

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

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**Yuji Miyatake, Tomokatsu Ikawa, Ryo Yanagiya, Ryutaro Kotaki, Kazuaki Kameda, Ryo Koyama-Nasu, Kazuki Okuyama, Ken-ichi Hirano, Hiroyuki Hosokawa, Katsuto Hozumi, Masato Ohtsuka, Takahiro Kishikawa, Chikako Shibata, Motoyuki Otsuka, Reo Maruyama, Kiyoshi Ando, Tomohiro Kurosaki, Hiroshi Kawamoto, Ai Kotani** 

Department of Innovative Medical Science, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193, Japan • Division of Immunology and Allergy, Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Chiba, 278-0022, Japan • Department of Immunology, Graduate School of Medicine, Chiba University, Chiba, Chiba, 260-0856, Japan • Department of Experimental Immunology, Graduate School of Medicine, Chiba University, Chiba, Chiba, 260-0856, Japan • Laboratory for Transcriptional Regulation, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa, 230-0045, Japan • Department of Immunology, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193, Japan • Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193, Japan • Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, 113-0033, Japan • Department of Gastroenterology and Hepatology, Okayama University Hospital, Okayama, Okayama, 700-8558, Japan • Project for Cancer Epigenomics, Cancer Institute, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, 135-8550, Japan • Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Hiroshima, 734-8551, Japan • Laboratory of Lymphocyte Differentiation, WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka, 565-0871, Japan • Laboratory for Infectious Disease Education and Research, Osaka University, Suita, Osaka, 565-0871, Japan • Laboratory for Lymphocyte Differentiation, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa, 230-0045, Japan • Laboratory of Immunology, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Kyoto, 606-8507, Japan

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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.

## 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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## Introduction

Developmental hierarchy in hematopoiesis has been widely researched and it is well-known that proper stimulation leads hematopoietic stem cell 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 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*<sup>-/-</sup> cell express B220 but is disabled 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. Conceivable 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

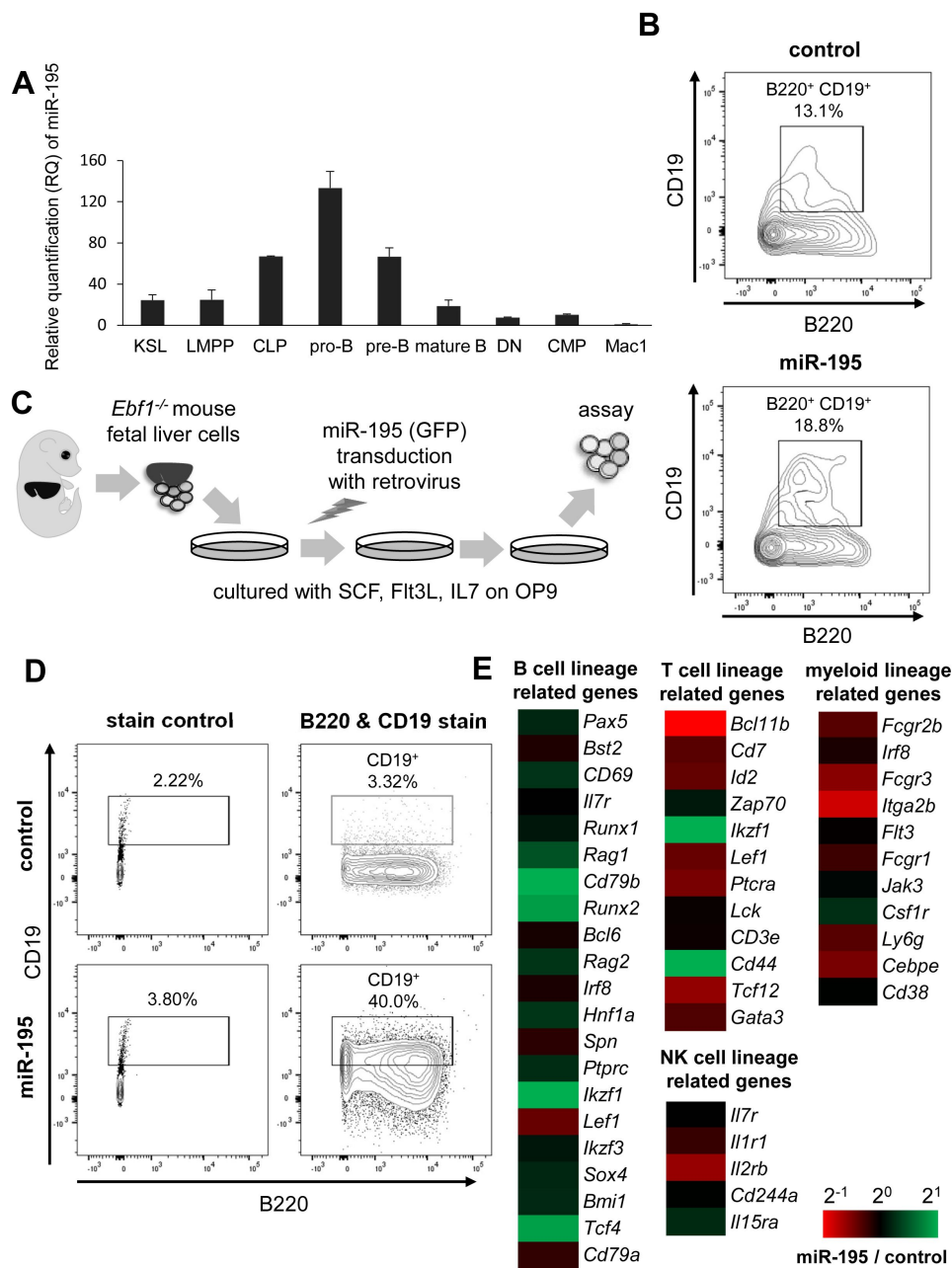
### miR-195 induces B cell character in EBF1 deficient HPCs

