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**Supplementary Material**

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MiR-210 Is Induced by Oct-2, Regulates B Cells, and Inhibits Autoantibody Production

Yingting Mok,*1 Vera Schwierzeczek,*1,2 David C. Thomas,* Elena Vigorito,† Tim F. Rayner,* Lorna B. Jarvis,* Haydn M. Prosser,‡ Allan Bradley,‡ David R. Withers,§ Inga-Lil Mürtensson,*3 Lynn M. Corcoran,¶ Cherie Blenkiron,¶∥ Eric A. Miska,∥ Paul A. Lyons,* and Kenneth G. C. Smith*  

MicroRNAs (MiRs) are small, noncoding RNAs that regulate gene expression posttranscriptionally. In this study, we show that MiR-210 is induced by Oct-2, a key transcriptional mediator of B cell activation. Germline deletion of MiR-210 results in the development of autoantibodies from 5 mo of age. Overexpression of MiR-210 in vivo resulted in cell autonomous expansion of the B1 lineage and impaired fitness of B2 cells. Mice overexpressing MiR-210 exhibited impaired class-switched Ab responses, a finding confirmed in wild-type B cells transfected with a MiR-210 mimic. In vitro studies demonstrated defects in cellular proliferation and cell cycle entry, which were consistent with the transcriptomic analysis demonstrating downregulation of genes involved in cellular proliferation and B cell activation. These findings indicate that Oct-2 induction of MiR-210 provides a novel inhibitory mechanism for the control of B cells and autoantibody production. The Journal of Immunology, 2013, 191: 3037–3048.

B cells are activated upon antigenic stimulation to mediate a variety of effector functions, including Ab production (1), and dysregulated B cell behavior has been implicated in autoimmunity and malignancy (1, 2). Our understanding of the genetic control of B cell maturation involving key transcriptional regulators, such as Oct-2, remains incomplete (3). Oct-2 is a POU domain–containing transcription factor required for normal humoral responses to T-dependent and T-independent Ags (4). Although recent studies identified important targets, including IL-6 and IL-5 receptor α-chain (5, 6), the full extent of the transcriptional complexity underlying Oct-2–mediated regulation of B cell responses remains to be elucidated.

MicroRNAs (MiRs) are small, noncoding RNAs that regulate gene expression posttranscriptionally. MiRs are processed by Dicer and form MiR-induced silencing complexes that base pair imperfectly with target mRNAs at sites located mainly in their 3′UTR (7). In mammalian cells, target repression occurs by decreasing mRNA levels and translation (8). MiRs are subjected to regulation at several levels, including transcription, precursor processing, and export, as well as by other MiRs (9). Early studies showed that MiRs are important in B cell development. Removal of Dicer at an early stage in B cell development resulted in an almost complete block at the pro- to pre-B transition and reduced B cell populations in the periphery (10). Dicer ablation in CD19+ B cells resulted in skewing of B2 cellular subsets, with increased transitional and marginal zone B (MZB) cells and reduced follicular B cells (11).

MiRs are also important in the B cell response to Ag (12). Dicer ablation at either the pro-B or CD19+ stage results in altered Ab repertoires (10, 11), and deletion of Dicer in murine Ag-activated B cells results in impaired production of high-affinity class-switched Abs, memory B cells, and long-lived plasma cells (13). MiR-155, an activation-induced MiR, enhances the formation of germinal centers in vivo and is essential for the generation of class-switched Ab-secreting cells via downregulation of Pu.1 (14, 15). Activation-induced cytokine deaminase, an enzyme important in somatic hypermutation and class-switch recombinaton, is targeted by both MiR-155 and MiR-181b to prevent activation...

References

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The microRNA sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE48186. The mRNA sequences presented in this article have been submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-1758.  
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The online version of this article contains supplemental material.  
Abbreviations used in this article: ChIP, chromatin immunoprecipitation; ES, embryonic cell; HIF, hypoxia-inducible factor; KLH, keyhole limpet hemocyanin; KO, knockout; MFI, median fluorescence intensity; MiR, microRNA; MZB, marginal zone B; NP, 4-hydroxy-3-nitrophenylacetyl; NTG, nontransgenic; TG, transgenic; WT, wild-type.  
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induced cytidine deaminase–mediated Myc–IgH translocations and malignant transformation (16–18).

