and phospho-FOXO1 band intensities from three independent experiments is shown in the bar graph. Data are presented as mean ± SD. Shown data is representative of n=3. (D) Flow cytometry analysis of control and *Foxo1*-expressing *Ebf1*$^{-/-}$ HPCs. Shown data is representative of n=3. Statical significance was tested using one-sample t test. *p<0.05, n=3.

wild type pro-B cells/ *Ebf1*$^{-/-}$ pro-B cells differentially accessible (DA) ATAC peaks were observed in 2809 sites, wild type CD19 positive/ CD19 negative early B cells DA ATAC peaks were in 904 sites. Then, 678 sites were overlapped, which were considered to be regulated by EBF1 as important locus for early B cell development. Moreover, some of them were overlapped with miR-195-transduced B220 and CD19 double positive *Ebf1*$^{-/-}$ cells (miR-195 CD19+) / B220 positive CD19 negative *Ebf1*$^{-/-}$ cells (control CD19-) DA ATAC peaks (73 out of 226 peaks), which were considered to be regulated by miR-195 (Fig. 5B). These peaks included important genes for early B cell development, such as *Pax5*, *Runx1*, *Erg*, *Ifr8*, and *Blnk*, and B cell-related genes, such as *Rarres1*, *Ciita*, and *Atg7* (Table S3). These results indicated that gene locus opened by miR-195 were fewer than by EBF1, but they included several key locus for B cell differentiation, and they were enough to differentiate the progenitor cells to mature B cells. Moreover, HOMER Motif Analysis revealed that enriched motives opened by EBF1 and by miR-195 were 198 and 111, respectively (Fig. 5C). The common motives were 104 which included critical genes for B cell development, such as E2A, Foxo1, and PAX5, and high ranked motives were very similar between EBF1 and miR-195 (Fig. 5D). These results suggested that miR-195 transduction opened important chromatin regions for early B cells, which were normally regulated by EBF1.

Finally, we concluded that miR-195 transduction was able to compensate EBF1 deficiency in B cell development through activation of FOXO1 and epigenetic regulation of several B cell-related genes.

Discussion

The canonical notion of hematopoietic fate determination implies that EBF1 is an indispensable factor for B lymphopoiesis. However, in this study, we showed that a single microRNA miR-195 rescued the arrest of pro-B cell differentiation induced by EBF1 deficiency. As miRNA plays roles in a bundle of their family, single miRNA deficient mice often do not show significant phenotype (37). Nevertheless, miR-195 deficient mice showed not much but sure decreased number of several hematopoietic cells including marginal zone B cells and peritoneal B-1 cells, which were reported to almost disappear in EBF1^{ihCd2} mice in which EBF1 was deficient in mature B cells (38). Considering that other miRNA deficient mice have subtle phenotype and miR-195 is one of the large family, including miR-15/16 and miR-195/497 (39), the remarkable potential of miR-195 is beyond a fine tuner as microRNA, at least as far as it is considered with regard to B cell lineage commitment.

A part of the mechanisms of the potent function of miR-195 was caused by inhibition of phosphorylation of FOXO1. FOXO1 is a transcriptional factor controlled by EBF1 and strongly promote differentiation of pre-B cell. FOXO1 activity is regulated by PI3K/AKT pathway and several miRNAs were reported to be involved in the regulation (40). We showed *Foxo1* transduction enable EBF1 deficient cell to express CD19.

However, the CD19 positive cells rapidly disappeared and couldn't be detected in transplanted mice (data not shown). It is presumable that FOXO1 activity was necessary to express CD19, but other factors undertake maintenance and proliferation of the developing cells. ATAC-seq analysis revealed that miR-195 was directly or indirectly involved in chromatin accessibility. As the chromatin regions and motives opened by miR-195 were critical for B cell differentiation and hematopoiesis, further investigation is needed for the mechanism.

Although our study indicates that miR-195 has the potential to promote B cell lineage commitment in the absence of EBF1, the precise downstream targets and mechanisms remain only partially defined. We hypothesize that the observed effects are mediated through the downregulation of multiple mRNA targets involved in opposing B-lineage differentiation, including kinases in the MAPK and PI3K-Akt pathways that modulate FOXO1 phosphorylation. While our microarray, qPCR, and luciferase assays support the regulation of specific targets such as *Mapk3* and *Akt3*, a more comprehensive identification of direct targets—especially those related to transcriptional and epigenetic regulation—would further strengthen our conclusions. We interpret our findings as revealing the potential of miR-195 to compensate for EBF1 deficiency, rather than a

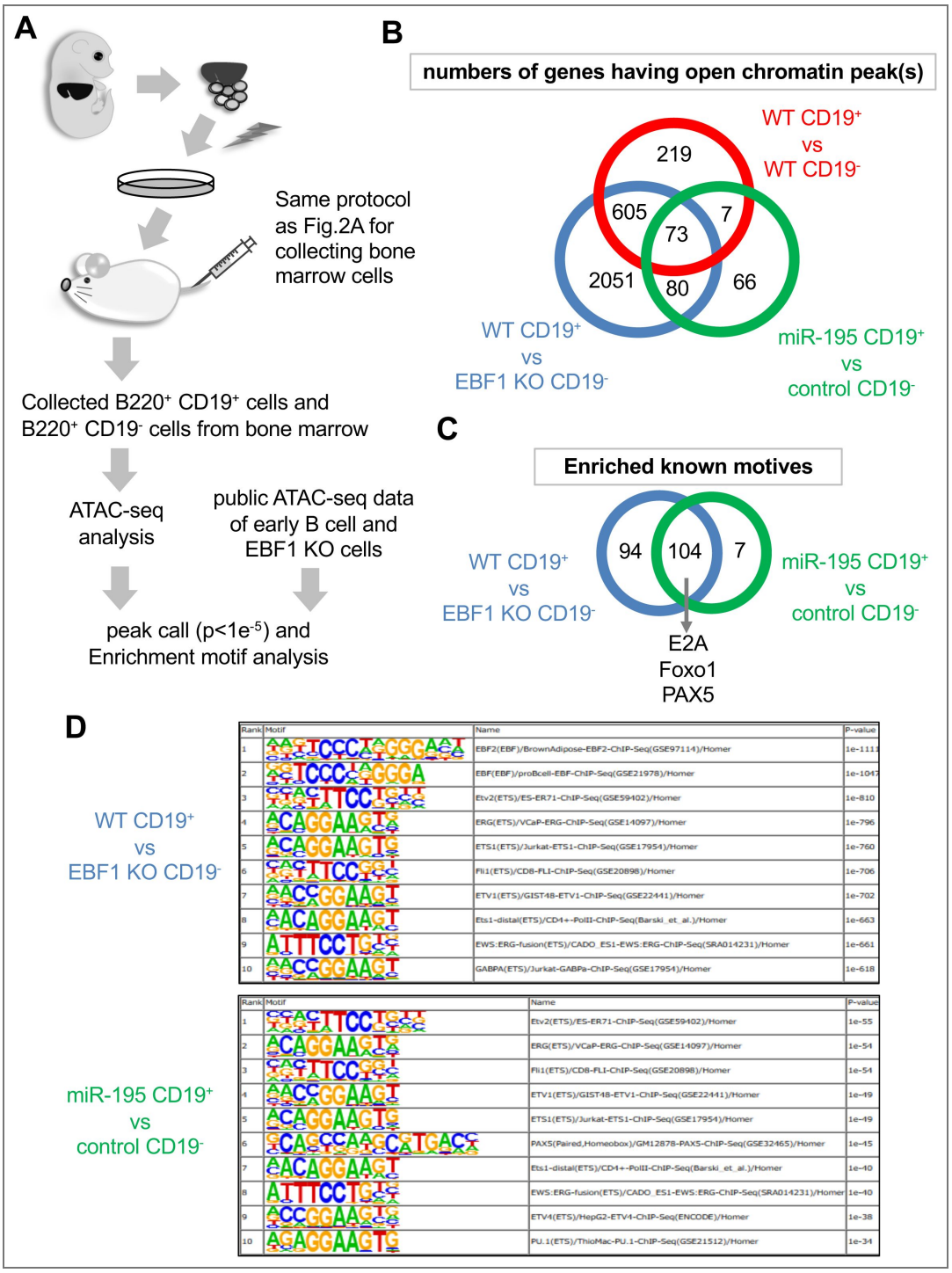

Fig. 5. ATAC-seq analysis of *Ebf1*$^{-/-}$ CD19-positive B cells differentiated by miR-195.