To test function of miR-195 in B cell development, we analyzed the expression levels of the miRNA in mouse early hematopoietic lineages mainly focused on B cells. Although the expression level was not high in mature B cell, upregulated in B cell developmental steps especially during early stages (Fig. 1A). This expression profile suggested the possibility of miR-195 as a potential

regulator in B cell lineage commitment. To assess the contribution of miR-195 on B cell development, miR-195 was transduced into mouse fetal liver (FL)-derived Lin<sup>-</sup> c-kit<sup>+</sup> 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. 1B**). This result suggests that miR-195 has ability to shift the HPCs differentiation toward B cells. Next, we attempted to differentiate *Ebf1*<sup>-/-</sup> FL HPC to B cell with miR-195 transduction (**Fig. 1C**). As by a previous study (6), control *Ebf1*<sup>-/-</sup> FL HPCs expressed B220 but did not express CD19. However, miR-195 transduced *Ebf1*<sup>-/-</sup> FL HPCs highly expressed CD19 (**Fig. 1D**). 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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. 1E**). 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*<sup>-/-</sup> HPCs induced differentiation toward B cell. However, a large part of the miR-195 transduced HPCs expressed CD19 but not B220 that implied they strayed from the canonical B cell differentiation steps (**Fig. 1D**). 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 controlled the maintenance and differentiation of lineage precursors by cytokines and chemokines (22). Then to explore the development of miR-195 transduced *Ebf1*<sup>-/-</sup> FL HPCs under bone marrow niche, we engrafted miR-195 transduced *Ebf1*<sup>-/-</sup> 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*<sup>-/-</sup> FL early B cells which suggest the normal stepwise B cell development occurred (**Fig. 2B**). In B cell development, after CD19 expression, most prominent steps are VDJ recombination and subsequential IgM expression on cell surface. In addition to CD19 expression, EBF1 is known to also essential for VDJ recombination especially V<sub>H</sub> to DJ<sub>H</sub> recombination (23). To determine whether miR-195 transduced *Ebf1*<sup>-/-</sup> cell rearranged the VDJ region, we attempt to detect V<sub>H</sub>-J<sub>H</sub> assembled gene segments in the engrafted mouse bone marrow cell by digital PCR. The data revealed that there were certain number of V<sub>H</sub>-J<sub>H</sub> segments in the bone marrow of mouse engrafted miR-195 transduced *Ebf1*<sup>-/-</sup> cells (**Fig. 2C**). 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 after 10 days from engrafted (**Fig. 2D**). Moreover, those IgM positive cells were also detected in splenocytes. These data suggested that engrafted cells had differentiated to IgM positive immature or mature B cells and has been recruited to spleen. The critical function of B cell is changing B cell receptor from IgM to IgG following class switch DNA recombination accompanied by cell proliferation by the stimuli. To clarify whether miR-195 transduced *Ebf1*<sup>-/-</sup> B cells have the function, whole splenocyte of the engrafted mouse 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



