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miR-191 modulates B-cell development and targets transcription factors E2A, Foxp1, and Egr1

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The interdependence of posttranscriptional gene regulation via miRNA and transcriptional regulatory networks in lymphocyte development is poorly understood. Here, we identified miR-191 as direct upstream modulator of a transcriptional module comprising the transcription factors Foxp1, E2A, and Egr1. Deletion as well as ectopic expression of miR-191 resulted in developmental arrest in B lineage cells, indicating that fine tuning of the combined expression levels of Foxp1, E2A, and Egr1, which in turn control somatic recombination and cytokine-driven expansion, constitutes a prerequisite for efficient B-cell development. In conclusion, we propose that miR-191 acts as a rheostat in B-cell development by fine tuning a key transcriptional program.

Keywords: B cells · Lymphocyte development · miRNA · miR-191 · Transcriptional factors

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Development of B cells from bone marrow derived progenitors as well as final differentiation of mature B cells are characterized by the execution of distinct and tightly controlled transcriptional networks. In consequence, dysregulation of these networks constitutes a frequent cause for generation of B lineage leukemia and lymphomas.

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In addition to a B-lineage determining transcriptional network comprising E2A (encoded by *Tcf3*), Ebf1, and Pax5, the temporally controlled action of other transcription factors, such as Egr1 and Foxp1 drives development of B cells from bone-marrow derived progenitors [1]. Developmental progression of B lineage cells is centered on somatic recombination of Ig heavy (IgH) and light (IgL) chain loci [2]. Expression of key elements of the recombination machinery, such as Rag1 and Rag2, is regulated by E2A, Ebf1, and Pax5 [3]. In addition, the transcription factor Foxp1 contributes to expression of Rag1 and Rag2 by binding to an enhancer region within the Rag locus [4]. Upon productive IgL rearrangement immature B cells undergo negative selection, which can be partially overcome by receptor editing, and finally exit from BM.

MicroRNAs (miRs) form an additional layer of regulation of B-cell development. Disruption of components of miRNA biosynthesis and concomitant total miRNA deficiency severely impairs B-cell development and differentiation [5]. Aberrant expression of miRNAs has also been linked to the development of B-cell malignancies. Thus, overexpression of miR-17~92, also termed oncomiR-1, resulted in lymphoproliferative disease and exacerbated B-cell lymphoma [6–9]. Furthermore, ectopic expression of miR-155 resulted in development of B-cell leukemia [10].

In the present study, we demonstrated that miR-191 targeted key transcriptional regulators of B-cell development and malignancy, E2A, Egr-1, and Foxp1, in mice and humans. Deletion as well as ectopic expression of miR-191 resulted in impaired early B-cell development, suggesting that expression of miR-191 is tightly controlled and serves to maintain exactly tuned transcriptional programs.

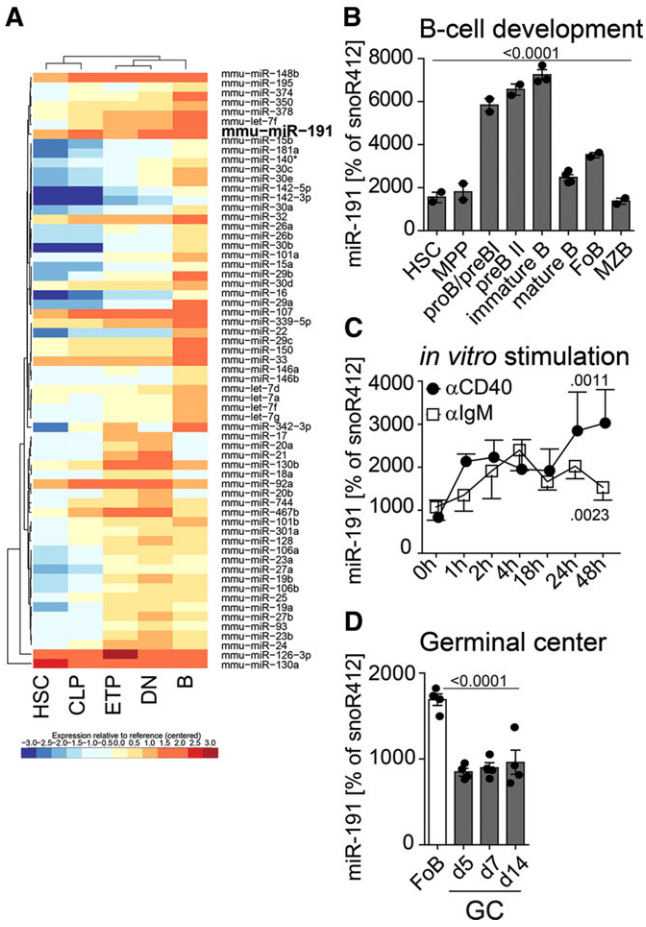

Figure 1. Expression of miR-191 is dynamically regulated during B-cell development and differentiation. (A) Unsupervised clustering of 63 miRNAs differentially expressed between HSC, common lymphoid progenitors (CLP), early T-cell rogenitors (ETP), double negative thymocytes (DN), and B-cell progenitors (B). (B–D) Expression levels of miR-191 relative to snoRNA412 determined by qRT-PCR: (B) in developing B cells, (C) in Fo B cells upon in vitro stimulation, (D) in GC B cells isolated at the indicated time points after immunization of mice with SRBC. Pooled data of four (B), three (C), two (D) independent experiments are depicted. Data are shown as mean relative expression + SD or +/− SD. Statistical analysis was performed using one-way ANOVA. Number above the graphs indicates *p* value.

Results

Expression of miR-191 is dynamically regulated during B-cell development and differentiation

We performed miRNA transcriptome analysis to identify miRNAs differentially expressed at the branching point of B- and T-cell development. Sixty-three miRNAs were differentially expressed between noncommitted and progressively committed progenitor cells of the B and T lineage (Fig. 1A). Validation by quantitative RT-PCR showed that expression of miR-191 was developmentally regulated in B cells reaching peak expression levels from proB to immature B cells followed by a decline toward mature B cells (Fig. 1B). In contrast, expression of miR-191 was about tenfold lower in early T-cell progenitors (ETPs) when compared to HSC and remained low during the course of intrathymic T-cell development (Fig. S1). This finding suggests that the thymic environment negatively regulated expression of miR-191. Next, we addressed whether expression of miR-191 was also regulated during activation and differentiation of B cells. Stimulation via the BCR resulted in a twofold upregulation of miR-191 after 1–2 h and remained elevated over a period of 48 h (Fig. 1C). Stimulation via CD40, mimicking T-cell interaction, had a similar but more pronounced effect. However, after immunization with SRBC, a thymus-dependent antigen, expression of miR-191 was reduced in germinal center derived B cells after extended periods of time (Fig. 1D). Taken together, dynamic regulation of miR-191 suggests that this miRNA constitutes a posttranscriptional regulatory factor of lymphocyte development and differentiation.