Previous studies showed that most murine MiRs are downregulated upon B cell activation (18, 19). In this study, we identify the highly conserved MiR-210 as an Oct-2–regulated MiR induced upon B cell activation. MiR-210 is widely expressed and has been implicated in the hypoxic response (20), oncogenesis (21), and angiogenesis (22). It is expressed in hematopoietic stem cells, myeloid cells, and lymphocytes (19). In macrophages, MiR-210 was reported to negatively regulate production of proinflammatory cytokines by targeting NF-κB (23). Its function in lymphocytes has not been defined, and, interestingly, MiR-210 was shown to be overexpressed in B cell malignancies (24, 25). In this study, we demonstrate that MiR-210 is a novel regulatory target of Oct-2 and has a physiologically important role in inhibiting the development of age-associated autoantibodies. Furthermore, overexpression of MiR-210 can result in B cell subset and functional abnormalities by downregulating genes involved in cellular proliferation and B cell activation. Our results reveal a novel, MiR-mediated mechanism for the control of B cell responses and autoantibody production.

Materials and Methods

Mouse strains

C57BL/6, MRL, MRL+/−, and NZB mice were purchased from Harlan Europe. NOD mice were provided by Sarah Howlett (Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research). All experiments were performed according to the regulations of the U.K. Home Office Scientific Procedures Act (1986). The animal experiments were approved by the U.K. Home Office.

Generation of MiR-210–deficient mice

Heterozygous knockouts (KOs) in the C57Black/6N embryonic stem (ES) cells were generated as part of the Wellcome Trust Sanger Institute’s mirKO initiative (26). Full details of the targeting vector and ES cell reagents are described at http://www.knockoutmouse.org/martsearch/search/query=mir-210. Briefly, a targeting vector with a Puro selection cassette was generated by recombineering in Escherichia coli. The Anc linearized vector was electroporated into JMS.F6 ES cells, and correctly targeted clones were identified by using long-range PCR across both of the targeting arms. The primer sequences for the 5′ arm are 5′-TGAGAATGATCTCAG-TCTTTGGAGGAAGTATT-3′ and 5′-CCAGTGAATCTCAGTCTAAATGTA-GTCAAT-3′, with a product of 5889 bp, and for the 3′ arm the primers are 5′-TCTCAGAAGATGAACTGGACCTCTGAGC-3′ and 5′-CCAAGGTCCTCTGAGAAGTATG-3′, with a product of 2885 bp. The Puro2Δt selection cassette was deleted from the targeted allele by transient transfection with a Cre recombinase expression plasmid, followed by selection with FIAU (200 nm). Surviving ES cells were pooled and microinjected into C57BL6/J-Tyr c-Brd blastocysts for the generation of chimeric mice. Germline transmission was tested by breeding with albino C57BL/6J-Tyr c-Brd mice, and the colony was maintained by backcrossing with C57BL/6N. Genotyping of the MiR-120 KO allele was by PCR across the deleted locus using primers 5′-AGGGTGAATAGGAG-GGTACAAAGGT-3′ and 5′-AACAATCTAATACCTAAGAGAAGTT-C3′, annealing at 56°C. The wild-type (WT) product was 527 bp, and the mutant product was 443 bp.

Generation of MiR-210–overexpressing mice and mixed chimeras

A 260-bp region including the MiR-210 precursor sequence was cloned into a construct containing the H chain (VH) promoter, IgH intronic enhancer (Ei), and the Igκ 3′ enhancer (27, 28) and injected into CBA-fertilized C57BL/6J eggs. Transgenic (TG) offspring were backcrossed onto C57BL/6J mice for at least five generations. The presence of the transgene was identified by tail DNA PCR assays using primers designed to amplify a 500-bp region spanning the Ei–Vp junction (MiR-210F: 5′-TGCGGAGTACGTTCTC-3′ and MiR-210R: 5′-TGCTATACGTTCTC-3′), with a product of 5489 bp. The wild-type product was 527 bp, and the mutant product was 443 bp.