**Fig. 1.**

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

(A) Endogenous expression levels of miR-195 were measured by means of quantitative RT-PCR. RNAs were collected from the following hematopoietic lineage cells in 8-week-old C57BL/6 mice. KSL (c-kit<sup>+</sup> Sca1<sup>+</sup> Lin<sup>-</sup>); lymphoid-primed multipotent progenitors (LMPP: C-kit<sup>+</sup> Sca1<sup>+</sup> Lin<sup>-</sup> Flt3<sup>+</sup> IL7Rα<sup>+</sup>); common lymphoid progenitors (CLP: CD43<sup>+</sup> IL7Rα<sup>+</sup>); pro-B (B220<sup>+</sup> CD43<sup>+</sup> IgM<sup>-</sup>); pre-B (B220<sup>+</sup> CD43<sup>+</sup> IgM<sup>-</sup>); mature B (B220<sup>high</sup> CD43<sup>+</sup> IgM<sup>+</sup>); double-negative T (DN: CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup>); common myeloid progenitor (CMP: c-kit<sup>+</sup> Sca1<sup>-</sup> CD11b<sup>+</sup>); myeloid cell (Mac1: c-kit<sup>-</sup> Sca1<sup>-</sup> CD11b<sup>+</sup>). Data are shown as mean ± S.D. (n=3). (B) Flow cytometry analysis of control and miR-195-expressing Lin<sup>-</sup> 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). (C) Outline of the *in vitro* culture system of *Ebf1*<sup>-/-</sup> HPCs. (D) Flow cytometry analysis of control and miR-195-expressing *Ebf1*<sup>-/-</sup> HPCs. Shown data is representative of n=3. (E) Microarray analysis of miR-195-expressing *Ebf1*<sup>-/-</sup> HPCs. Log<sub>2</sub> 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.

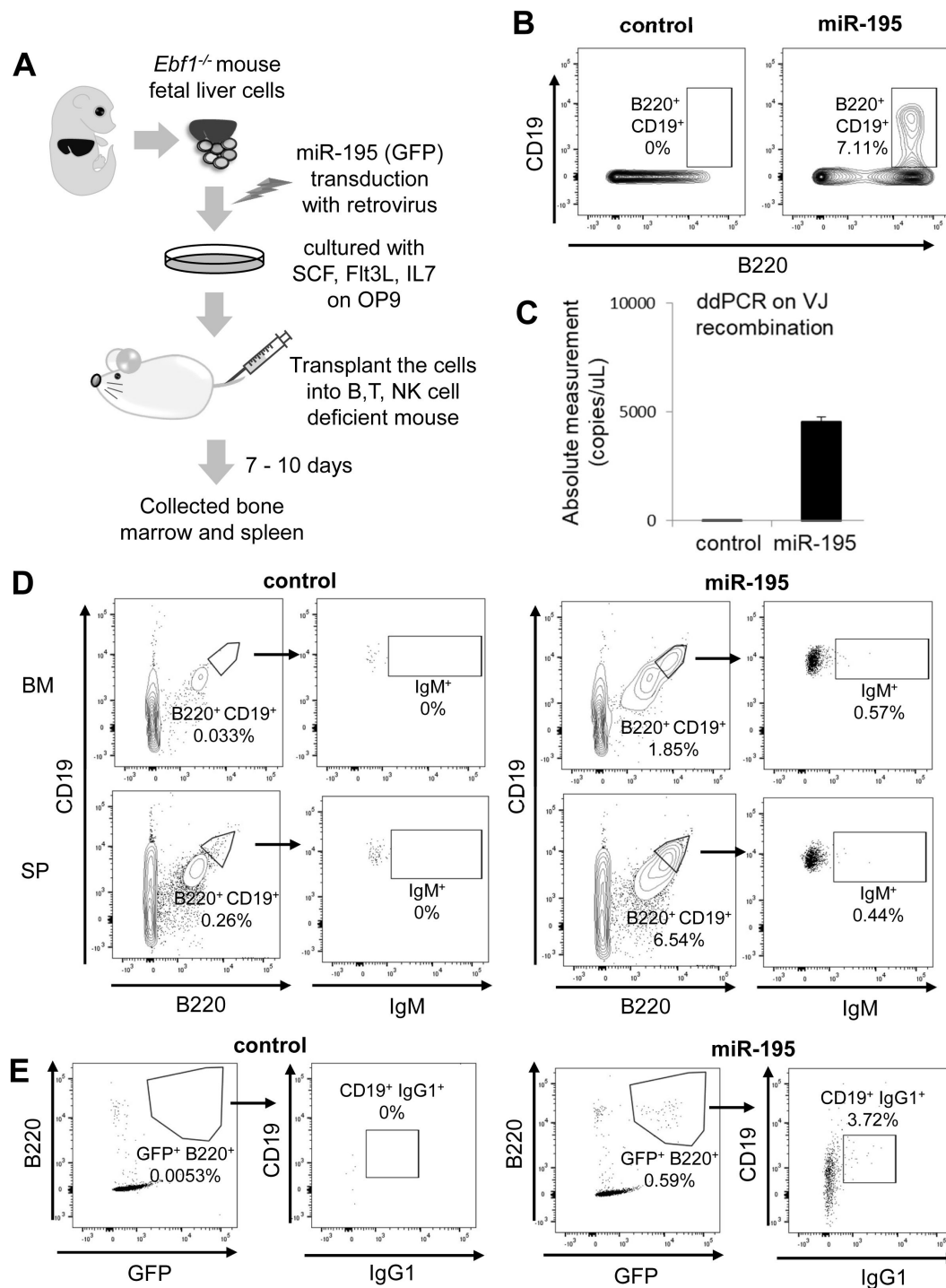
stimuli, miR-195 transduced GFP positive cells expanded enough to be surely detected and importantly a part of them expressed IgG1 (**Fig. 2E**). These data suggested that miR-195 has potential to induce B cell differentiation from HPCs to mature B cells to show class switch recombination even when critical 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 mouse in which the genome around miR-195-5p was eliminated by CRISPR/CAS9 system was established. The analysis of HPC lineage populations in the bone marrow revealed that several B cell related progenitor were relatively reduced in miR-195<sup>-/-</sup> mouse. Sca-1<sup>-</sup> c-kit<sup>+</sup> common myeloid progenitor cell population was increased but controversially, Sca-1<sup>+</sup> c-kit<sup>-</sup> (LSK) cells was decreased in miR-195<sup>-/-</sup> mouse (**Fig. 3A**). As LSK<sup>-</sup> 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<sup>+</sup> IgM<sup>-</sup> pre B cell populations was slightly increased in the BM of miR-195<sup>-/-</sup> mouse (**Fig. 3B**). In the splenic B cells, marginal zone B (MZB) cells was reduced in miR-195<sup>-/-</sup> mouse (**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<sup>-/-</sup> mouse, B-1 cells was 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. Together, those results were in line with the results obtained from ectopic expression of miR-195.