(A) Outline of analysis of open chromatin regions in miR-195-expressing *Ebf1*$^{-/-}$ cells. (B) Venn diagram of numbers of genes in which DNA regions of open chromatin peaks were detected by means of peak call analysis. The analyses were examined between CD19-negative (FrA) and-positive (FrB, FrC, and FrD) stages of B cell development (GSE100738; upper red circle); wild-type and *Ebf1*$^{-/-}$ pro-B cells (GSE92434; left-lower blue circle); B220^{+} CD19^{-} cells of control and B220^{+} CD19^{+} positive miR-195-expressing *Ebf1*$^{-/-}$ cells (right-lower green circle). Overlapping regions in the Venn diagram are interpreted as follows: the intersection of WT and Rescue represents canonical EBF1-regulated regions; the overlap between Rescue and miR-195 indicates partial mimicry by miR-195; and regions unique to miR-195 may reflect EBF1-independent chromatin changes. (C and D) Venn diagram of numbers of enriched known motifs detected using HOMER find motif analysis (C) and lists of high *p*-value motifs, up to rank 10 (D).

demonstration of its physiological role. Future studies using global transcriptome, proteome, and chromatin-binding assays will be essential to fully elucidate the mechanisms underlying this observation.

To compensate for the lack of transcriptome data from sorted *miR-195*-transduced pre-pro-B or CD19+ *Ebf1*$^{-/-}$ cells, we compared our microarray data with publicly available RNA-seq profiles of *Ebf1*$^{-/-}$ pro-B cells (GSE92434). This analysis revealed that several B-lineage defining genes downregulated in *Ebf1* deficiency were upregulated upon miR-195 expression, suggesting that miR-195 may partially restore transcriptional programs disrupted by the loss of EBF1.

Although direct evidence of FOXO1 binding to B-lineage gene loci (e.g., via ChIP-seq or CUT&RUN) is currently lacking due to technical limitations in cell numbers, our results suggest that FOXO1 plays a key functional role. This is supported by its increased protein level upon miR-195 expression, the partial phenocopy by FOXO1 overexpression, and the enrichment of FOXO1 motifs in open chromatin regions identified by ATAC-seq. Future studies incorporating FOXO1 chromatin profiling will be important to validate its direct regulatory role in this context.

While ddPCR provided a sensitive means to detect *VH-JH* rearranged fragments, it does not offer resolution of specific V, D, or J gene usage or recombination completeness.

Therefore, the full extent and diversity of V(D)J recombination in *Ebf1*$^{-/-}$ miR-195-induced CD19^{+} cells remains to be clarified. Future studies incorporating high-throughput sequencing approaches will be important to fully characterize the immunoglobulin repertoire and confirm progression through the pre-BCR checkpoint.

While our data support the B-lineage identity of miR-195-induced *Ebf1*$^{-/-}$ CD19^{+} cells based on gene expression, chromatin accessibility, and immunoglobulin expression, we have not directly tested their lineage plasticity under alternative differentiation conditions. Whether these cells retain responsiveness to myeloid cytokines or exhibit residual multipotency remains to be determined. Future studies using single-cell fate mapping or in vitro differentiation assays will be required to fully define the lineage commitment status of this population.

While our results demonstrate that ectopic expression of miR-195 can compensate for the loss of EBF1 in promoting B cell development, we acknowledge that this does not necessarily reflect a physiological role for miR-195. The miR-195 knockout mice exhibited only mild alterations in B cell populations, suggesting that under normal conditions, miR-195 is not essential for B lymphopoiesis. Therefore, our findings should be interpreted as highlighting the potential of miR-195 to modulate B cell fate under specific conditions, rather than indicating its requirement in physiological B cell development. Further studies will be needed to determine whether miR-195 plays a more prominent role under stress or disease contexts, or in cooperation with other miRNAs.

The luciferase activity was markedly reduced in the presence of the Akt3 3'UTR, even in cells transduced with a control vector (Fig. 4A). We hypothesize that the Akt3 3'UTR contains strong post-transcriptional regulatory elements—such as AU-rich elements or binding sites for endogenous miRNAs or RNA-binding proteins—which may suppress mRNA stability or translation independent of miR-195. Alternatively, the secondary structure or length of the UTR may inherently reduce luciferase expression.

Materials and Methods

Plasmid construction

To construct MDH1-PGK-GFP-miR-195, genomic DNA was first extracted from RS4;11 using the DNeasy Tissue Extraction Kit (Qiagen). Next, a segment around miR-195 was amplified by means of PCR, using Pfx polymerase (Invitrogen) and the oligonucleotides, 5'-AGATCTCTCGAGAAGGAGAGGGTGGGGTAT-3' and 5' - GGGGCGGAATTCGCTATTCCCGCATAAGCA-3'. The obtained PCR product was then cloned into the XhoI-EcoRI site of MDH1-PGK-GFP 2.0 (Addgene #11375). To construct pMYs-RFP-Foxo1, first, pEX-Foxo1 (in which mouse Foxo1 is optimized for gene synthesis; Eurofins Genomics K.K.) was synthesized and inserted into the

EcoRI-XhoI site of pEX. Next, the Foxo1 region was extracted using the restriction enzymes and inserted into pMYs-RFP retroviral vector (kindly provided by Prof. T. Kitamura, Tokyo University). For in vitro transcription of small-guide RNA (sgRNA), pUC57-195sg-upstream and-downstream were generated. Both plasmids originated from the pUC57-sgRNA expression vector (Addgene #51132), and the annealed oligonucleotides were inserted into a BsaI site (For the former, 5' - TAGGCCCACAAAGGCAGGGACCTA-3' and 5' - AAACTAGGTCCCTGCCTTTGTGGG-3' were annealed, while for the latter, 5' - TAGGGGAAGTGAGTCTGCCAATAT-3' and 5' - AAACATATTGGCAGACTCACTTCC-3' were annealed). For the Dual-Luciferase® assay, psiCHECK-2 vector was purchased from Promega and the 3'-UTRs of Akt3 and Mapk3 were inserted between the XhoI and NotI sites. MDH1-PGK-GFP-miR-195-mut was generated by mutating 6 bases, from the second to seventh bases of the mature miR-195 and complimentary regions of the stem loop structure in MDH1-PGK-GFP-miR-195. In detail, normal miR-195 stem loop sequence 5' - AGCUUCCCUGGCUCUAGCAGCACAGAAAUAUUGGCACAGGGAAGCGAGUC UGCCAAUAUUGGCUGUGCUGCUCCAGGCAGGGUGGUG-3' (mature miR-195-5p sequence 5'-UAGCAGCACAGAAAUAUUGGC-3') was mutated to 5' - AGCUUCCCUGGCUCUgcgccgACAGAAAUAUUGGCACAGGGAAGCGAGUCU GCCAAUAUUGGCUGUcggcgcCCAGGCAGGGUGGUG-3' (mature sequence 5' - UgcgccgACAGAAAUAUUGGC-3').

Animals

C57BL/6 mice were purchased from CLEA Japan Inc. NOD/Shi-scid, IL-2RγKO (NOG) and B6RG mice were purchased from Central Institute for Experimental Animals (CIEA). The Ebf1$^{-/+}$ mice were originally generated by R. Grosschedl (41). miR-195-deficient mice were generated based on the CRISPR/Cas9 system established by C. Gurumurthy (42), using pUC57-195sg-upstream and-downstream for sgRNA expression and pBGK (Addgene #65796) for Cas9 mRNA expression. Sanger sequencing confirmed a deletion of 5,103 base pairs at chromosome 11 (GRCm38/mm10 chr11:70,234,425–70,235,103), encompassing the entire *miR-497* sequence upstream and 61 bp of the 93-bp *miR-195* precursor. The deletion was validated using genomic DNA and aligned to the mouse reference genome. All transgenic mice used for experiments were backcrossed to the C57BL/6 background for at least eight generations to minimize off-target effects. The obtained mice were subsequently bred and housed at Tokai University. All the animal experiments in this study complied with the Guidelines for the Care and Use of Animals for Scientific Purposes at Tokai University. To reduce the number of sacrificed animals, the sample sizes for each animal experiment were empirically determined from previous studies or the results of the first littermate mice.

Flow cytometry analysis

Cells were collected and washed in FACS buffer (phosphate-buffered saline supplemented with 2% fetal bovine serum) and subsequently stained with the following antibodies purchased from BioLegend: anti-c-kit (2B8),-Sca-1 (D7),-IL7Rα (A7R34),-B220 (RA3-6B2),-IgM (RMM-1),-CD3ε (145-2C11),-CD4 (GK1.5),-CD8 (53-6.7),-CD11b (M1/70),-CD19 (1D3),-CD23 (B3B4), and-IgG1 (RMG1-1) and Thermo Fisher: anti-Flt3 (A2F10),-CD43 (eBioR2/60), and-CD21/35 (eBio8D9). All samples were analyzed on the BD FACSVerse™ system and the data obtained was analyzed using FlowJo. FACSAria™ III was used for cell sorting.