Defined expression levels of miR-191 are essential for efficient B-cell development

To understand the role of miR-191 in lymphocyte development we generated mice carrying a targeted deletion of miR-191 using CRISPR/Cas9 technology. To minimize the effect of genetic manipulation on neighboring loci we designed sgRNAs to cut only critical fragments of miR-191 stem-loop. We obtained two mouse lines, in

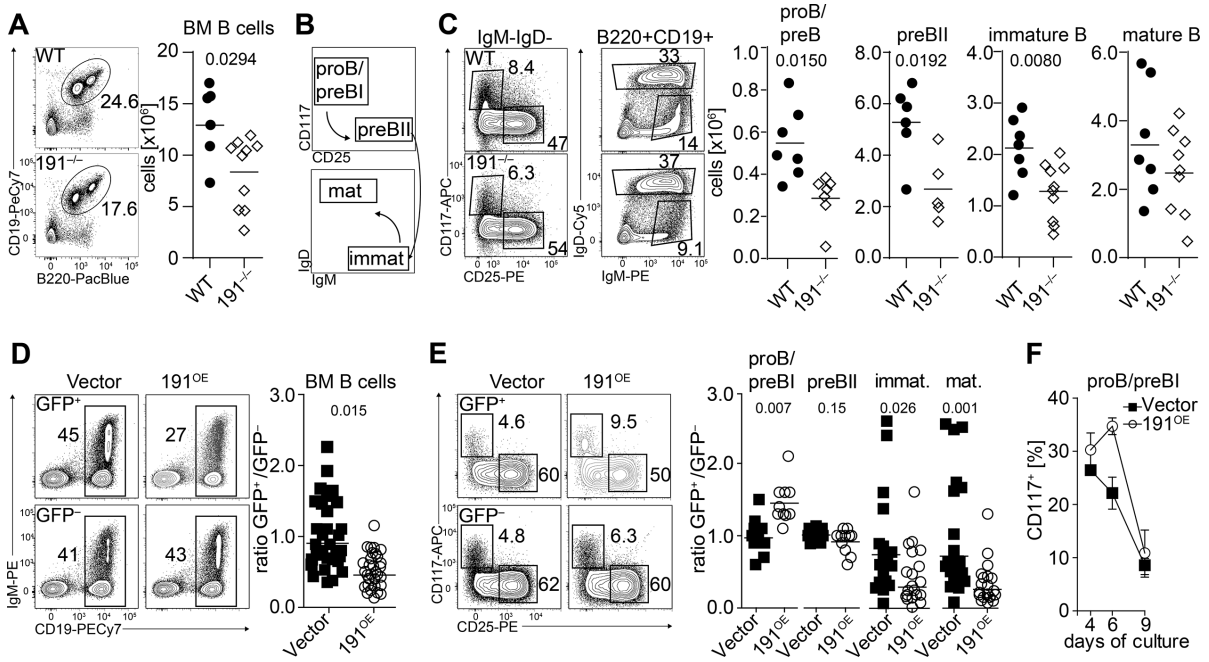

Figure 2. Defined expression levels of miR-191 are essential for efficient B-cell development. (A–C) Analysis of B-cell development in C57BL/6 (WT) and miR-191$^{-/-}$ mice (191$^{-/-}$). (A) Representative contour plots of bone marrow resident B cells indicating frequencies of B cells given as % of total nucleated cells (left) and total number of B cells (right). (B) Schematic gating strategy to identify proB/preBI, preBII, immature, and mature bone marrow B cells. (C) Representative contour plots of bone marrow resident B-cell stages from WT and 191$^{-/-}$ mice and quantification given as % of total nucleated cells (left) and total number (right). (D–F) Analysis of B-cell development of chimeric mice reconstituted with LSK cells transduced with either empty vector (Vector) or miR-191 overexpressing vector (191^{OE}). Approximately 50% of LSK cells were transduced with either of the vectors (GFP$^+$ cells), and remaining nontransduced (GFP$^-$ cells) served as competitor. Eight to ten weeks after transplantation cells were analyzed by FACS. (D) Representative contour plots of bone marrow resident B cells and quantification given as ratio of GFP$^+$/GFP$^-$. (E) Representative contour plots indicating development of early B cells upon overexpression of miR-191 (left) and quantification of two independent experiments with six mice per group (right). ProB/preBI cells were B220$^+$CD19$^{+/-}$CD117$^+$, preBII cells were B220$^+$CD19$^+$CD25$^+$, immature B cells were CD19$^+$IgM$^+$IgD$^-$ and mature were CD19$^+$IgM^{lo}IgD^{hi}. (F) Ex vivo development of proB/preBI cells cocultured with ST-2 stroma cells. Frequency of CD19$^+$CD117$^+$ cells is shown. Each dot represents mean value of three wells ±SD. Data shown are representative of two independent experiments. (A–E) Numbers next to the gates indicate percentages. Numbers above the plots indicate *p* values. (A, C–E) Each dot represents an individual mouse. Pooled data of three (A, C) or five (D, E) independent experiments with minimum three mice per group are shown. Statistical analysis was performed using unpaired Student's t test.

which either 36 bp or 7 and 15 bp of critical region were deleted resulting in complete loss of mature miR-191 (Fig. S2A). Of note, expression of *Daldr3* and *Ndufa3*, genes located in close proximity to miR-191 (< 2 kb), was not affected (Fig. S2B). Cellularity of BM, thymus and spleen from miR-191$^{-/-}$ mice was indistinguishable from littermate controls (Fig. S2C). Furthermore, T-cell development in these mice was comparable to that in WT littermates (Fig. S2D). However, loss of miR-191 resulted in a 1.4-fold reduction in frequencies and total number of B cells in BM (Fig. 2A). Analysis of BM B-cell subsets showed that B-lineage deficiency became apparent starting at the proB/preBI stage (defined as B220$^+$CD19$^+$CD117$^+$CD25$^-$), which represents the earliest subset of B-lineage committed progenitors (Fig. 2B, C). Of note, B-cell frequencies and total numbers recovered at the transition between the immature and mature stage in BM from miR-191$^{-/-}$ mice (Fig. 2C). Thus, the effect of deletion of miR-191 directly correlates with physiological expression levels of miR-191 in B-cell progenitors.

In a complementary approach we next addressed the function of miR-191 in lymphocyte development upon overexpression in competitive BM chimeras. Ectopic expression using a retroviral vector resulted in an approximately twofold overexpression of miR-191 in B lineage cells isolated from chimeric mice (Fig. S2E). Surprisingly, we observed a reduction in frequencies of bone marrow B cells by 40% within the subset of cells ectopically expressing miR-191 (Fig. 2D). At the same time, we did not observe any significant changes in developing T cells and myeloid cells (Fig. S2F and not depicted). Analysis of recipient mice after 8 weeks revealed no differences in B-cell ratios upon overexpression of unrelated miRNAs miR-126 and miR-22 (Fig. S2G). Next, we examined B-cell progenitors in BM of chimeric mice. We detected an accumulation of GFP$^+$ proB/preBI cells upon overexpression of miR-191 indicating a developmental block induced by miR-191 (Fig. 2E). Ratios of preBII cells in chimeric mice remained unaltered upon overexpression of miR-191, presumably due to compensatory effects (Fig. 2E). Furthermore, a significant (~twofold) reduction in both immature and postselection mature B cells overexpressing miR-191 was detected (Fig. 2E).