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

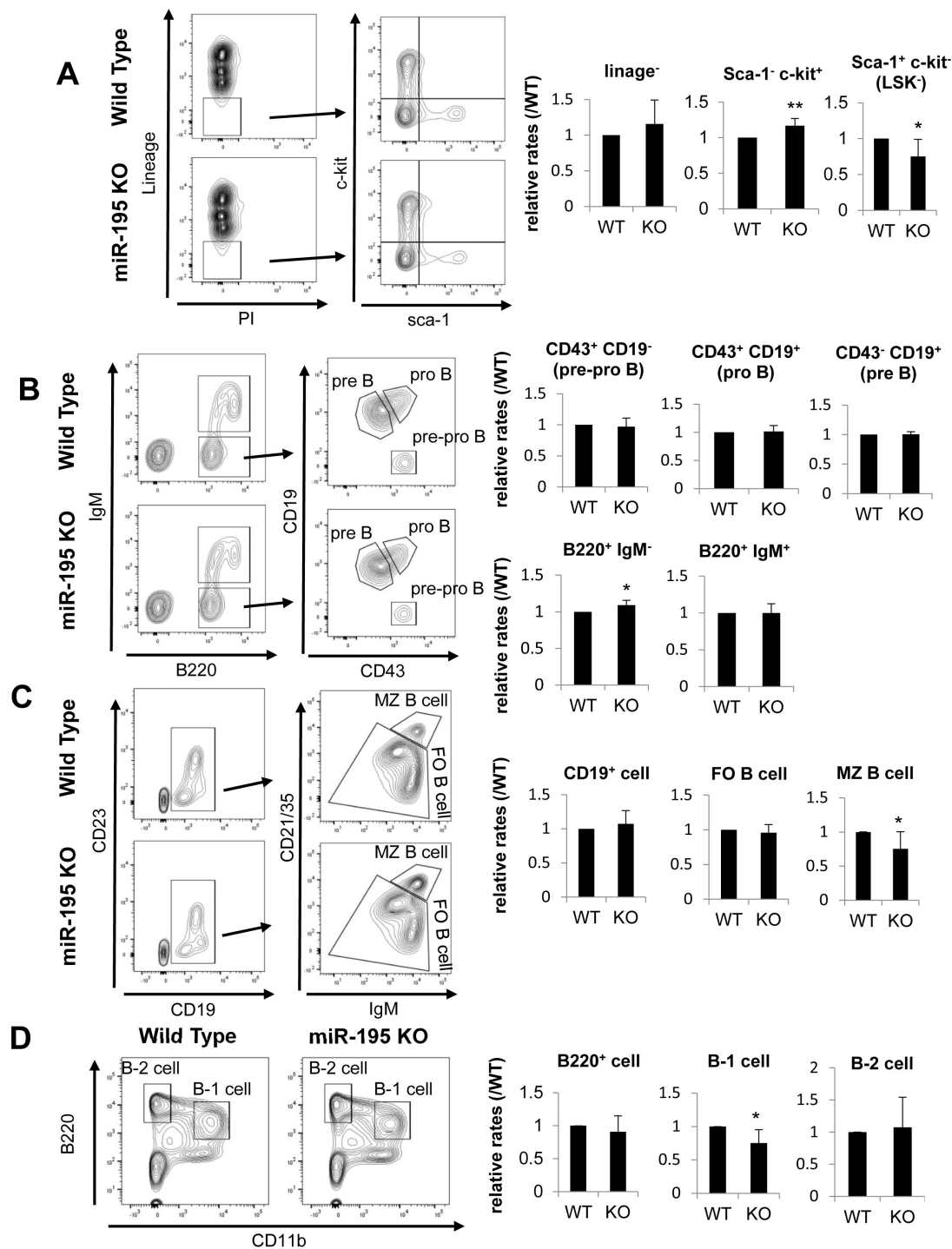
To elucidate how miR-195 promote B cell development in EBF1 deficient HPC, we analyzed regulatory networks of predicted miR-195 target genes by using starBase\_v2.0 and David Bioinformatics Resources 6.8 in KEGG pathway database (27–33). Several gene regulation networks were detected as candidates of responsible pathways on the miR-195 function. Remarkably, MAPK signaling pathway and PI3K-Akt signaling pathway included various targets of miR-195. Both MAPK and Akt were known to phosphorylate and degrade 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 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, although the luciferase activity was down-regulated by miR-195 transduction, 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*<sup>-/-</sup> HPCs and qPCR analysis showed that miR-195 transduction certainly decreased the mRNA levels (**Fig. 4B**). 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*<sup>-/-</sup> HPCs. The western blotting results revealed that miR-195 transduction decreased pFOXO1 levels and increased relative FOXO1 protein levels (**Fig. 4C**). Finally, to determine whether FOXO1 accumulation is enough to enable *Ebf1*<sup>-/-</sup> HPCs to differentiate into pro-B cells, *Ebf1*<sup>-/-</sup> HPCs were transduced *Foxo1* and cultured with the B cell differentiating condition. As a result, similar to miR-195 transduction, *Foxo1* transduction arose B220 and CD19 double positive *Ebf1*<sup>-/-</sup> cells 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*<sup>-/-</sup> HPCs to differentiate into B cell lineage.