Culture of lineage-negative (Lin$^-$) cells from the fetal liver

Fetal livers were harvested from pregnant C57BL/6 or *Ebf1*$^{+/-}$mice (mated with *Ebf1*$^{+/-}$male) at 13.5 days after vaginal plug formation and minced gently by means of pipetting. The cell suspensions were filtered through a 67-μm pore nylon mesh and Lin$^-$ cells were collected using the Lineage Cell Depletion Kit, mouse and AutoMACS® Pro Separator (Miltenyi Biotec), according to the manufacturer's instructions. Subsequently, the collected Lin$^-$ cells were transduced with miR-195 or *Foxo1* by means of retroviral transfection. In brief, Platinum-E cells were transfected with MDH1-PGK-GFP (for EMPTY sample) or MDH1-PGK-GFP-miR-195 or pMYs-RFP-Foxo1 using PEI

MAX® (Polysciences Inc.), and retroviral supernatants were harvested 48 hours later. Lin$^-$ cells were infected with the supernatants using 10 μg/mL Polybrene (Sigma-Aldrich). The infected and transduced Lin$^-$ cells were cultured and differentiated into B cells on OP9 cells in IMDM (Thermo Fisher) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution, 50 μM 2-mercaptoethanol, 100 units/mL penicillin G, 100 μg/mL streptomycin (all from Wako), and 10 ng/mL recombinant SCF, IL-7, and Flt3-ligand (R&D Systems). Cells were cultured on OP9 cells for 7 days before analysis unless otherwise specified. For *in vivo* analysis of B cell development of EBF1$^{-/-}$ Lin$^-$ cells, 1×10^6 cells were injected into the NOG or B6RG mice after >7 days of culture and expansion *in vitro*.

Microarray analysis

Total RNA was isolated using the RNeasy MINI Kit (Qiagen), and its quality was analyzed using the 2100 Bioanalyzer (Agilent Technologies). Approximately 100 ng RNA was labeled, and gene expression microarray analysis was performed using the Agilent Whole Mouse Genome Microarray 4×44K v2 (Agilent Technologies), according to the manufacturer's instructions. The processed data was analyzed using GeneSpring GX version 14.9 (Agilent Technologies). Raw intensity values were normalized using the 75th percentile and transformed to the Log_2 scale. All experiments were carried out in duplicates.

Droplet digital PCR (ddPCR)

To carry out ddPCR for VJ recombination analysis, total DNA was isolated from whole cells of the bone marrow in miR-195-transduced *Ebf1*$^{-/-}$ FL HPCs-engrafted NOG mice, using the Wizard® Genomic DNA Purification Kit (Promega). ddPCR was conducted using QX100 Droplet Digital PCR system (Bio-Rad). Briefly, 3.3 μL of template cDNA with 20× primer and a TaqMan™ probe set was partitioned into approximately 20,000 droplets using the QX100 Droplet Generator, for amplification. The cycling conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min, and a final 10-min incubation at 98°C. The droplets were subsequently read automatically using the QX10 droplet reader. The data were analyzed with QuantaSoft analysis software (ver. 1.3.2.0; Bio-Rad). The primers used were as follows: forward primer – 5'-GAGGACTCTGCRGTCTATTWCTGTGC-3'; reverse primer – 5'-CCCTGACCCAGACCCATGT-3'; and probe – 5'-6FAM-TTCAACCCCTTTGTCCCAAAGTT-TAM-3'.

Class-switch stimulation

EBF1$^{-/-}$ Lin$^-$ cells were transduced with EMPTY and miR-195-expressing vector and transplanted into B6RG mice. At 10 days post-transplantation, the spleens were collected from the mice, minced with slide glasses, and filtered through a 67-μm pore nylon mesh. IgM$^+$ cells were sorted and stimulated for 3 days with 12.5 μg/mL lipopolysaccharide (Sigma-Aldrich) and 7.5 ng/mL IL-4 (Peprotech) in RPMI-1640 (Wako) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin.

Gene Ontology analysis

The miR-195 targetomes were gathered from the miR-195 target mRNAs identified from three databases (Targetscan, miRDB, and *microRNA.org*) and by comparing the microarray data of the targets in control-and miR-195-transduced Ebf1$^{-/-}$ FL HPCs. To investigate the biological functions, these genes were applied to the Gene Ontology classification using GeneSpringGX11.

Quantitative real-time PCR

For mRNA quantification, total RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque) and cDNA was synthesized from it using the ReverTra Ace™ qPCR RT Master Mix (TOYOBO). qPCR was performed using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO) on the StepOnePlus™ Real-Time PCR System (Thermo Fisher). The following primers were used for qPCR: Pik3r1 – 5'-AAACTCCGAGACACTGCTGA-3' and 5'-GAGTGTAATCGCCGTGCATT-3'; Pdpk1 – 5'-CTGGGCTCTGCTCTAGTGTT-3' and 5'-CCCAGGTTCAGGACAGGATT-3'; Akt3 – 5'-

GTGGACCACTGTTATAGAGAGAACAT-3' and 5'-TTGGATAGCTTCCGTCCACT-3'; Raf1 – 5'-TCTTCCATCGAGCTGCTTCA-3' and 5'-GGATGTAGTCAGCGTGCAAG-3'; Sos2 – 5'-AACTTTGAAGAACGGGTGGC-3' and 5'-TTTCCTGCAGTGCCTCAAAC-3'; and Mapk3 – 5'-ACTACCTGGACCAGCTCAAC-3' and 5'-TAGGAAAGAGCTTGGCCCAA-3'. For miR-195 quantification, TaqMan™ MicroRNA Assay (ABI) was used. Briefly, total RNA was isolated using Sepasol-RNA I Super G and cDNA was synthesized from it using the microRNA TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher) and a specific primer, 5'-UAGCAGCACAGAAAUAUUGGC-3'. The expression levels were measured using the TaqMan™ Fast Advanced Master Mix (Thermo Fisher) on the StepOnePlus™ Real-Time PCR System. Given the high sequence similarity among miR-15/16 family members, the TaqMan assay for miR-195 may detect related miRNAs such as miR-16. Therefore, we interpreted miR-195 qPCR results as approximate estimates rather than precise quantification. GAPDH was used for normalization to maintain consistency with other qPCR assays in this study. All reagents and kits in this section were used according to the manufacturer's instructions. Target RNA expression levels were compared with those of GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Dual-Luciferase® assay

293T cells were co-transfected with 20 ng psiCHECK-2 of *Akt3* or *Mapk3* and 100 ng MDH1-PGK-GFP-miR-195 or MDH1-PGK-GFP-miR-195-mut. At 48 hrs post-transfection, the relative amounts of Renilla and firefly luciferase were analyzed using a Dual-Luciferase® Reporter Assay System (Promega). The Renilla/firefly luciferase ratio was calculated and normalized against the control.

Western blot

Total proteins were collected from whole cells using radioimmunoprecipitation assay buffer (Wako) with protease inhibitor cocktail (Sigma-Aldrich) and SDS sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 50 mM dithiothreitol). The proteins were separated using SDS-PAGE and the western blot signal was detected and analyzed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) on Ez-Capture MG AE-9300 (ATTO). The following antibodies were used: anti-FOXO1 (C29H4, Cell Signaling Technology),-phospho-FOXO1(Ser256) (9461, Cell Signaling Technology), and-GAPDH (G9545, Sigma-Aldrich). Signal intensities were quantified using ImageJ version 1.54g (43).

ATAC-seq analysis

For ATAC-seq analysis, $B220^+$ $CD19^+$ and $B220^+$ cells were sorted from the bone marrow of NOG mice transplanted with miR-195-transduced $EBF1^{-/-}$ Lin^- cells. $B220^+$ cells were also sorted from the empty transduced sample. The collected cells were resolved using CELLBANKER® (Takara Bio) and temporarily preserved at –20°C.

ATAC-seq libraries were prepared from the cryopreserved cells according to the Omni-ATAC protocol (44). Briefly, >5,000 cells were lysed and subjected to a transposition reaction. The transposed fragments were pre-amplified, quantitated using RT-PCR, and then amplified again. The prepared libraries were sequenced on the NextSeq 550 platform (Illumina) with paired-end reads (read 1, 75 bp; index 1, 8 bp; index 2, 8 bp; read 2, 75 bp). Short-read data were trimmed using sickle 1.33 (*https://github.com/najoshi/sickle*) and mapped onto a mm10 reference genome using bowtie2. Unmapped, multi, chrM mapping, and duplicate reads were eliminated using samtools 1.16.1 and Picard Tools (Picard MarkDuplicates; *http://broadinstitute.github.io/picard*). Peak summits in all populations were determined using the MACS3 functions (-callpeak-p 1e-5 *https://github.com/macs3-project/MACS*). Motif enrichment analysis was carried out using HOMER, with default settings.