Luciferase assay and mutagenesis

The 2-kb MiR-210 promoter was amplified from C57BL/6 genomic DNA and cloned into pGL4.14 and pGL4.26.HLuc vectors (Promega), using the primers D1 (5′-AAGCTTACACCCAGAGAGCT-3′) and D2 (5′-AAAGAGTCTTGACGGCTTAGGAACTG-3′). Deletion of the 2-kb or hypoxia-inducible factor (HIF)-1α consensus binding site was generated using QuikChange mutagenesis (Agilent Technologies) with primers E1 (5′-CATGTCACCCGAGCACTGATT-3′) and E2 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) or E3 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) and E4 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′) and E5 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′). Deletion of the 2-kb or hypoxia-inducible factor (HIF)-1α consensus binding site was generated using QuikChange mutagenesis (Agilent Technologies) with primers E1 (5′-CATGTCACCCGAGCACTGATT-3′) and E2 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) or E3 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) and E4 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′) and E5 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′). Deletion of the 2-kb or hypoxia-inducible factor (HIF)-1α consensus binding site was generated using QuikChange mutagenesis (Agilent Technologies) with primers E1 (5′-CATGTCACCCGAGCACTGATT-3′) and E2 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) or E3 (5′-GCTGGAAATAGAAGGCAAACCAAGTATT-3′) and E4 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′) and E5 (5′-ACCCGCGCAGCAAGGGGCGTTT-3′).

Immunizations

Mice were immunized i.p. with 100 μg 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll or NP-keyhole limpet hemocyanin (KLH) (Biosearch Technologies) emulsified in alu thermo (Thermo), according to the manufacturer’s instructions.

ELISA and ELISPOT assay

Serum anti-NP, anti-chromatin, and anti-dsDNA ELISAs were carried out as previously described (25). Serum IgM, IgG, IgG2a, IgG2b, and IgG3 were assayed using paired capture and HRP-conjugated Abs (Southern Biotechnology Associates, Inc.) and MAdCAM1-biotin (clone MECA-367; eBioscience). Anti-mouse B220-biotin (clone RA3-6B2; eBioscience), IgD–FITC (clone 2G12), and Pgp-1–FITC (clone 28-14-8; Immunotech) were used as bivalent Abs specific for B cells. Antibody analysis was performed using Zeiss LSM software. Anti-nuclear serum anti-NP, anti-chromatin, and anti-dsDNA ELISAs were carried out as previously described (24). Briefly, proteins were cross-linked to DNA using formaldehyde (0.4%), followed by cell lysis. Chromatin was fragmented using a Bioruptor sonicator (Diagenode), and separate immunoprecipitations were produced using polyclonal anti-Oct-2 serum, pemimunconjugated control serum (24), and Abs to H3K4me3, H3K9Ac (Millipore), and control IgG (Sigma). Primers A1 (5′-TCAAGGTTGGGGCCCTCAGGACTG-3′) and A2 (5′-GATCAGTCTTGTGGCAAGCA-3′) were used to detect the MiR-210 promoter, and primers B1 (5′-GAGCTGTCTTTTGCTTCT-3′) and B2 (5′-GAGCTGTCTTTTGCTTCT-3′) were used to amplify regions 1 kb 5′ or 3′ of the Oct-2 binding site, respectively. Data were normalized to genomic regions 1 kb upstream (5′ 1 kb) and downstream (3′ 1 kb) of the Oct-2 binding site to demonstrate localized enrichment, and the CD36 promoter was used as a positive control, as previously described (5).

Immunofluorescence

Spleens were embedded in Tissue-Tek OCT compound (Bayer Healthcare), cut into 6-μm sections, and then goat anti-rabbit IgG FITC (Southern Biotechnology) and MAdCAM1-biotin (clone MECA-367; eBioscience). Confocal images were obtained using an LSM 510 Meta microscope (Zeiss). Chromatin immunoprecipitation (ChIP) was carried out as previously described (24). Briefly, proteins were cross-linked to DNA using formalde-
**MiR-210 mimics and luciferase assay**

The target site of CD23 was amplified using primers F1 (5′-AAACTC-GAGCCACAGCAATGGGTCG-3′) and F2 (5′-AAAGCGGCCC-GCTACTGAGGCGAAGTCTG-3′) and cloned into psiCheck-2 Renilla luciferase reporter plasmid (Promega). HeLa cells cultured in 96-well plates were transfected with plasmid and MiR-210 mimic or control (80 nM; Dharmacon) using Lipofectamine 2000 (Invitrogen). Reporter Renilla luciferase activity was measured after 24 h with a Dual-Luciferase Reporter Assay System (Promega) and normalized to firefly luciferase activity.