**Fig. 2.**

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

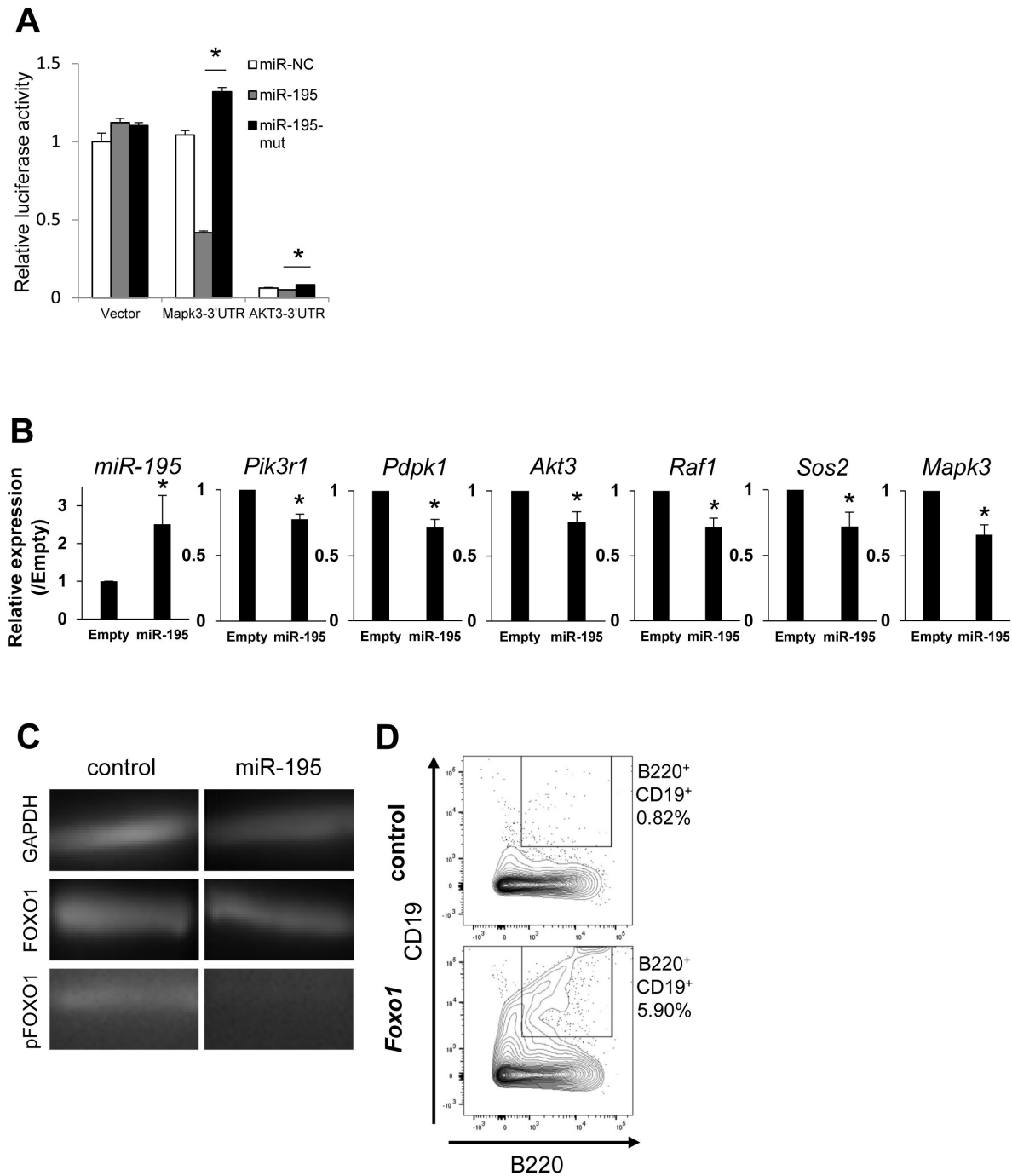
(A) *In vivo* analysis of B cell development of *Ebf1*<sup>-/-</sup> HPCs. (B) Flow cytometry analysis of control and miR-195-expressing *Ebf1*<sup>-/-</sup> HPCs in the bone marrow collected at 7 days after transplantation. (C) Using droplet digital PCR, VJ region fragments were amplified from the genomic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