Accumulation of early B-cell precursors might be a result of delayed B-cell development or augmented proliferation of proB/preBI cells. To be able to distinguish between these alternative scenarios, we differentiated preBI precursors on ST-2 stromal

cells. Declining surface expression of CD117 served as signature marker for differentiation. Control cells lost CD117 more rapidly while not declining in numbers when compared to cells overexpressing miR-191. This observation further supported the notion that elevated expression of miR-191 induces a partial developmental arrest at the preBI to preBII cell transition (Fig. 2F). Taken together, these data show that neither complete loss nor even moderate overexpression of miR-191 are conducive to efficient early B-cell development. In contrast, development of other lymphocyte lineages is completely independent of miR-191.

Overexpression but not loss of miR-191 affect B cells in the periphery

Next, we tested whether perturbed development of B cells in the absence of miR-191 penetrates to the periphery. Frequencies and total number of splenic B cells were unaffected by loss of miR-191 (Fig. S3A). Given the dynamic regulation of miR-191 upon BCR stimulation and during germinal center formation we studied the response of miR-191$^{-/-}$ mice to SRBC, a well-characterized T-cell dependent model antigen. Seven and 14 days postimmunization both WT and miR-191$^{-/-}$ mice generated comparable numbers of germinal center B cells (Fig. S3B).

Analysis of peripheral lymphocytes from competitive overexpression chimeras revealed a reduction in frequencies of splenic B cells, equally affecting follicular B cells (Fo B) and marginal zone B cells (MZ B), upon overexpression of miR-191 (Fig. S3C). This finding is consistent with the observed miR-191-dependent retardation of BM B-cell development, given that T-cell development remained unaffected by overexpression of miR-191 (Fig. S2C). Antigen-specific B-cell expansion and class switch of B cells upon immunization of chimeras with the T-cell dependent antigen TNP-KLH as well as overall production of IgM and IgG by Fo B cells were unaffected by overexpression of miR-191 (Fig. S3D). Furthermore, stimulation of follicular B cells overexpressing miR-191 and respective control cells in vitro revealed a similar proliferation potential (Fig. S3E). These findings imply that once B-cell development is completed, elevated levels of miR-191 do not impair activation, proliferation, or class switch of mature B cells. Taken together, these results indicate that miR-191 controls development but not peripheral activity of B cells. Consistent with its expression pattern across the B lineage, ectopic expression, but not deletion of miR-191 resulted in developmental defects penetrating into the periphery.

Transcription factors Foxp1, Egr1, and E2A are bona fide targets of miR-191

The sequence of mature miR-191 is broadly conserved across species (Fig. S4A). Using the target prediction tools miRanda and TargetScan, we identified the transcription factors Egr1, Foxp1, and E2A as potential targets. All of them contain at least two conserved potential-binding sites for miR-191 (Fig. S4B–D) and

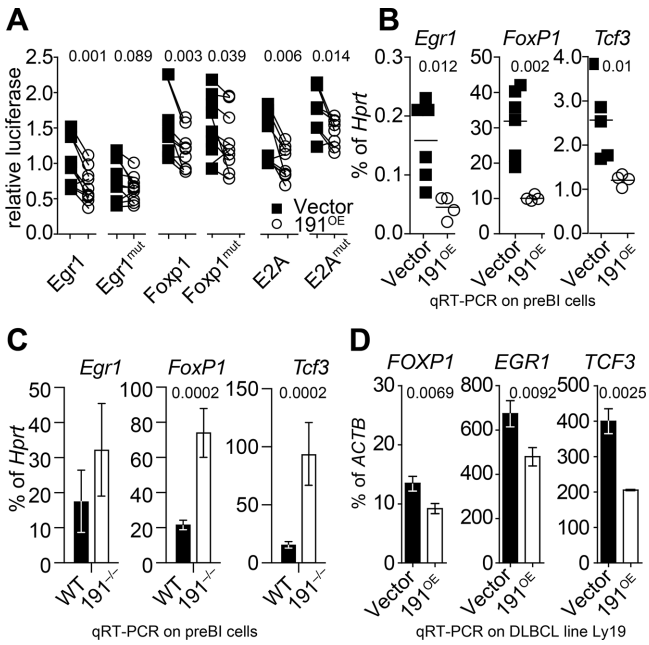

Figure 3. Transcription factors Egr1, Foxp1, and E2A are bona fide targets of miR-191. (A) Dual luciferase assay in 3T3 cells with 3'UTR of Egr1, Foxp1, and E2A and respective mutated 3'UTRs lacking miR-191 binding sites. Pairwise comparison of relative luciferase activity in control (Vector) and cells overexpressing miR-191 (191^{OE}). Pooled data of a minimum nine independent experiments are shown. (B–C) Relative expression of *Egr1*, *Foxp1*, and *Tcf3* (E2A) assessed by qRT-PCR in sorted preBI cells from (B) chimeric mice expressing Vector or 191OE vector or from (C) miR-191$^{-/-}$ mice (191$^{-/-}$) or littermate controls (WT). Expression levels are shown as percentage of *Hprt*. Each point represents a pool of cells from two to three mice. (D) Relative expression of *FOXP1*, *EGR1*, and *TCF3* in in vitro cultured DLBCL Ly19 line assessed after transduction with Vector or 191^{OE} vector. (B) Each dot represents data from an individual mouse. (C–D) Pooled data of two independent experiments (mean ± SD). Statistical analysis was performed using paired Student's *t* test. Numbers above the charts indicate *p* values.

sequences surrounding putative miR-191 binding sites in the 3'UTRs of *Egr1* and *Foxp1* were enriched in CLIP-Seq data obtained from naïve CD4 T cells [11]. To assess whether miR-191 directly targeted these transcription factors, we established 3T3 cell lines overexpressing miR-191 by using a retroviral vector that encodes miR-191 along with GFP (Fig. S4E). Dual-luciferase assays using psiCheck vectors encoding a luciferase cDNA fused to the respective 3′UTRs revealed that luciferase activity was lower in 3T3 cells that overexpressed miR-191 when compared to control cells (Fig. 3A). Deletion of candidate binding sites resulted in derepression of luciferase activity. Partial derepression in case of Foxp1 and E2A can be attributed to additional noncanonical-binding sites in the 3′UTR of those genes (Fig. S4B–D).