Statistical analysis

One-sample *t*-test was used to analyze differences between groups, and *p*-values<0.05 were considered statistically significant. All analyses were performed using Excel (Microsoft). Statistical significance was determined using the Fisher's exact test, followed by multiple test corrections

using the Benjamini and Yekutieli false discovery rate method.

Data availability

The microarray data was deposited in Gene Expression Omnibus with the identifier GSE246669, and the ATAC-seq data was also deposited with the identifier GSE246530. The other data generated in this study are available in the manuscript or supplementary materials.

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Additional information

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Author contributions

A.K. designed the research; Y.M., T.K., R.Y., R.K., K.K., R.-K.N., and K.O. performed the research; T.I., K. Hirano, H.H., K. Hozumi, M. Ohtsuka, T. Kishikawa, C.S., M. Otsuka, R.M., K.A., and T. Kurosaki contributed new reagents and analytic tools; H.K. analyzed the data; Y.M., T.K., and A.K. wrote the paper.

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Additional files

Supplementary Figure 1-3

Supplementary Table 1-3

Supplementary Table 4-5

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Peer reviews

Reviewer #1 (Public review):

Summary:

Here, the authors are proposing a role for miR-196, a microRNA that has been shown to bind and enhance degradation of mRNA targets in the regulation of cell processes, has a novel role in allowing the emergence of CD19+ cells in cells in which Ebf1, a critical B-cell transcription factor, has been genetically removed.

Strengths:

That over-expression of mR-195 can allow the emergence of CD19+ cells missing Ebf1 is somewhat novel.

Their data does perhaps support to a degree the emergence of a transcriptional network that may bypass the absence of Ebf1, including the FOXO1 transcription factor, but this data is not strong or definitive.

Weaknesses:

It is unclear whether this observation is in fact physiological. When the authors analyse a knockout model of miR-195, there is not much of a change in the B-cell phenotype. Their findings may therefore be an artefact of an overexpression system.

The authors have provided insufficient data to allow a thorough appraisal of the step-wise molecular changes that could account for their observed phenotype.

On review of the resubmitted manuscript, while I note the authors have attempted to address several of my comments, unfortunately, their resubmission is not sufficient to address several of the comments I had previously made.

In particular, in the resubmitted data that includes western blots for PAX5 and ERG in their EBF1-/- model, Supp Fig S3, the bands they show infer that that PAX5 and ERG expression can still be significantly detected in their EBF1-/- early B-cell model. This should not be the case, as no expression of PAX5 or ERG should be seen, as has been shown in prior literature.

https://doi.org/10.7554/eLife.101510.2.sa3

Reviewer #2 (Public review):

Summary:

The authors investigate miRNA miR-195 in the context of B-cell development. They demonstrate that ectopic expression of miR-195 in hematopoietic progenitor cells can, to a considerable extent, override the consequences of deletion of Ebf1, a central B-lineage defining transcription factor, in vitro and upon short-term transplantation into immunodeficient mice in vivo. In addition, the authors demonstrate that the reverse experiment, genetic deletion of miR-195, has virtually no effect on B-cell development. Mechanistically, the authors identify Foxo1 phosphorylation as one pathway partially contributing to the rescue effect of miR-195. An additional analysis of epigenetics by ATACseq adds potential additional factors that might also contribute to the effect of ectopic expression of miR-195.

Strengths:

The authors employ a robust assay system, Ebf1-KO HPC, to test for B-lineage promoting factors. The manuscript overall takes on an interesting perspective rarely employed for analysis of miRNA by overexpressing the miRNA of interest. Ideally, this approach may reveal, if not the physiological function of this miRNA, the role of distinct pathways in developmental processes.

Weaknesses:

At the same time, this approach constitutes a major weakness: It does not reveal information on the physiological role of miR-195. In fact, the authors themselves demonstrate in their KO approach, that miR-195 has virtually no role in B-cell development, as has been demonstrated already in 2020 by Hutter and colleagues. While the authors cite this paper, unfortunately, they do so in a different context, hence omitting that their findings are not original.

Conceptually, the authors stress that a predominant function of miRNA (in contrast to transcription factors, as the authors suggest) lies in fine-tuning. However, there appears to be a misconception. Misregulation of fine tuning of gene expression may result in substantial biological effects, especially in developmental processes. The authors want to highlight that miR-195 is somewhat an exception in that regard, but this is clearly not the case. In addition to miR-150, as referenced by the authors, also the miR-17-92 or miR-221/222 families play a significant role in B-cell development, their absence resulting in stage-specific developmental blocks, and other miRNAs, such as miR-155, miR-142, miR-181, and miR-223 are critical regulators of leukocyte development and function. Thus, while in many instances a single miRNA moderately affects gene expression at the level of an individual target, quite frequently targets converge in common pathways, hence controlling critical biological processes.

The paper has some methodological weaknesses as well: For the most part, it lacks thorough statistical analysis and only representative FACS plots are provided. Many bar graphs are based on heavy normalization making the T-tests employed inapplicable. No details are provided regarding statistical analysis of microarrays. Generation of the miR-195-KO mice is insufficiently described and no validation of deletion is provided. Important controls are missing as well, the most important one being a direct rescue of Ebf1-KO cells by re-expression of Ebf1. This control is critical to quantify the extent of override of Ebf1-deficiency elicited by miR-195 and should essentially be included in all experiments. A quantitative comparison is essential to support the authors' main conclusion highlighted in the title of the manuscript. As the manuscript currently stands, only negative controls are provided, which, given the profound role of Ebf1, are insufficient, because many experiments, such as assessment of V(D)J recombination, IgM surface expression, or class-switch recombination, are completely negative in controls. In addition, the authors should also perform long-term reconstitution experiments. While it is somewhat surprising that the authors obtain splenic IgM+ B cells after just 10 days, these experiments would certainly be much more informative after longer periods of time. Using "classical" mixed bone marrow chimeras using a combination of B-cell defective (such as mb1/mb1) bone marrow and reconstituted Ebf1-KO progenitors would permit much more refined analyses.

With regard to mechanism, the authors show that the Foxo1 phosphorylation pathway accounts for the rescue of CD19 expression, but not of other factors, and mentioned in the discussion. The authors then resort to epigenetic analysis, but their rationale remains somewhat vague. It remains unclear how miR-195 is linked to epigenetic changes.

https://doi.org/10.7554/eLife.101510.2.sa2

Reviewer #3 (Public review):

Summary:

In this study, Miyatake et al. present the interesting finding that ectopic expression of miR-195 in EBF1-deficient hematopoietic progenitor cells can partially rescue their developmental block and allows B cells to progress to a B220+ CD19+ cells stage. Notably, this is accompanied by an upregulation of B cell specific genes and, correspondingly, a downregulation of T,

myeloid and NK lineage-related genes, suggesting that miR-195 expression is at least in part equivalent to EBF1 activity in orchestrating the complex gene regulatory network underlying B cell development. Strengthening this point, ATAC sequencing of miR-195-expressing EBF1-deficient B220+CD19+ cells and a comparison of these data to public datasets of EBF1-deficient and -proficient cells suggest that miR-195 indirectly regulates gene expression and chromatin accessibility of some, but not all regions regulated by EBF1.

Mechanistically, the authors identify a subset of potential target genes of miR-195 involved in MAPK and PI3K signalling. Dampening of these pathways has previously been demonstrated to activate FOXO1, a key transcription factor for early B cells downstream of EBF1. Accordingly, the authors hypothesize that miR-195 exerts its function through FOXO1. Supporting this claim, also exogenous FOXO1 expression is able to promote the development of EBF1-deficient cells to the B220+CD19+ stage and thus recapitulates the miR-195 phenotype.

Strengths:

The strength of the presented study is the detailed assessment of the altered chromatin accessibility in response to ectopic miR-195 expression. This provides insight into how miR-195 impacts on the gene regulatory network that governs B cell development and allows the formation of mechanistic hypotheses.

Weaknesses:

The key weakness of this study is that its findings are based on the artificial and ectopic expression of a miRNA out of its normal context, which in my opinion strongly limits the biological relevance of the presented work.

While the authors performed qPCRs for miR-195 on different B cell populations and show that its relative expression peaks in early B cells, it remains unclear whether the absolute miR-195 expression is sufficiently high to have any meaningful biological activity. In fact, other miRNA expression data from immune cells (e.g. DOI 10.1182/blood-2010-10-316034 and DOI 10.1016/j.immuni.2010.05.009) suggest that miR-195 is only weakly, if at all, expressed in the hematopoietic system.

Update to this part after revision: The authors now state in the discussion that their study does not aim to uncover and characterize a physiological role of miR-195 in lymphocytes development, but rather reveals "the potential of miR-195 to compensate for EBF1 deficiency". However, in my opinion, the absence of any physiological context still limits this study's relevance.