**Fig. 3.**

### Several B cell populations are disturbed in the miR-195-deficient mouse.

Flow cytometry data of B cell lineage populations in miR-195<sup>-/-</sup> and littermate WT mice. Representative plots (left side) and mean  $\pm$  S.D. of relative population rates in each littermate WT mouse (right side) are shown. (A) Analysis of early B cell populations in the bone marrow. Pre-pro-B (B220<sup>+</sup> IgM<sup>-</sup> CD43<sup>+</sup> CD19<sup>-</sup>); pro-B (B220<sup>+</sup> IgM<sup>-</sup> CD43<sup>+</sup> CD19<sup>+</sup>); pre-B (B220<sup>+</sup> IgM<sup>-</sup> CD43<sup>-</sup> CD19<sup>+</sup>);  $n=5$ . (B) Analysis of hematopoietic progenitor populations in the bone marrow;  $n=5$ . (C) Analysis of B cell populations in the spleen. FO B (CD19<sup>+</sup> IgM<sup>+</sup> CD21/35<sup>low-middle</sup>); MZ B (CD19<sup>+</sup> IgM<sup>+</sup> CD21/35<sup>high</sup>);  $n=8$ . (D) Analysis of B cell populations in the peritoneal cavity: B-1 (B220<sup>+</sup> CD11b<sup>+</sup>); B-2 (B220<sup>+</sup> CD11b<sup>-</sup>);  $n=7$ . Statistical significance was tested using two-tailed Student's  $t$  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*<sup>-/-</sup> 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*<sup>-/-</sup> HPCs. Shown data is representative of *n*=3. (D) Flow cytometry analysis of control and *Foxo1*-expressing *Ebf1*<sup>-/-</sup> HPCs. Shown data is representative of *n*=3. Statistical significance was tested using two-tailed Student's *t* test. \**p*<0.05, *n*=3.