To validate that miR-191 was effectively repressing its targets Foxp1, Egr1, and E2A in vivo we ectopically expressed miR-191 together with GFP as reporter in hematopoietic progenitors and generated developing B cells by injection into irradiated mice. Ten days after reconstitution, we examined expression of Foxp1, Egr1, and E2A in FACS-sorted proB/preBI subsets by qRT-PCR. Ectopic expression of miR-191 resulted in a two to threefold decrease in expression of all three transcription factors (Fig. 3B). In contrast,

expression of other key mediators of B-cell development, such as Ebf1 and Pax5, was not affected by ectopic expression of miR-191 (Fig. S4F). Conversely, expression levels of Foxp1 and E2A were elevated in preBI cells isolated from miR-191$^{-/-}$ mice when compared to WT controls, whereas a trend toward, but no statistically significant elevation of expression of Egr1 was observed (Fig. 3C). The high degree of conservation of miR-191 across species (Fig. S4A) and preserved miR-191-binding sites in the 3'UTRs of the three target genes (Fig. S4B–D) implied a functional relevance for miR-191 in human B cells. Consistent with our data from murine hematopoietic cells, expression of the three target genes *FOXP1*, *EGR1,* and *TCF3* was decreased upon overexpression of miR-191 in Ly19 DLBCL cells (Fig. 3D). Collectively, these data indicate that Foxp1, Egr1, and E2A are conserved targets of miR-191.

miR-191 regulates V(D)J recombination and IL-7-dependent expansion of preBI cells

Next, we addressed the molecular mechanisms through which miR-191 modulates B-cell development. First we tested the hypothesis that *Igh* rearrangement, which occurs prior to the preBI–preBII transition, might be impaired upon overexpression of miR-191. To this end, we assessed expression of *Rag1*, *Rag2,* and *Dntt* (encoding TdT), which constitute key components of the recombination machinery. Expression of these genes, but not other components of the recombination machinery, such as *Xrcc6* and *Lig1*, in preBI cells was substantially decreased upon overexpression of miR-191 (Fig. 4A and Fig. S5A). Furthermore, expression of surrogate light chain components was not affected (Fig. S5B). Direct analysis of V-to-DJ$_H$ rearrangements in preBI cells revealed that the occurrence of complete V-DJ rearrangements in miR-191 overexpressing preBI progenitors was substantially lower than in controls (Fig. 4B, Fig. S5C), irrespective of the V gene position in the *Igh* locus.

Clonal expansion and developmental progression at the preBI stage are driven by IL-7 signaling. We observed reduced expression of IL-7Rα (CD127) on preBI cells overexpressing miR-191 (Fig. 4C). To test whether this reduction affected expansion of B-cell progenitors, purified preBI cells were cultured for ten days in methylcellulose supplemented with IL-7. Expansion of preBI cell colonies was reduced by approximately 2.5-fold upon miR-191 overexpression (Fig. 4D).

In summary, our findings indicate that elevated levels of miR-191 impair development of early B-cell progenitors by inhibiting *Igh* recombination as well as by reducing their responsiveness to IL-7.

Overexpression of E2A and Foxp1 rescue the developmental block induced by miR-191

Next, we directly assessed the interdependence of miR-191 and transcription factors E2A and Foxp1. To this end, we generated

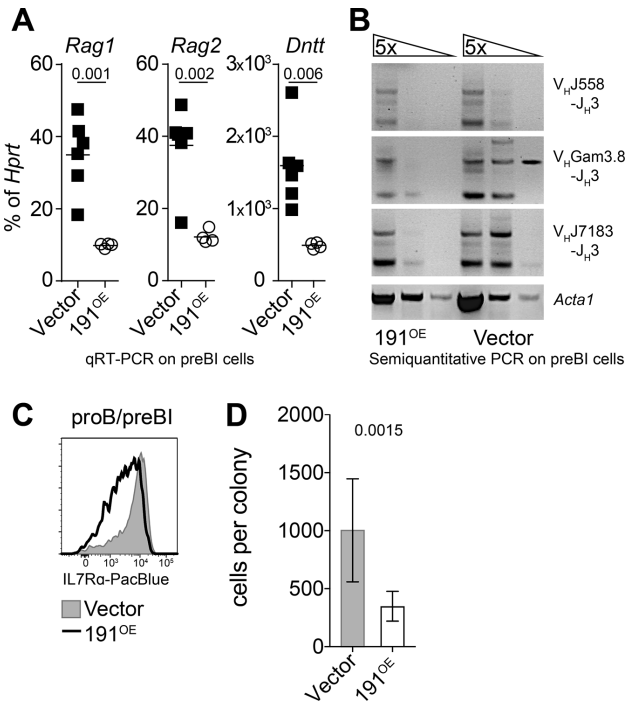

Figure 4. miR-191 regulates V(D)J recombination and IL-7-dependent expansion of preBI cells. (A) Relative expression of *Rag1*, *Rag2,* and *Dntt* in proB/preBI cells isolated from chimeric mice assessed by qRT-PCR. Expression levels are shown as percentage of *Hprt*. Pooled data of two individual experiments. Each point represents a pool of sorted cells from two to three mice. (B) Semiquantitative PCR analysis of genomic DNA for V_HJ558, $V_HGam3.8$, or $V_HJ71833$ to J_H3 rearrangements from sorted proB/preBI cells. Expression of recombination-independent *Acta1* serves as loading control. Data shown are representative of 3 independent experiments. (C) FACS analysis of IL-7Rα (CD127) expression on surface of proB/preBI cells isolated from Vector and 191^{OE} bone marrow chimeras. Representative histogram of two independent experiments is shown. (D) Sorted proB/preBI cells from Vector and 191^{OE} chimeras were cultured in methyl cellulose supplemented with 10 ng/mL of IL-7. After ten days colonies were harvested and the number of cells per colony was assessed by flow cytometry. Pooled data of two independent experiments are shown ($n = 46$ and $n = 49$), indicating median cellularity and 95% CI. (A, D) Statistical analysis was performed using unpaired Student's *t* test. Numbers above the plots indicate *p* values.

chimeras with cells cooverexpressing miR-191 and its targets E2A or Foxp1. Cells overexpressing miR-191, its targets or both could be detected based on GFP, hCD25 or both reporter genes, respectively. In order to avoid development of leukemia by enforced expression of E2A, we assessed B-cell development 10 days after transfer of BM precursors. B-cell development in these short-term chimeras was arrested at the same developmental check point as in long-term chimeras upon ectopic expression of miR-191 resulting in an even more pronounced accumulation of proB/preBI precursors (Fig. 5A). Development of preBII cells was delayed in short-term chimeras, an effect that was presumably compensated by the increased pool of proB/preBI cells at steady state in long-term chimeras. Ectopic expression of E2A alone strongly promoted B-cell development indicated by increased frequencies of preBII cells at the expense of the preBI subset (Fig. 5B). Interestingly, combined expression of E2A and miR-191 resulted in a distribution of preBI and preBII subsets comparable to that