The authors support their finding by a CRISPR-derived miR-195 knockout mouse model which displays mild but significant differences in the hematopoietic stem cell compartment and in B cell development. However, they fail to acknowledge and discuss a lymphocyte-specific miR-195 knockout mouse that does not show any B cell defects in the bone marrow or spleen and thus contradicts the authors' findings (DOI 10.1111/febs.15493). Of note, B-1 B cells in particular have been shown to be elevated upon loss of miR-15-16-1 and/or miR-15b-16-2, which contradicts the data presented here for loss of the family member miR-195.

A second weakness is that some claims by the authors appear overstated or at least not fully backed up by the presented data. In particular, the findings that miR-195-expressing cells can undergo VDJ recombination, express the pre-BCR/BCR and can class switch need to be strengthened. It would be beneficial to include additional controls to these experiments, e.g. a RAG-deficient mouse as a reference/negative control for the ddPCR and the surface IgM staining, and cells deficient in class switching for the IgG1 flow cytometric staining.

Moreover, the manuscript would be strengthened by a more thorough investigation of the hypothesis that miR-195 promotes the stabilization and activity of FOXO1, e.g. by comparing the authors' ATACseq data to the FOXO1 signature.

https://doi.org/10.7554/eLife.101510.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This useful study reports that the exogenous expression of the microRNA miR-195 can partially compensate in early B cell development for the loss of EBF1, one of the key transcription factors in B cells. While this finding will be of interest to those studying lymphocyte development, the evidence, particularly with regard to the molecular mechanisms that underpin the effect of miR-195, is currently incomplete.*
>
> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Here, the authors are proposing a role for miR-196, a microRNA that has been shown to bind and enhance the degradation of mRNA targets in the regulation of cell processes, and has a novel role in allowing the emergence of CD19+ cells in cells in which Ebf1, a critical B-cell transcription factor, has been genetically removed.*
>
> *Strengths:*
>
> *That over-expression of mR-195 can allow the emergence of CD19+ cells missing Ebf1 is somewhat novel.*
>
> *Their data does perhaps support to a degree the emergence of a transcriptional network that may bypass the absence of Ebf1, including the FOXO1 transcription factor, but this data is not strong or definitive.*
>
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>
> *It is unclear whether this observation is in fact physiological. When the authors analyse a knockout model of miR-195, there is not much of a change in the B-cell phenotype. Their findings may therefore be an artefact of an overexpression system.*
>
> *The authors have provided insufficient data to allow a thorough appraisal of the stepwise molecular changes that could account for their observed phenotype.*
>
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>
> *Summary:*
>
> *The authors investigate miRNA miR-195 in the context of B-cell development. They demonstrate that ectopic expression of miR-195 in hematopoietic progenitor cells can, to a considerable extent, override the consequences of deletion of Ebf1, a central Blineage defining transcription factor, in vitro and upon short-term transplantation into immunodeficient mice in vivo. In addition, the authors demonstrate that the reverse experiment, genetic deletion of miR-195, has virtually no effect on B-cell development. Mechanistically, the authors identify Foxo1 phosphorylation as one pathway partially*

contributing to the rescue effect of miR-195. An additional analysis of epigenetics by ATACseq adds potential additional factors that might also contribute to the effect of ectopic expression of miR-195.

Strengths:

The authors employ a robust assay system, Ebf1-KO HPC, to test for B-lineage promoting factors. The manuscript overall takes on an interesting perspective rarely employed for the analysis of miRNA by overexpressing the miRNA of interest. Ideally, this approach may reveal, if not the physiological function of this miRNA, the role of distinct pathways in developmental processes.

Weaknesses:

At the same time, this approach constitutes a major weakness: It does not reveal information on the physiological role of miR-195. In fact, the authors themselves demonstrate in their KO approach, that miR-195 has virtually no role in B-cell development, as has been demonstrated already in 2020 by Hutter and colleagues. While the authors cite this paper, unfortunately, they do so in a different context, hence omitting that their findings are not original.

Conceptually, the authors stress that a predominant function of miRNA (in contrast to transcription factors, as the authors suggest) lies in fine-tuning. However, there appears to be a misconception. Misregulation of fine-tuning of gene expression may result in substantial biological effects, especially in developmental processes. The authors want to highlight that miR-195 is somewhat of an exception in that regard, but this is clearly not the case. In addition to miR-150, as referenced by the authors, also the miR-17-92 or miR-221/222 families play a significant role in B-cell development, their absence resulting in stage-specific developmental blocks, and other miRNAs, such as miR-155, miR-142, miR-181, and miR-223 are critical regulators of leukocyte development and function. Thus, while in many instances a single miRNA moderately affects gene expression at the level of an individual target, quite frequently targets converge in common pathways, hence controlling critical biological processes.

The paper has some methodological weaknesses as well: For the most part, it lacks thorough statistical analysis, and only representative FACS plots are provided. Many bar graphs are based on heavy normalization making the T-tests employed inapplicable. No details are provided regarding the statistical analysis of microarrays. Generation of the miR-195-KO mice is insufficiently described and no validation of deletion is provided. Important controls are missing as well, the most important one being a direct rescue of Ebf1-KO cells by re-expression of Ebf1. This control is critical to quantify the extent of override of Ebf1-deficiency elicited by miR-195 and should essentially be included in all experiments. A quantitative comparison is essential to support the authors' main conclusion highlighted in the title of the manuscript. As the manuscript currently stands, only negative controls are provided, which, given the profound role of Ebf1, are insufficient, because many experiments, such as assessment of V(D)J recombination, IgM surface expression, or class-switch recombination, are completely negative in controls. In addition, the authors should also perform long-term reconstitution experiments. While it is somewhat surprising that the authors obtained splenic IgM+ B cells after just 10 days, these experiments would be certainly much more informative after longer periods of time. Using "classical" mixed bone marrow chimeras using a combination of B-cell defective (such as mb1/mb1) bone marrow and reconstituted Ebf1-KO progenitors would permit much more refined analyses.

With regard to mechanism, the authors show that the Foxo1 phosphorylation pathway accounts for the rescue of CD19 expression, but not for other factors, as mentioned in

the discussion. The authors then resort to epigenetics analysis, but their rationale remains somewhat vague. It remains unclear how miR-195 is linked to epigenetic changes.

Reviewer #3 (Public review):

Summary:

In this study, Miyatake et al. present the interesting finding that ectopic expression of miR-195 in EBF1-deficient hematopoietic progenitor cells can partially rescue their developmental block and allow B cells to progress to a B220+ CD19+ cells stage. Notably, this is accompanied by an upregulation of B-cell-specific genes and, correspondingly, a downregulation of T, myeloid, and NK lineage-related genes, suggesting that miR-195 expression is at least in part equivalent to EBF1 activity in orchestrating the complex gene regulatory network underlying B cell development. Strengthening this point, ATAC sequencing of miR-195-expressing EBF1-deficient B220+CD19+ cells and a comparison of these data to public datasets of EBF1-deficient and -proficient cells suggest that miR-195 indirectly regulates gene expression and chromatin accessibility of some, but not all regions regulated by EBF1.

Mechanistically, the authors identify a subset of potential target genes of miR-195 involved in MAPK and PI3K signaling. Dampening of these pathways has previously been demonstrated to activate FOXO1, a key transcription factor for early B cells downstream of EBF1. Accordingly, the authors hypothesize that miR-195 exerts its function through FOXO1. Supporting this claim, also exogenous FOXO1 expression is able to promote the development of EBF1-deficient cells to the B220+CD19+ stage and thus recapitulates the miR-195 phenotype.

Strengths:

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10.1182/blood-2010-10-316034 and DOI 10.1016/j.immuni.2010.05.009) suggest that miR-195 is only weakly, if at all, expressed in the hematopoietic system.

The authors support their finding by a CRISPR-derived miR-195 knockout mouse model which displays mild, but significant differences in the hematopoietic stem cell compartment and in B cell development. However, they fail to acknowledge and discuss a lymphocyte-specific miR-195 knockout mouse that does not show any B cell defects in the bone marrow or spleen and thus contradicts the authors' findings (DOI

10.1111/febs.15493). Of note, B-1 B cells in particular have been shown to be elevated upon loss of miR-15-16-1 and/or miR-15b-16-2, which contradicts the data presented here for loss of the family member miR-195.

A second weakness is that some claims by the authors appear overstated or at least not fully backed up by the presented data. In particular, the findings that miR-195expressing cells can undergo VDJ recombination, express the pre-BCR/BCR and class switch needs to be strengthened. It would be beneficial to include additional controls to these experiments, e.g. a RAG-deficient mouse as a reference/negative control for the ddPCR and the surface IgM staining, and cells deficient in class switching for the IgG1 flow cytometric staining.