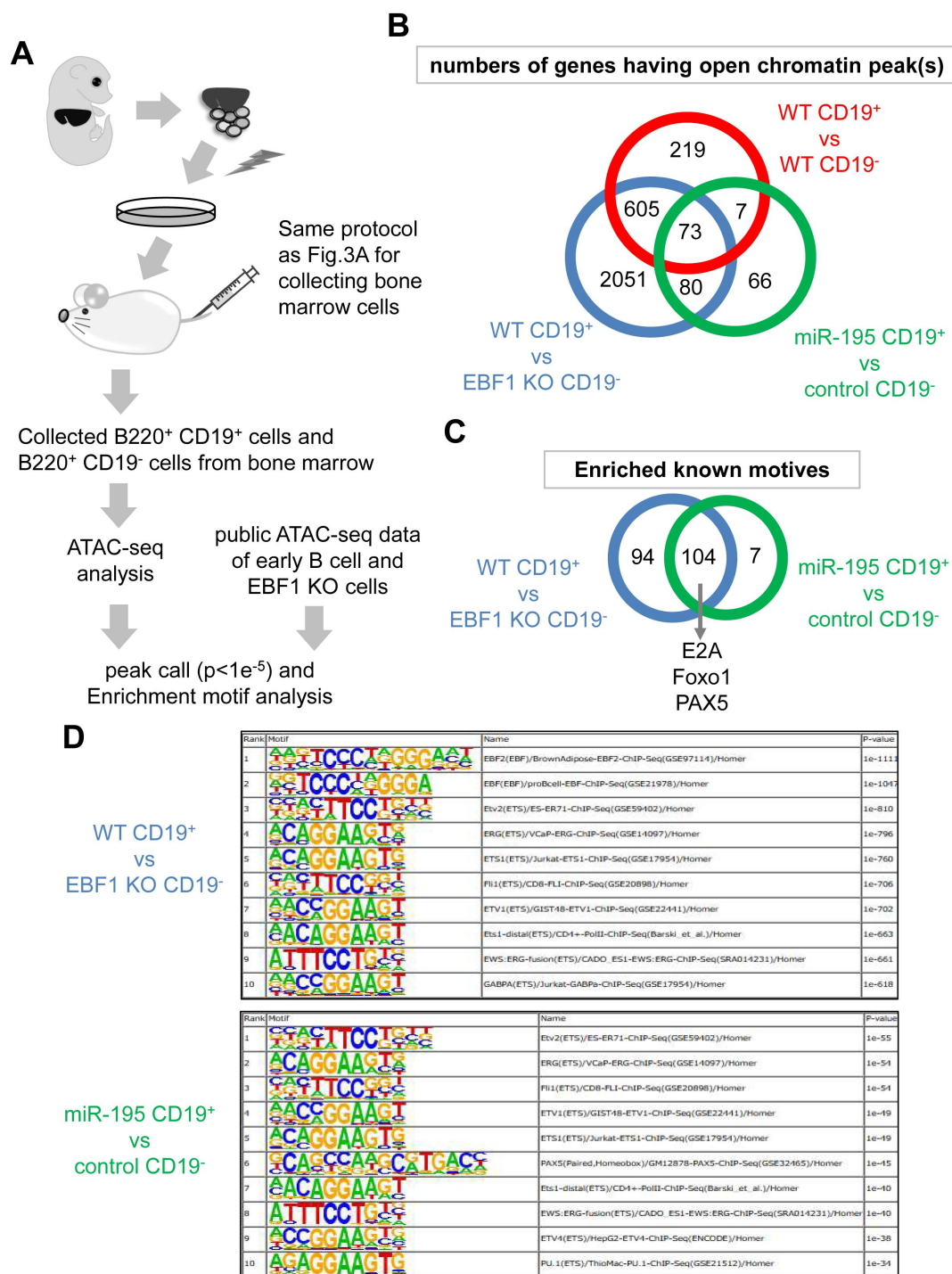
## Less genes are epigenetically activated in pro-B cells by miR-195 than EBF1

In B cell development, epigenetic changing of transcription factors and differentiation molecules is crucial for proper development, which is mainly regulated by EBF1 (35, 36). We investigated transposase-accessible chromatin using sequencing (ATAC-seq) data of *Ebf1*<sup>-/-</sup> pro-B cells and wild type pro-B cells from GSE92434 and cells in early B cell lineages from GSE100738. While wild type pro-B cells/ *Ebf1*<sup>-/-</sup> pro-B cells differentially accessible (DA) ATAC peaks were observed in 2809 sites, early B cells wild type CD19 positive/ CD19 negative 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, among them, some peaks were overlapped with miR-195 induced B220 and CD19 double positive *Ebf1*<sup>-/-</sup> cells(miR-195 CD19+) / B220 positive CD19 negative *Ebf1*<sup>-/-</sup> cells(control CD19-) DA ATAC peaks (73 out of 226 peaks) which were considered to be regulated by miR-195 (Fig. 5B). The 73 genes included *Pax5*, *Runx1*, *Erg*, *Irf8*, and *Blkl* which were well-known important genes for early B cell development and several B cell related genes for example, *Rarres1*, *Ciita*, *Atg7* (fig S1, S2). These results indicated that miR-195 opened less gene locus than EBF1 but several key locus for B cell differentiation were involved in them 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). The 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 by FOXO1 activation, 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 mouse often doesn't show significant phenotype (37). Nevertheless, miR-195 deficient mouse 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* mouse in which EBF1 was deficient in mature B cells (38). Therefore, miR-195 considered to have similar function with EBF1 to some extent. Considering that other miRNA deficient mice have subtle phenotype, and miR-195 is one of the large family, miR-15/16 and miR-195/497 (39), the remarkable potential of miR-195 is beyond a fine tuner as microRNA considered at least 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 were rapidly disappeared and couldn't be detected in transplanted mouse (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.



**Fig. 5.**

### ATAC-seq analysis of *Ebf1*<sup>-/-</sup> CD19-positive B cells differentiated by miR-195.

(A) Outline of analysis of open chromatin regions in miR-195-expressing *Ebf1*<sup>-/-</sup> 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*<sup>-/-</sup> pro-B cells (GSE92434; left-lower blue circle); B220<sup>+</sup> CD19<sup>-</sup> cells of control and B220<sup>+</sup> CD19<sup>+</sup> positive miR-195-expressing *Ebf1*<sup>-/-</sup> cells (right-lower green circle). (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).