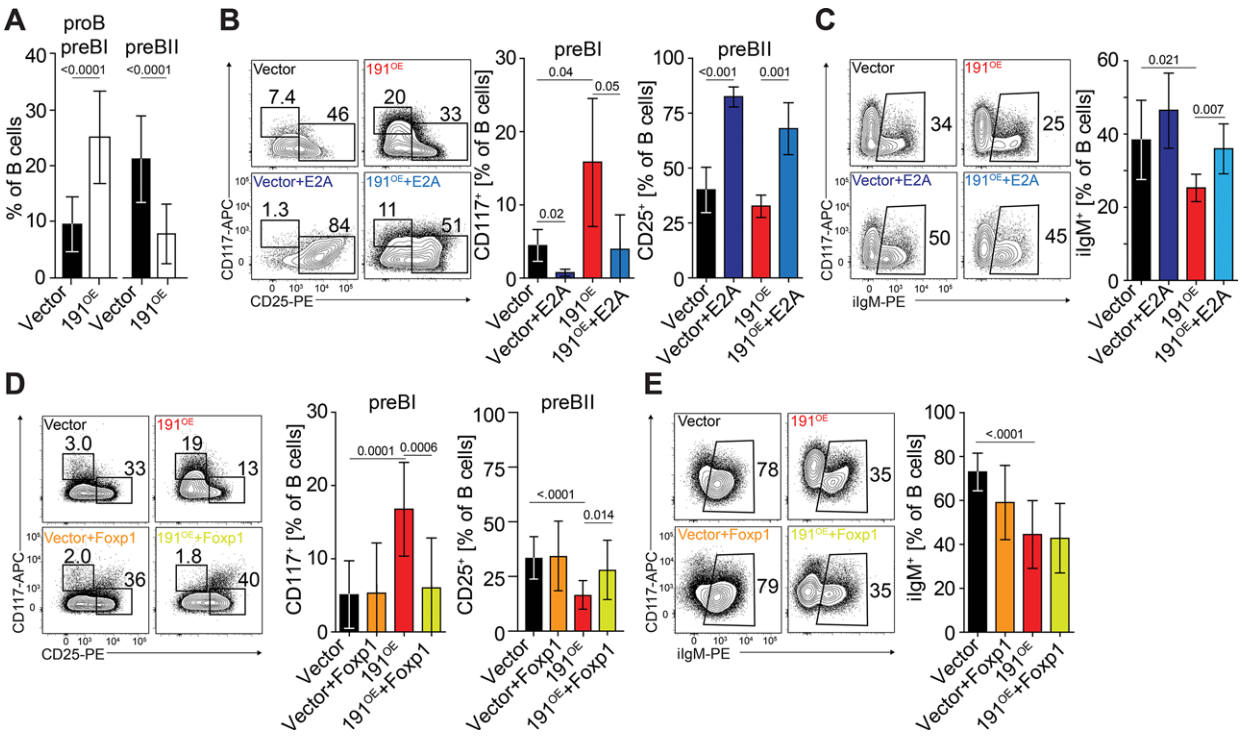

Figure 5. Overexpression of E2A and Foxp1 rescue the developmental block induced by miR-191. (A) LSK precursors were transduced with Vector or 191^{OE} vector and transfer red into lethally irradiated recipient mice. Development of early B-cell progenitors was assessed ten days after transplantation. Representative data of five independent experiments are shown. (B) Similar to A, LSK precursors were transduced with Vector or 191^{OE} vectors with or without E2A vector and transfer into lethally irradiated host. Ten days after transplantation development of proB/preBI and preBII precursors was assessed by FACS. (C) Bone marrow cells of chimeric mice depicted in (B) were fixed and intracellularly stained for IgM. Representative contour plots of B-cell progenitor. (D–E) Analog to B and C but replacement of E2A with Foxp1 vector. (B–C) Representative contour plots of early B-cell progenitors (left panel) and statistical analysis of one of three independent experiments (right panel) is shown ($n = 4$, average + SD). (D–E) Representative contour plots of early B-cell progenitors (left panel) and statistical analysis of two independent experiments (right panel) is shown ($n = 10$–12, average + SD). Statistical analysis was performed using unpaired Student's *t* test. Numbers above the charts indicate *p* values.

observed in control chimeras (Fig. 5B). Furthermore, coexpression of E2A and miR-191 rescued expression of intracellular IgM, indicating that restoration of E2A expression is sufficient to overcome miR-191-induced inhibition of *Igh* rearrangement (Fig. 5C). Ectopic expression of Foxp1 alone did not result in alterations in B-cell development in short-term chimeras (Fig. 5D). However, coexpression of Foxp1 and miR-191 was able to specifically overcome the B-lineage developmental block induced by ectopic expression of miR-191 alone (Fig. 5D). Of note, expression of Foxp1 did not restore *Igh* rearrangement (Fig. 5E). In summary, we conclude that coexpression of E2A as well as Foxp1 were sufficient to rescue early B-cell development in cells ectopically expressing miR-191, albeit in part via distinct mechanisms. These data establish that miR-191 acts opposingly to E2A and Foxp1 and, together with in silico prediction, luciferase assays and expression analyis, suggest that both transcription factors are functionally relevant targets of miR-191.

Discussion

In this study, we have identified miR-191 as an upstream regulator of key B lineage transcription factors, E2A, Foxp1, and Egr1. We demonstrated that, paradoxically, both deletion as well as elevated levels of miR-191 prevented efficient developmental progression prior to and at the pre-B cell stage. Thus, our data suggest that miR-191 acts as a rheostat to create a tight window of transcription factor expression to robustly execute the B lineage developmental program. This notion is consistent with earlier studies investigating B-cell development in models with graded levels of transcription factor expression. Thus, lowering the gene dosage of E2A in heterozygous gene-targeted mice affects B-cell development by reducing expression of the E2A targets *Rag2* and *mb-1* [12]. Concomitant heterozygosity for E2A and Ebf1 results in a virtually complete block in B-cell development, underscoring the synergistic function of these transcription factors. Although no gene dosage effect of Foxp1 on B-cell development has been described, at least part of its function overlaps with that of E2A. Both transcription factors bind to the Erag enhancer and control expression of Rag genes [4, 13]. Interestingly, expression of all three transcription factors identified as targets was substantially increased or downregulated in preBI cells deficient in miR-191 or ectopically expressing miR-191, respectively. In contrast, luciferase assays indicated only a rather modest effect of miR-191 on posttranscriptional modulation via the 3'UTRs. Although these assay systems cannot be compared directly, our data suggest that miR-191 may

interfere with positive feedback regulation of at least some of its target transcription factors. Furthermore, we noticed that the substantial repression of target mRNA showing two- to threefold lower levels resulted in comparatively moderate effects. Thus, the developmental block observed here is of a similar magnitude, if not smaller, than heterozygous deletion of E2A alone [12].Given a miRNA's propensity to act on multiple targets simultaneously this apparent discrepancy might be explained by engagement of other, yet to be identified, targets that might account for compensatory effects. That such effects might play a role here is supported by our finding that in short-term chimeras the B-lineage developmental block is stronger when compared to the steady-state in long-term chimeric mice. In addition, still comparatively little is known about complex gene regulatory networks and the direct interplay of multiple transcription factors. Combined ChIPseq analysis has only recently resulted in identification of regulatory networks and new modes of regulation, such as redirection and capture of transcription factors by competing factors, are only beginning to emerge [14, 15]. Ultimately, we cannot exclude that expression of Foxp1, E2A, and/or Egr1 mRNA does not completely reflect protein expression, which we were not able to directly test due to a lack of suitable reagents. Such divergence might occur in case of uncoupling of transcription and translation due to posttranscriptional mechanisms or posttranslationally due to targeted degradation or selective stabilization of protein. Such means of selective regulation on the protein level have not been reported for the three transcription factors in question. Nevertheless, restriction of expression analysis to mRNA levels represents a limitation to our study.