Moreover, the manuscript would be strengthened by a more thorough investigation of the hypothesis that miR-195 promotes the stabilization and activity of FOXO1, e.g. by comparing the authors' ATACseq data to the FOXO1 signature.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

Miyatake et al., present a manuscript that explores the role of miR-195 in B cell development.

Their data suggests a role for this microRNA:

Using an Ebf1 fetal liver knockout of B-cell differentiation that a small population of CD19 expressing with some evidence of V(D)J recombination capable of class switch can be derived by transduction of miR-195.

In the emergent CD19+ Ebf1-/- cells, the authors provide some evidence that Mapk and Akt3 may be miR-195 targets that are downregulated allowing FOXO1 transcription factor pathway may be involved in the emergent CD19+ cells arising from miR-195 transduction.

Perhaps less compelling data is provided with regards to a role for miR-195 in normal Bcell development through analysis of a miR-195 knockout model.

While there are some interesting preliminary data presented for a role for miR-195 in the context of Ebf1-/- cells, there are some questions I think the authors could consider.

Comments:

(1-1) It is difficult to ascertain the potential role of miR-195 transduction in allowing the emergence of CD19+ cells from the data provided. miR-195 has been generally shown to destabilize mRNA transcripts by 3' UTR binding that targets mRNA transcripts for degradation. The effect of transduction of miR-195 would therefore be expected to be related to the degradation of factors opposing aspects of B-lineage specification or maintenance. I would be particularly interested in transcriptional or epigenetic regulators that may be modified in this way, at an mRNA as well as protein level.

We appreciate the reviewer's thoughtful comments and agree that miRNAs often exert their effects through the degradation or translational repression of mRNAs encoding regulatory factors. In our study, we attempted to address this point by combining predictive analysis (using TargetScan and starBase) with luciferase reporter assays and qPCR to validate several potential targets of miR-195, including Mapk3 and Akt3. We acknowledge that this is not a comprehensive mechanistic analysis. We agree that a broader and systematic identification of direct targets of miR-195, particularly those involved in transcriptional and epigenetic regulation, would further clarify the mechanisms involved. However, due to limitations in resources and time, we are currently unable to perform global proteomic or ChIP-based validations. Nevertheless, our ATAC-seq and microarray data indicate that miR-195 overexpression leads to increased accessibility and expression of several key B-lineage

transcription factors (Pax5, Runx1, Irf8), suggesting that miR-195 indirectly activates transcriptional programs relevant to B cell commitment. We have now clarified this limitation in the revised Discussion section (lines 505–524), and we emphasize that our current findings represent the potential of miR-195 rather than its physiological role. We hope that this clarification addresses the concern.

> *(1-2) While I acknowledge the authors have undertaken TargetScan and starBase analysis to try and predict miR-195 interactions, they do not provide a comprehensive list of putative targets that can be referenced against their cDNA data. Though they postulate Mapk3 and Akt3 as putative miR-195 targets and assay these in luciferase reporter systems (Figure 4), these were not clearly differentially regulated in the microarray data they provided (Figure 1E) as being downregulated on miR-195 transduction in Ebf1-/- cells.*

We thank the reviewer for pointing out the need for a more comprehensive list of predicted miR-195 targets. In response, we have now included a supplementary table 4 (human) and 5 (mouse) listing all putative miR-195 targets predicted by TargetScan and starBase. As noted, Mapk3 expression was indeed downregulated upon miR-195 transduction, consistent with our luciferase reporter and qPCR results. For Akt3, we observed variability in the microarray data depending on the probe used, resulting in inconsistent expression levels. We acknowledge this and have added a clarification in the revised manuscript (lines 335–339), noting that the regulation of Akt3 by miR-195 is potentially probe-dependent and may require further validation. We hope this clarification resolves the concern.

> *(1-3) The authors should provide a more comprehensive analysis of transcriptional changes induced by miR-195 Ebf1-/- specifically in the preproB cell stage of development in Ebf1-/- and miR-195 Ebf1-/- cells. The differentially expressed gene list should be provided as a supplemental file. The gene expression data should be provided for the different B-cell differentiation stages, eg. Ebf1-/- preproB cells, and Ebf1-/- miR-195 preproB cells, CD19+ cells and more differentiated subsets induced by miR-195 transduction.*

We appreciate the reviewer's suggestion to provide a more comprehensive transcriptomic analysis at different B-cell differentiation stages. Unfortunately, due to the limited availability of cells and technical constraints, we were unable to perform RNA-seq on miR-195 transduced Ebf1$^{-/-}$ pre-pro-B or CD19+ cells. However, to address this point, we referenced publicly available RNA-seq data (GEO accession: GSE92434), which includes transcriptomic profiles of Ebf1$^{-/-}$ pro-B cells and wild-type controls. By comparing our microarray data from miR-195 transduced Ebf1$^{-/-}$ cells with this dataset, we found partial restoration of expression for several key B-lineage genes, such as Pax5, Runx1, and Irf8, which are normally downregulated in the absence of EBF1. This comparison supports the notion that miR-195 partially reactivates the transcriptional network essential for B cell development. We have added this interpretation to the Discussion section (lines 528–533).

> *(1-4) More replicates (at least 3 of each genotype) are required for their Western Blots for FOXO1 and pFOXO1 (Fig 4C, D). Western blots should also be provided for other known B-lineage transcriptional regulators such as PAX5 and ERG.*

We thank the reviewer for these valuable suggestions. In response, we have now quantified and added the relative band intensities of FOXO1 and pFOXO1 from three independent experiments in the revised Figure 4C, and we include statistical analysis to support the reproducibility of these results. Additionally, as requested, we performed western blotting for PAX5 and ERG using the same samples. The results showed no significant change in these protein levels between miR-195-transduced and control Ebf1$^{-/-}$ cells, consistent with the modest upregulation observed in our microarray data. We have included the PAX5 and ERG

western blot images in Supplementary Figure S3 and have revised the text in the Results section (lines 351−35)

> *(1-5) The authors have not shown a transcriptional binding by ChIPseq or other methods such as cut and tag/ cut and run for FOXO1 binding to B-lineage genes in their Ebf1-/- miR-195 CD19+ cells to be able to definitively show this TF is critical for the emergence of the C19+ cell phenotype by demonstrating direct binding to "upregulated" genes cis-regulatory regions in the Ebf1-/- miR-195 CD19+ cells*

We appreciate the reviewer's suggestion regarding the use of ChIP-seq or related methods to demonstrate direct FOXO1 binding to cis-regulatory regions of B-lineage genes in Ebf1$^{-/-}$ miR-195 CD19^{+} cells. We agree that such data would provide definitive evidence of FOXO1's direct involvement in promoting the B cell-like transcriptional program. However, due to current technical limitations, including the scarcity of CD19^{+} cells derived from Ebf1$^{-/-}$ miR-195 transduction and the requirement for large cell numbers in ChIP-seq or CUT&RUN protocols, we were unable to perform these assays in this study. Nevertheless, our current data provide multiple lines of indirect evidence supporting the involvement of FOXO1:

miR-195 transduction leads to reduced phosphorylation and increased accumulation of FOXO1 protein (Fig. 4C).

Overexpression of FOXO1 in Ebf1$^{-/-}$ HPCs partially recapitulates the miR-195 phenotype (Fig. 4D).

ATAC-seq data show increased chromatin accessibility at known FOXO1 target gene loci (e.g., Pax5, Runx1, Irf8) in miR-195-induced CD19^{+} cells, many of which overlap with FOXO1 motifs(Fig.5)

These observations collectively suggest that FOXO1 activity is functionally important for the emergence of CD19^{+} cells, even though direct binding has not been confirmed. We have added this limitation to the Discussion (lines 531−537), and we note that future studies using FOXO1 CUT&RUN in this system would be valuable to further define the underlying mechanism.

> *(1-6) The authors have not shown significant upregulation of expression of other critical B-cell regulatory transcription factors in their Ebf1-/- miR-195 CD19+ cells that could account for the emergence of these cells such as Pax5 or Erg. The legend in Figure 1E suggests for example the change in expression of Pax5 is modest if anything at best as no LogFC or western blot data is presented.*

We thank the reviewer for raising this point. In our microarray analysis (Figure 1D, original Figure 1E), we observed that both Pax5 and Erg mRNA levels were upregulated in Ebf1$^{-/-}$ cells upon miR-195 transduction. Specifically, Pax5 showed an increase of approximately $\log_2$FC 1.2, and Erg was also consistently elevated across biological replicates. These changes, although modest, were statistically significant and consistent with the upregulation of other B-lineage-associated transcription factors, such as Runx1 and Irf8. We agree that the magnitude of Pax5 upregulation is not as high as typically seen during full B cell commitment, and therefore may not have been immediately apparent in Figure 1D (original Figure 1E). To clarify this point, we have now revised the text in the Results section (lines 170−174) to highlight the observed changes in Pax5 and Erg expression. We believe that the upregulation of these transcription factors, together with increased FOXO1 activity and changes in chromatin accessibility (Figure 5), contributes to the partial reactivation of the B cell gene regulatory network in the absence of EBF1.