## 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'-GGGGCGGAATTCGCTATTCGCCATAAGCA-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'-TAGGCCACAAAGGCAGGGACCTA-3' and 5'-AACTAGGTCCTGCCTTTGTGGG-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'-AGCUUCCUGGCUCUAGCAGCACAGAAUAUUGGCACAGGGAAGCGAGUCUGCCAAUAUUGGCUGUGCUGCUCCAGGCAGGGUGGUG-3' (mature miR-195-5p sequence 5'-UAGCAGCACAGAAUAUUGGC-3') was mutated to 5'-AGCUUCCUGGCUCUgcgccgACAGAAUAUUGGCACAGGGAAGCGAGUCUGCCAAUAUUGGCUGUcgcgccCAGGCAGGGUGGUG-3' (mature sequence 5'-UgcgccgACAGAAUAUUGGC-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<sup>-/+</sup> mouse was originally generated by R. Grosschedl (39). miR-195-deficient mice were generated based on the CRISPR/Cas9 system established by C. Gurumurthy (40), using pUC57-195sg-upstream and -downstream for sgRNA expression and pBKG (Addgene #65796) for Cas9 mRNA expression. 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<sup>-</sup>) cells from the fetal liver

Fetal livers were harvested from pregnant C57BL/6 or *Ebf1*<sup>+/-</sup> mice (mated with *Ebf1*<sup>+/-</sup> 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<sup>-</sup> 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<sup>-</sup> 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<sup>-</sup> cells were infected with the supernatants using 10 μg/mL Polybrene (Sigma-Aldrich). The infected and transduced Lin<sup>-</sup> 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). For *in vivo* analysis of B cell development of *EBF1*<sup>-/-</sup> Lin<sup>-</sup> cells, 1×10<sup>6</sup> cells were injected into the NOG or B6RG mice after >7 days of culture and expanded *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 75<sup>th</sup> percentile and transformed to the Log<sub>2</sub> 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*<sup>-/-</sup> 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*<sup>-/-</sup> Lin<sup>-</sup> 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<sup>+</sup> 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](https://www.microrna.org/)) and by comparing the microarray data of the targets in control- and miR-195-transduced Ebf1<sup>-/-</sup> 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<sup>TM</sup> qPCR RT Master Mix (TOYOBO). qPCR was performed using THUNDERBIRD<sup>TM</sup> SYBR<sup>®</sup> qPCR Mix (TOYOBO) on the StepOnePlus<sup>TM</sup> Real-Time PCR System (Thermo Fisher). The following primers were used for qPCR: Pik3r1 – 5'-AAACTCCGAGACACTGCTGA-3' and 5'-GAGTGTAATCGCCGTGCATT-3'; Pdk1 – 5'-CTGGGCTCTGCTCTAGTGTT-3' and 5'-CCCAGGTTTCAGGACAGGATT-3'; Akt3 – 5'-GTGGACCACTGTTATAGAGAGAACAT-3' and 5'-TTGGATAGCTCCGTCCACT-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<sup>™</sup> 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<sup>™</sup> MicroRNA Reverse Transcription Kit (Thermo Fisher) and a specific primer, 5'-UAGCAGCACAGAAAUAUUGGC-3'. The expression levels were measured using the TaqMan<sup>™</sup> Fast Advanced Master Mix (Thermo Fisher) on the StepOnePlus<sup>™</sup> Real-Time PCR System. 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<sup>-ΔΔCt</sup> method.

## Dual-Luciferase<sup>®</sup> 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<sup>®</sup> 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).

## ATAC-seq analysis

For ATAC-seq analysis, B220<sup>+</sup> CD19<sup>+</sup> and B220<sup>+</sup> cells were sorted from the bone marrow of NOG mice transplanted with miR-195-transduced EBF1<sup>-/-</sup> Lin<sup>-</sup> cells. B220<sup>+</sup> cells were also sorted from the empty transduced sample. The collected cells were resolved using CELLBANKER<sup>®</sup> (Takara Bio) and temporarily preserved at -20°C. ATAC-seq libraries were prepared from the cryopreserved cells according to the Omni-ATAC protocol (41). 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

Student's *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.

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## Author contributions

A.K. designed the research; Y.M., 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. and A.K. wrote the paper.

## Competing interests

The authors declare that they have no conflict of interest.

## Data and materials 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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## Editors

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## 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<sup>+</sup> cells in cells in which Ebf1, a critical B-cell transcription factor, has been genetically removed.

Strengths:

That over-expression of miR-195 can allow the emergence of CD19<sup>+</sup> 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.

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#### 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 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<sup>+</sup> 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.

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### 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<sup>+</sup> CD19<sup>+</sup> 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<sup>+</sup>CD19<sup>+</sup> 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<sup>+</sup>CD19<sup>+</sup> 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 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.

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 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.

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