In conclusion, targeting of this transcriptional module by miR-191 is well in line with a proposed role of miRNAs in fine-tuning and stabilizing transcriptional networks or signaling pathways. The rescue of B-cell development by cooverexpression of E2A and miR-191 supports this model. Of note, whereas rescue by E2A restored pre-BCR expression it did not affect expression levels of IL-7R, which is line with previous reports [12]. The contribution of Egr1 as a third target of miR-191 identified in this study is more difficult to assess. Whereas the role of Egr family members in B-cell development has been well established through transgenic expression of dominant-negative Egr mutants, deletion of individual family members including Egr1 had no apparent effect on B-cell development. These data suggest that other Egr family proteins can compensate for loss of Egr1 [16, 17]. Egr proteins function downstream of BCR signaling [18]. Thus, it is conceivable that miR-191 not only acts upstream of pre-BCR signaling via regulation of somatic recombination but also downstream by limiting the expression of Egr1.

Cooperativity of transcription factor action and thus the formation of transcription factor coregulatory networks has been well established at the level of loss-of-function mutations and cotargeting of critical effector genes of B-cell development [14]. In contrast, little is known about the consequences of compound overexpression of transcription factors in the course of B-cell development. An increase in Foxp1 expression alone due to downregulation of miR-34 did not abrogate B-cell development but rather displayed a B-lineage promoting function [19]. In contrast, deletion of miR-191 paradoxically showed a similar phenotype as overexpression of the same miRNA. Analysis of miR-191-deficient developing B cells revealed elevated expression of Foxp1, E2A, and Egr1, which was consistent with overexpression data, but cannot be easily reconciled with the phenotype observed. To date, combined transcription factor action is difficult to analyze quantitatively and combined ectopic expression of transcription factors acting in parallel is usually not analyzed. Thus, there is currently no precendence for a combined negative effect of individually positively acting transcription factors. Combined ChIPseq experiments might provide insight into such scenarios. In addition, we cannot exclude that although overexpression and deletion of miR-191 result in corresponding changes in expression of the three targets identified here, other targets yet to be identified might act predominantly upon deletion of miR-191. Accordingly, it has recently been proposed for miR-17~92 that not all targets react equally sensitive to deletion or overexpression of the miRNA cluster [20].

Upon ectopic expression of miR-191 developing B-cell precursors displayed limited responsiveness to survival cues from the IL-7R as well as reduced expression levels of components of the recombination machinery resulting in impaired IgH rearrangement. Computational analysis indicated that the 3'UTRs of *Rag1*, *Rag2,* and *Dntt* (encondingTdt) lack candidate-binding sites for miR-191, suggesting that reduced expression of these genes is likely to be an indirect effect. In fact, it has been shown that both E2A and Foxp1 transcriptionally regulate Rag genes by binding to the Erag enhancer region [19, 21, 22]. In conclusion, we propose that miR-191 is at the top of a hierarchy of transcriptional control of the recombination machinery comprising the transcription factors E2A and Foxp1 as key intermediates. Interestingly, restoring expression of Foxp1 alone did not rescue somatic recombination, suggesting that repression of E2A by miR-191 plays a dominant role in this regulatory network.

Expression levels of miR-191 increased with developmental progression toward B cells, but also substantially decreased once progenitors entered the T lineage. These findings are in good agreement with an earlier study describing the overall dynamics of miRNA expression during hematopoiesis [23]. Therefore, it was somewhat surprising that ectopic expression of miR-191 did not influence T-cell development to any detectable degree. Our data suggest a model, in which miR-191 downmodulates E2A and Foxp1, thus limiting IgH rearrangement. Interestingly, both transcription factors regulate Rag expression by binding to an enhancer element (Erag), which is only required for optimal Rag gene expression in cells of the B lineage but not in T lineage cells [24]. Furthermore, Foxp1 has no apparent function during early T-cell development. Thus, the effect on Foxp1 may best explain why ectopic expression of miR-191 constrains B cell but not T-cell development. In addition, it is possible that alternative transcripts of E2A required at different stages of B and T-cell development are selectively targeted by miR-191 [25–28]. Further work is required to test this hypothesis.

Materials and methods

Mice

All animal experiments were conducted in accordance with local and institutional regulations (Nds. Landesamt f. Verbraucherschutz und Lebensmittelsicherheit, 33.14-42502-04-08-1480, −11-0533, −14/1431). C57BL/6J and NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1}Wjl/SzJ (NSG) mice were purchased from Charles River or bred at the animal facility of Hannover Medical School. Animals were maintained under specific-pathogen-free conditions. To generate miR-191$^{-/-}$ mice two single guide RNAs (sgRNAs) targeting miR-191 locus were designed with an online CRISPR design tool (http://crispr.mit.edu/) to disrupt hairpin structure and minimize off-target effects. sgRNAs were generated via in vitro transcription based on previously published protocol [29]. Briefly, T7 promoter sequence was added to the forward primers containing sgRNA sequence. PCR was performed with T7-sgRNA forward primer (5′-TAATACGACTCACTATAGGG N20GTTTAAGAGCTATGCTGGAAACAGC-3′) and reverse primer (5′-AAAAAAAGCACCGACTCGGTG-3′), using the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene #42230, a gift from Feng Zhang) [30] as template, to generate a 133bp PCR product. The product was purified and in vitro transcribed (MEGAshortscipt T7 Transcription Kit, Thermofisher Scientific). Two T7-sgRNAs and Cas9 Nuclease (IDT) were combined to generate an RNP complex. Microinjection of embryos was performed as previously described [31].Two mouse lines carrying a 36 bp or 7 + 15 bp deletion in the miR-191 locus were established. Deletions resulting in disruption of miR-191 hairpin structure in both founder lines were confirmed by Sanger sequencing after purification of PCR product using (forward) AACACCTACTCCTTCCTACTCAGC and (reverse) CTTCAGAGAGAGGACCCAGGACC primers. Quantitative RT-PCR with primers recognizing mature miR-191 showed that both deletions in miR-191 locus resulted in essentially complete absence of miR-191. To minimize off-target effects founder F0 mice were bred to heterozygocity with wild-type C57BL/6 mice. miR-191$^{-/-}$ mice were maintained under specific-pathogen-free conditions at Central Animal Facility of LMU. To exclude off-target deletions potential targeting sites were assessed using the MIT online webtool. Five sites with the highest score were selected and subjected to Sanger sequencing using the following primer sets:

191-T1-OT1-F-GTTGCTGAGCATGGCCATCTTTC	365bp
191-T1-OT1-R-CTACCAGGGACACACAGGTCTG	
191-T1-OT2-F-GCTGAGGGTGGTGTGTTCTTTG	284bp
191-T1-OT2-R-TAACGTGGAGTTCTGGTTGGC	
191-T1-OT3-F-ACCCTTTTCCGTCTTTAGCCCA	426bp
191-T1-OT3-R-AGCATCATACTGATCCCCCAGC	
191-T1-OT4-F-AGGTGGGAAACAGGTTAAGGGG	341bp
191-T1-OT4-R-GCCCCCTCTACCCATCTCATTC	
191-T1-OT5-F-ACTGCTTCCTCTCTGCTTCCTG	351bp
191-T1-OT5-R-GCTGGAAGCCCTCAACAAGTTT	
191-T2-OT1-F-AGAAACGGCCTCCACTTACCTC	391bp
191-T2-OT1-R-CTCTGATATGGTCTCTGGGGCG	
191-T2-OT2-F-TTGCTGTTGACTGGAGCATTGC	305bp
191-T2-OT2-R-GACACACTTAGTCCACCAGGCT	
191-T2-OT3-F-TTTCCGAAAACAGAGTGCGCTC	283bp
191-T2-OT3-R-AAGGTGGAGGCGATCTGTTTCA	
191-T2-OT4-F-GCTGCTAAGCCAGTAGTGTCTG	363bp
191-T2-OT4-R-CCTGTGCTTGGCATGTCTGTTT	
191-T2-OT5-F-AGCTTTTTGTGCCTGAACCTCG	257bp
191-T2-OT5-R-TCTTCCTCTCCTCCCTGACACT	

Bone marrow chimeras

Bone marrow chimeras were established as previously described [32]. Irradiated C57BL/6 mice (9 Gy) were analyzed 10 days or 8–10 weeks after reconstitution.

Purification of lin$^-$ BM cells and enrichment of developing thymocytes

Lin$^-$ cells were isolated from total BM by staining cell suspensions with a lineage-specific antibody cocktail including TCRβ, CD19, CD11b, CD11c, Gr-1, Ter-119, and NK1.1, all eBioscience or BioLegend (followed by incubation with sheep anti-rat IgG conjugated to magnetic beads (Dynal, Invitrogen) and magnetic bead depletion of mature lineages. DN thymocytes were enriched by complement lysis of DP and SP cells using anti-CD4 and anti-CD8 antibodies (clones RL1.72 and 31M) and incubation with LowTox-M rabbit complement (Cedarlane) followed by gradient separation using Lympholyte M (Cedarlane).

Cloning and production of retroviruses

HEK293T cells were transfected using the Calcium Phosphate Method (CalPhos, Clontech). As a packaging plasmid pCLEco (coexpressing MLV gag, pol and env) was used. MDH1-PGK-GFP_2.0, a gift from Chang-Zheng Chen [33], served as empty vector control (Addgene plasmid # 11375) and was used to generate MDH1-PGK-GFP-2.0-miR-191 and MDH1-PGK-GFP-2.0-miR-126 containing pre-miRNAs and flanking regions of miR-191 and miR-126, respectively, under control of an H1 promoter. pHuE47TAC – encoding human E47 cDNA in combination with an IRES-linked hCD25 reporter [34, 35] and MSCV-mFoxp1-IRES-Thy1.1 were based on Addgene plasmids # 17442 and # 35170. MSCV-IRES-Thy1.1 DEST was a gift from Anjana Rao (Addgene plasmid # 17442) [36]; pCMV10-mFoxp1 was a gift from Benjamin Blencowe (Addgene plasmid # 35170) [37].

Transduction of cells

For competitive bone marrow chimeras, bead-enriched lin$^-$ bone marrow cells were cultured overnight in complete α-MEM medium

(Life Technologies) supplemented with 10% heat inactivated FCS (GE Healthcare), 50 U/mL and 50 μg/mL Penicillin-Streptomycin, 1 mM Sodium Pyruvate (both Life Technologies), 60 μM β-mercaptoethanol and SCF (50 ng/mL), IL-7 (25 ng/mL), Flt3L (25 ng/mL) and IL-6 (20 ng/mL) (all cytokines from Peprotech). Spin infections were conducted with retrovirus supernatants in 24 well plates in the presence of 8 μg/mL of polybrene (Sigma Aldrich). For short-term and most long-term chimeras, $lin^-Sca\text{-}1^+CD117^{hi}$ (LSK) cells were sorted from lineage-depleted BM and cultured overnight in complete α-MEM medium supplemented with SCF (50 ng/mL), IL-7 (25 ng/mL), Flt3L (25 ng/mL), and IL-6 (20 ng/mL). LSK cells were then transferred into 96-well plates (Sarstedt) preloaded with retroviral vector attached to RetroNectin (Takara) according to the manufacturer's protocol.

Immunization

Germinal center B cells ($CD95^+GL7^+$) were sorted from spleen on day 5, 7, and 14 after induction by i.v. injection of 200 μL PBS-washed SRBC suspension (Acila). For ELISPOT analysis of class switch recombination, mice were immunized i.p. with 200 μL TNP-KLH (Biosearch Technologies) emulsified in Complete Freund's Adjuvant (Sigma Aldrich). After 7 and 14 days splenic B cells were analyzed for TNP specific IgM^+ or IgG^+ B cells. Transduced and nontransduced cells were seeded onto plates coated with TNP. Cells were incubated overnight at 37°C in 5% CO_2. After washing, biotin-conjugated rat anti–mouse IgM (LO-MM-9; AbDSerotec) or goat-anti-mouse IgG (Sigma Aldrich) were added and developed with streptavidin- HRP (BD Biosciences) using 3-amino- 9-ethylcarbazole (Sigma Aldrich) in N,N-dimethylformamide (Sigma Aldrich) diluted in 0.1 M acetate solution and with H_2O_2 as substrate [38].

Cells

Primary cells from wild-type or chimeric mice were sorted and cultured at 37°C, 5% CO_2. Mature B cells were stimulated in complete α-MEM supplemented with cross-linking 20 μg/mL $F(ab')_2$ fragment goat anti-mouse IgM (Jackson Immuno Research), anti-CD40 (clone FGK4T) and fully thiolated CpG 2006 type B (24mer 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′, TIB MOLBIO). Proliferation was assessed by staining of cells with Cell Proliferation Dye eFluor 670 according to manufacturer's instructions (eBioscience).

Clonal expansion of preB1 cells was assessed in methyl cellulose containing IL-7 (MethoCult M3630, Stemcell Technologies) according to the manufacturer's instructions with a starting cell number of 5000–20 000 sorted cells. Cellularity was assessed by picking single colonies and flow cytometric cell counting after 7 days.

Cell death of immature and transitional B cells was assessed in plain α-MEM (Life Technologies) supplemented with cross-linking $F(ab')_2$fragments of goat anti-mouse IgM antibodies (20 μg/mL) overnight. Dead cells were identified by flow cytometry by loss of GFP expression and reduction in size (FSC) and increased granularity (SSC).