> *(1-7) Which V(D)J transcripts have been produced? A more detailed analysis other than ddPCR is required to help understand the emergence of this population that can presumably proceed through the preBCR and BCR checkpoints.*

We appreciate the reviewer's interest in understanding the nature of the V(D)J rearrangements in Ebf1$^{-/-}$ miR-195 CD19^{+} cells. As noted, our current data rely on droplet digital PCR (ddPCR), which was used to detect rearranged VH-JH segments in the bone marrow of engrafted mice. While this approach does not allow for detailed mapping of specific V, D, or J gene usage, it provides a sensitive and quantitative measure of V(D)J recombination activity. The detection of rearranged VH-JH fragments in miR-195-transduced Ebf1$^{-/-}$ cells suggests that at least partial recombination of the immunoglobulin heavy chain locus is occurringan essential checkpoint for progression past the pro-B cell stage. Given the lack of such rearrangements in control-transduced Ebf1$^{-/-}$ cells, we interpret this as evidence that miR-195 enables cells to initiate the recombination process. We acknowledge the limitations of ddPCR and agree that a more detailed analysis using VDJ-seq or singlecell RNA-seq would be valuable in determining the diversity and completeness of the V(D)J transcripts produced. This is a direction we intend to pursue in future work. We have added this limitation to the Discussion section (lines 538–543).

> *(1-8) The authors reveal that the Foxo1 transduced Ebf1-/- cells (Fig. 4D) do not persist in vitro or be detected via transplant assay (line 256) and therefore does not represent a truly "rescued" B cell, suggesting that CD19+ cells Ebf1-/- miR-195 transduced cells have more B-cell potential. Further characterisation is therefore warranted of this cell population. For instance, can these cells be induced to undergo myeloid differentiation in myeloid cytokine conditions? What other B-lineage transcriptional regulators are expressed in this cell population that could account for VDJ recombination and expression of a B-lineage transcriptional program (see comments 1, 3, and 5) that allow transition through preBCR and BCR checkpoints as well as undergo class switching?*

We thank the reviewer for this insightful comment. We agree that the persistence and lineage potential of the CD19^{+} cells emerging from Ebf1$^{-/-}$ miR-195-transduced progenitors deserve further characterization. Although we were unable to perform additional lineage re-direction assays, our current data provide several lines of evidence suggesting that these cells are stably committed toward the B-lineage:

Gene expression profiling revealed upregulation of multiple B cell transcriptional regulators, including Pax5, Runx1, and Irf8.

ATAC-seq analysis showed increased chromatin accessibility at B cell–specific loci and enrichment of motifs bound by key B-lineage factors such as FOXO1 and E2A.

The cells express surface IgM and undergo class switch recombination to IgG1 upon stimulation, indicating successful transition through the pre-BCR and BCR checkpoints and acquisition of mature B cell functions.

Importantly, no upregulation of myeloid- or T-lineage genes was detected in the microarray analysis, arguing against multipotency at this stage.We acknowledge that functional tests for lineage plasticity under altered cytokine conditions would provide important insights and plan to address this question in future studies. This limitation has now been noted in the revised Discussion (lines 544–550).

> *(1-9) In the original Ebf1-/- miR-195 CD19+ experiments, a wild-type control should be provided for each experiment.*

We appreciate the reviewer's suggestion to include wild-type controls in all experiments. While we did not include wild-type samples side-by-side in every assay, we carefully designed our experiments to include biologically appropriate and informative comparisons. For example, in the bone marrow transplantation experiments (Figure 2), Ebf1$^{-/-}$ cells transduced with empty vector served as negative controls, clearly lacking CD19 expression, V(D)J recombination, IgM surface expression, and class switch capability. This allowed us to

specifically assess the gain-of-function effects of miR-195 in the EBF1-deficient background. In several analyses—such as the ATAC-seq and microarray comparisons—we did incorporate or refer to existing wild-type datasets (e.g., GSE92434), providing context for the extent of recovery toward a WT-like profile. We agree, however, that including parallel WT controls across all experimental platforms would enhance interpretability.

> *(1-10) For ATACseq data, a comparison between Ebf1-/- preproB cells and Ebf1-/- miR-195 CD19+ cells should be undertaken.*

We thank the reviewer for this important point. As suggested, we have performed a direct comparison of chromatin accessibility between Ebf1$_{-/-}$ pre-pro-B–like cells (CD19$_{-}$, control transduction) and Ebf1$_{-/-}$ miR-195–transduced CD19^{+} cells. This comparison is shown in green in Figure 5B and represents the ATAC-seq peaks differentially accessible between these two populations.

> *(1-11) I cannot agree with the authors with some of their statements such as Line 242 - "therefore miR-195 considered to have similar function with EBF1 to some extent" - how can this be the case when miR-195 is a miRNA and EBF1 is a transcription factor with pioneering transcriptional activity? Surely the effects of miR-195 must be secondary.*

We thank the reviewer for pointing out the inappropriateness of comparing miR-195 to EBF1 in terms of functional similarity. We agree that miR-195, as a microRNA, operates through post-transcriptional regulation and does not possess the pioneering transcriptional activity characteristic of EBF1. To avoid confusion or overstatement, we have removed the sentence in line 242 ("therefore miR-195 is considered to have similar function with EBF1 to some extent").

> *(1-12) It is unclear whether this observation is in fact physiological. When the authors analyse a knockout model of miR-195, there is not much of a change in the B-cell phenotype. Their findings may therefore be an artefact of an overexpression system. The authors should comment on this observation in their discussion.*

We thank the reviewer for this important observation. We agree that the mild phenotype observed in our miR-195 knockout mice suggests that miR-195 is not essential for B cell development under steady-state physiological conditions. Accordingly, we do not claim a physiological requirement for miR-195. Rather, our study demonstrates that miR-195 possesses the potential to activate a B-lineage program in the absence of EBF1 when ectopically expressed. This functional potential—rather than its endogenous necessity—is the main focus of our work. We have now clarified this distinction in the revised Discussion section (lines 551–560), and we emphasize that our findings highlight an alternative regulatory pathway that can be artificially engaged under specific conditions.

> *(1-13) I recommend the authors check spelling and grammar throughout their manuscript.*

We thank the reviewer for the suggestion. In response, we have carefully reviewed the manuscript for spelling, grammar, and clarity. Minor corrections have been made throughout the text to improve readability and ensure consistency. We hope that the revised version addresses any language-related concerns. In addition, the manuscript has been reviewed by professional editing service to improve the language quality.

> *(1-14) In general, I recommend more comprehensive primary data be presented in the manuscript or supplementary files to add value to their submission.*

We thank the reviewer for this helpful suggestion. In response, we have revised the manuscript and supplementary materials to include additional primary data wherever possible. The bar graphs have been updated to include individual data points to show

variability and replicate information. Uncropped western blot images are now provided in Supplementary Figure S2. We hope these additions provide greater transparency and value to the manuscript.

> ***Reviewer #2 (Recommendations for the authors):***
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> *I have a number of suggestions with regard to inclusion of details and controls:*
>
> *(2-1) The authors need to provide more details on in vitro differentiation, especially culture times.*

Thank you for your comment. The culture conditions for in vitro differentiation of Ebf1$^{-/-}$ hematopoietic progenitor cells are described in the Methods section (lines 648– 649) under "Culture of lineage-negative (Lin−) cells from the fetal liver." As stated, cells were cultured more than 7 days under the specified conditions.

> *(2-2) In Figure 1E, the authors need to provide information on statistics (FDR or similar).*

I thank the reviewer for the suggestion. In Figure 1D (Original Figure 1E) (the microarray analysis), only two biological replicates were available for each condition (n = 2 per group). Due to this limited sample size, we did not perform statistical testing, as the power would be insufficient to produce reliable p-values or adjusted FDRs. Instead, we focused on genes with consistent and biologically meaningful changes in expression, and presented representative examples based on fold change values.