For T-cell differentiation OP9-DL1 stromal cells transduced LSK cells were cultured in complete α-MEM supplemented with IL-7 (1 ng/mL) and Flt3-L (5 ng/mL) (both Peprotech). At day 4, the culture medium was exchanged and at day 7 cells were transferred onto fresh OP9-DL1 monolayers. Differentiation was assessed every 2–3 days by flow cytometry.

OCI-Ly19 were cultured in RPMI (Life Technologies) supplemented with 1% PenStrep, 1% Pyruvate and 20% (FCS #080150, Lot# 115654, Wisent INC).

Flow cytometry

Flow cytometry and cell sorting were performed on LSRII and FACSAriaIIu (BD), respectively, and followed the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" [39]. Monoclonal antibodies specific for CD4 (RM4-5, GK1.5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), Gr-1 (RB6-8C5), erythroid cell marker (Ter-119), CD19 (1D3), CD11b (M1/70), NK1.1 (PK136), CD11c (N418), CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), CD117 (ACK2), Sca-1 (E13-161.7), CD135 (A2F10), CD127 (A7R34), CD95 (15A7), Thy-1.1 (OX-7), T- and B-cell activation marker (GL7), human CD25 (BC96) were used purified or as various fluorescent or biotin conjugates. Antibodies were purified from hybridoma supernatants or were purchased from eBioscience, BD Biosciences, Biolegend, or Miltenyi Biotech. Flow cytometry was conducted on an LSRII (Becton Dickinson) and analyzed in FlowJo (9.3 or X, TreeStar). Cell sorting was done using a FACSAriaIIu (Becton Dickinson).

Bone marrow B-cell stages were defined as follows: proB ($B220^+CD19^-$), preBI ($B220^+CD19^+CD117^+CD25^-$), preBII ($B220^+CD19^+CD25^+CD117^-$), immature B ($CD19^+IgM^{lo}IgD^-$), transitional ($CD19^+IgM^{hi}IgD^{+/-}$), mature ($CD19^+IgM^{int/-}IgD^+$). Splenic B-cell stages were defined as: Fo B cells ($CD19^+CD23^+CD21/35^-$), MZ B cells ($CD19^+CD23^-CD21/35^+$). Thymic T-cell stages: ETP ($CD44^+$ $CD117^+CD25^-$), DN2 ($CD44^+CD117^+CD25^+$), DN3 ($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$), DP ($CD4^+CD8^+$), SP4 ($CD4^+$), SP8 ($CD8^+$). Intracellular staining, preserving GFP fluorescence, was carried out according to Heinen et al. [40].

miRNA microarrays

miRNA microarray (miRCURY LNA Arrays v. 10.0) transcriptional profiling was carried out by Exiqon. RNA samples from progenitor populations (200 ng each) were labeled with Hy3 and compared to Hy5-labeled skewed common reference sample consisting of an RNA mixture from total BM and thymus. Populations of interest were defined as follows: HSC, $lin^-Sca\text{-}1^+CD117^{hi}CD135^-$; CLP, $lin^-Sca\text{-}1^+CD117^+CD127^+CD135^+$; pre-pro-B, $lin^-B220^+CD19^-$; ETP, $lin^-CD44^+CD117^{hi}CD25^-$; DN2, $lin^-CD44^+CD117^{hi}CD25^+$. Analysis of scanned microarray images was performed by Exiqon

using the ImaGene software. Downstream data analysis was performed the "R" statistical analysis software package. Normalization and computation of significantly differentially expressed genes analysis was performed with the "limma" package from the Bioconductor suite. Genes whose absolute log2 fold change exceeded 1 and the adjusted p-value (using "Benjamini-Hochberg" method) was lower than 0.05 were assumed to be differentially expressed in a given pair-wise comparison. For hierachical clusterings only those probes were used that in a particular dataset were rated as differentially expressed in at least one pairwise comparison and to which a murine miRNA identifier was assigned.

Gene expression and rearrangement PCR

Total RNA was extracted with miRNeasy (Qiagen) followed by RT of mRNA (SuperScript II and random hexamers, Life Technologies) and miRNAs (TaqMan® MicroRNA Reverse Transcription Kit). Real-Time quantification was conducted using TaqMan Universal PCR master mix, MicroAmp™ 96-well fast reaction plates and optical adhesive covers in a StepOnePlus™ Instrument (Applied Biosystems) with TaqMan-probes for hsa-miR-191 (000490), snoR412 (001243), U6 (001973), *Rag1* (Mm01270936_m1), *Rag2* (Mm00501300_m1), *Dntt*(encoding TdT) (Mm00493500_m1), *Ebf1* (Mm00432648_m1), *Egr1* (Mm00656724_m1), *EGR1* (Hs00152928_m1), *Foxp1* (Mm00474848_m1), *FOXP1*(Hs00212860_m1), *Tcf3* (encoding E2A) (Mm01175588_m1), *TCF3* (Hs00413032_m1), *Pax5* (Mm00435502_m1), *Hprt* (Mm00446968_m1), *ACTB* (Hs99999903_m1). Heavy chain rearrangement was assessed on three V_H gene families: V_HJ558, V_HGam3.8 and V_HJ7183 to DJ_H3. The genes belonging to the V_HJ558 family are located at the most 5′ end of the V_H locus and belong to the most frequently used V_H families. Genes belonging to the V_HGam3.8 are located at the center of the V_H locus; the genes belonging to V_HJ7183 family are located at the most 3' end of the V_H locus and are the first used to rearrange with DJ_H [4, 41, 42] Genomic DNA was isolated using QIAamp micro kit (Qiagen) and the PCR was carried out on 20 000; 4000; and 800 cells using 1 nM of primers as previously described [43, 44] in 1x PCR reaction buffer (B9004S, Thermopol) for 40 cycles (annealing 59°C).

Luciferase Assay

Wild type and mutated 3'UTRs (Fig. S2) of Egr1, FoxP1, and E2A were synthesized by GeneArt (Regensburg) and cloned into PsiCheck2.0. 3T3 cells overexpressing miR-191 or control cells were cultured in DMEM (Life Technologies) (10% FCS, 1% Pen-Strep, 1% Na-Pyruvate, 25 g/mL Geneticin (Life Technologies)). A total of 250 000 cells were electroporated with 0.1 μg Plasmid (250 V, 950 μF, Biorad Gene Pulser II) and cultured for 24 h in six-well plates. Dual-Luciferase Reporter assay (Promega) was conducted according to manufacturer's instructions. Luciferase activities were normalized to empty PsiCheck2.0 to control for variances between cell lines.

Statistics

Statistical significance of differences between two groups was analyzed using paired or nonpaired *t*-tests where applicable. For comparison of multiple groups statistical significance was determined using ANOVA followed by *t*-tests to assess significance between groups.

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Abbreviations: **ETP**: early T-cell progenitor · **MZ**: marginal zone

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