> *(2-3) For in vivo experiments (Figure 2) the authors should comment on their use of two different recipient mouse strains despite very low n numbers. As described above, classical mixed BM chimeras would be much more informative. In these experiments, the authors should also show the formation of other lymphoid lineages. This would answer the question of whether miR-195 redirects cells to the B lineage. Most importantly, absolute numbers need to be provided, especially in conjunction with Ebf1 rescue as described above.*

We thank the reviewer for the thoughtful and detailed suggestions regarding our in vivo experiments. Regarding the use of different recipient mouse strains, our initial intention was to perform the transplantations in BRG mice; however, due to facility restrictions and animal husbandry considerations, we had to switch to NOG mice. All in vivo experiments were performed with n = 3 per group, in accordance with ethical guidelines and efforts to minimize animal use while still ensuring reproducibility. With respect to the suggestion of mixed bone marrow chimeras, we agree that this approach can provide valuable information on lineage competitiveness. However, in our system, miR-195 confers only a very limited B cell developmental potential in Ebf1$^{-/-}$ progenitors. In such a setting, the inclusion of wild-type competitor cells would overwhelmingly dominate the B cell compartment, likely masking any measurable effect of miR-195. Therefore, we opted to assess the gain-of-function potential of miR-195 in a noncompetitive setting. Regarding the assessment of other lymphoid lineages, we focused our analysis on the emergence of B-lineage cells, as the frequency of CD19^{+} cells induced by miR-195 is quite low. Given this low efficiency, we consider it unlikely that miR-195 significantly alters the development of non-B lineages, and thus did not observe substantial lineage diversion effects. Our aim was not to demonstrate lineage redirection, but rather to show that miR-195 can confer partial B cell potential in the absence of EBF1.

Finally, we acknowledge the importance of presenting absolute cell numbers. However, the cell number collected from the mice were so few that we did not get the reliable results, we described it in the manuscript. (lines 498-501)

> *(2-4) The statistics in Figure 3 are inadequate. No S.D. is provided for WT. How then was normalization performed? Student's T-test cannot be applied to ratios.*

We thank the reviewer for highlighting the need for more appropriate statistical analysis. Due to considerable inter-batch variability in absolute measurements, we normalized the KO values to their paired WT counterparts from the same experimental batch. Specifically, for each replicate, we calculated the KO/WT ratio to control for batch-specific variation. We then applied a one-sample t-test (against a null hypothesis of ratio = 1) to determine statistical significance. We have now revised the figure to show individual ratio values for each replicate and updated the legend and Methods to clearly explain the statistical approach. We hope this addresses the concern and improves the clarity and rigor of the analysis.

> *(2-5) In Figure 4A, the authors should comment on the strong repression of the Akt3UTR.*

We appreciate the reviewer's observation regarding the strong repression observed with the Akt3 3'UTR construct. Indeed, we also noted that luciferase activity was markedly reduced in the presence of the Akt3 3'UTR, even in cells transduced with a control vector. We hypothesize that the Akt3 3'UTR contains strong post-transcriptional regulatory elements—such as AU-rich elements or binding sites for endogenous miRNAs or RNA-binding proteins—which may suppress mRNA stability or translation independent of miR-195. Alternatively, the secondary structure or length of the UTR may inherently reduce luciferase expression. We have added this limitation to the Discussion section (lines 561−569).

> *(2-6) The Western blot in Figure 4C is of insufficient quality. The authors need to provide unspliced versions of the bands including markers.*

We thank the reviewer for this important comment. In response, we have included the unprocessed, full-length Western blot images corresponding to Figure 4C as Fig. S2. This provides a transparent view of the original data and addresses the concern about image cropping.

> *(2-7) The ATACseq experiment in Figure 5 is difficult to comprehend. A simpler design including Ebf1 rescue controls would clearly improve this part.*

We thank the reviewer for this valuable feedback. We agree that the original presentation of the ATAC-seq data may have been difficult to interpret. To address this, we have included a clear interpretation of the overlapping regions in the revised figure legend (lines 1018-1022). We hope this improves the clarity of the data and facilitates understanding of the chromatin changes mediated by EBF1 and miR-195.

> *(2-8) The miR-195 KO mouse lacks validation (RT-PCR, genomic PCR) as well as a clear description of the deleted region and whether miR-497 is affected. In addition, the genetic background and number of backcrosses for the removal of potential off-target effects need to be mentioned.*

We thank the reviewer for this important comment. The miR-195 knockout mouse was generated via CRISPR/Cas9, and Sanger sequencing confirmed a 628 bp deletion on chromosome 11 (GRCm38/mm10 chr11:70,234,425−70,235,103). This deletion includes the entire miR-497 locus and part of the miR-195 precursor sequence. Although we do not show PCR gel images, the deletion was validated by sequencing, and the results are now clearly described in the revised Methods section (lines 607619). All transgenic mice in this study were backcrossed to the C57BL/6 background for at least eight generations.

> *(2-9) The manuscript requires extensive editing for language.*

We appreciate the reviewer's comment. The manuscript has now been revised and professionally edited for language by a native English-speaking editor. We believe clarity and readability have been significantly improved.

> ***Reviewer #3 (Recommendations for the authors):***

> *(3-1) What is the expression level of miR-195 after viral overexpression? In Figure 4B, the authors show a 2.5-fold increase, but this appears very low for the experimental system (expression through the MDH1 retroviral construct) and the observed repressive effects (e.g. Figure 4A and B).*

We thank the reviewer for this insightful comment. We agree that the apparent ~2.5fold increase in miR-195 levels (Figure 4B) may seem modest in the context of retroviral overexpression and the associated functional effects. However, due to the high sequence similarity within the miR-15/16/195/497 family, it is technically challenging to measure mature miR-195 levels with complete specificity. The baseline signal observed in control samples likely reflects cross-reactivity with endogenous miRNAs such as miR-497 or miR-16, which share similar seed sequences. Therefore, the reported fold-change may underestimate the true level of ectopic miR-195 expression. Despite this, we observed robust repression of validated targets (e.g., Mapk3, Akt3) in both qPCR and luciferase assays, indicating that functionally effective levels of miR-195 were achieved. We have now clarified this limitation and interpretation in the revised Results sections (lines 332−335).

> *(3-2) In alignment with the transparency of the data, I would encourage the authors to display the individual data points for all bar graphs.*

We thank the reviewer for this helpful suggestion. In the revised manuscript, we have updated bar graphs to include individual data points to increase transparency and allow better visualization of data variability. In the ddPCR experiments, we provided the raw data in Fig. S1 for full transparency. In Fig. 1A, we have confirmed miR-195 expression profiles using the deposit data which the reviewer suggested, but miR-195 expression was very lower than we expected. We also performed scRNA-seq using hematopoietic lineage cells in 8-week-old C57BL/6 mice, but we could not get the reproducibility of miR-195 expression profiles. Therefore, we determined that this is an artifact caused by the miR-195 probe used for qPCR, and deleted Fig. 1A.

> *(3-3) The references appear to be compromised. For example, the authors state that "The Ebf1−/+ mouse was originally generated by R. Grosschedl (39)" (line 297), but this is not the respective paper. Likewise, the knockout mouse was generated "based on the CRISPR/Cas9 system established by C. Gurumurthy (40)" (line 299), but he/she is not involved in the referenced study.*

We thank the reviewer for pointing out the discrepancies in the reference citations. Upon revising the Methods section to integrate it with the main text, the reference numbering became misaligned. We have corrected the reference in the revised manuscript, and we thank the reviewer for bringing this to our attention.

> *(3-4) Given that the miRNA Taqman assays the authors used here have difficulties to discriminate closely related miRNAs such as e.g. miR-16 (highly expressed in the hematopoietic system) and miR-195, I would suggest that the authors test their qPCR in an appropriate setup, e.g. in their knockout mouse model. In this context, did the authors use another small RNA as a reference for the qPCR analysis? In the methods, only GAPDH is mentioned, but in my opinion, another RNA that uses the same stemloop-based cDNA synthesis protocol would be better suited.*

We thank the reviewer for this valuable and technically insightful comment.

As correctly pointed out, TaqMan-based qPCR assays for miRNAs such as miR-195 can show cross-reactivity with closely related family members, particularly miR-16, which is abundantly expressed in hematopoietic cells. Indeed, due to this limitation, we do not treat the qPCR results shown in the original Figures 1A and 4B as definitive quantification of miR-195 expression. Rather, these data are used to provide a suggestion and a rough estimate of

overexpression efficiency, while our core functional analyses rely on phenotypic and molecular outcomes such as target gene repression and lineage emergence. With this in mind, although we acknowledge that a small RNA reference based on the same stem-loop cDNA synthesis would offer a more compatible normalization in principle, the inherent variability and lack of absolute specificity in such assays also limits their interpretive value. Therefore, we used GAPDH as a normalization control for consistency with other qPCR analyses in the manuscript. We have now clarified this rationale and limitation in the revised Methods sections (lines 712–716), and we thank the reviewer again for highlighting this important technical consideration.

> *(3-5) The Western blot data used to support the hypothesis that FOXO1 phosphorylation is reduced upon overexpression of miR-195 are not convincing. The authors should not crop everything but the band.*

We thank the reviewer for the helpful comment. In response, we have now provided the full-length, uncropped Western blot images corresponding to Figure 4C, including both total FOXO1 and phospho-FOXO1 blots. These images are included in Fig. S2.

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