**Cell separation and culture**

B cells were isolated by magnetic cell purification using anti-CD19 beads (Miltenyi Biotec), according to the manufacturer’s instructions, and routinely sorted to >95% purity. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 1 nM sodium pyruvate, 10 nM HEPEs (pH 7.5), and 0.1 mM nonessential amino acids. All reagents were from Sigma-Aldrich, unless otherwise specified.

**Cell stimulation and transfection**

For MiR expression–profiling studies, cells were cultured at 2 × 10^6 cells/ml and stimulated with either goat anti-mouse IgM μ-chain–specific F(ab′)2 (10 μg/ml; Jackson ImmunoResearch Laboratories) with recombinant murine CD40L (1 μg/ml; PeproTech) or with LPS from E. coli (10 μg/ml; Dharmacon) and stimulated with either goat anti-mouse IgM or control (10 μg/ml; PeproTech) or with LPS from E. coli (10 μg/ml; Sigma-Aldrich).

**Oct2+/− and Oct2−/− B cells were obtained from RAG1−/− mice, because the Oct-2 mutation is lethal when homozygous, and stimulated with CpG for 48 h, as previously described (6). For transfection, primary B cells were prestimulated with LPS and IL-4 overnight and electroporated with MiR-210 mimic or control (10 μM; Dharmacon) using the Mouse B cell Nucleofector kit (Amaxa), according to the manufacturer’s instructions. In vitro class-switching and plasma cell–dissertation assays, cells were cultured at 1 × 10^6 cells/ml for 72 or 96 h, as previously described (15).

**B cell proliferation and cell cycle analysis**

For CFSE labeling, 5 × 10^5 purified B cells/ml were loaded with 5 μM CFSE (Invitrogen) in protein-free media or PBS by incubating for 10 min at room temperature. Cells were washed with complete media, and the extent of CFSE labeling was analyzed by flow cytometry. Cell cycle analysis with propidum iodide was assayed as previously described (29).

**RNA extraction and RT-PCR**

Total RNA was extracted with TRIZol reagent (Invitrogen), and real-time quantitative PCR was performed using TaqMan MiRNA Assays (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Data were acquired on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and normalized to snRNAU6. For mRNA expression assays, cDNA was generated using SuperRT reverse transcriptase (HT Biotechnology, Cambridge, U.K.) before quantification with TaqMan probes (Applied Biosystems).

**Gene ontology analysis**

Gene ontology overrepresentation analysis was performed using the Bioconductor topGO package, using the Fisher exact test in conjunction with the “elim” algorithm, as described previously (30).

**MiR target prediction**

MiR-210–predicted targets were generated from the miRwalk database (http://mirwalk.uni-hd.de/) using five algorithms—miRwalk (31), RNA22 (32), TargetScan (33), Miranda (34), and RNAHybrid (35)—with a minimum seed length of 7 nt (p = 0.05).

**Statistical analysis**

Statistical analysis was performed with a two-tailed unpaired Student t test using GraphPad Prism 4 software, unless otherwise indicated.

**MiR-expression profiling**

MiR-expression profiling was carried out as previously described (36). Briefly, MiRs were extracted from 5 μg total RNA, and adaptors were ligated at the 3′ and 5′ ends using T4 RNA ligase (Fermentas, Burlington, ON, Canada). These ligated products underwent reverse transcription using an adaptor-specific primer and were amplified and labeled using PCR. PCR products were precipitated and hybridized overnight to oligonucleotide probes coupled to color-coded polystyrene beads. Unbound sample was removed from beads by washing, and streptavidin-PE (Invitrogen) was added to the beads to bind biotin moieties on the cDNA. Samples were processed on a Luminex 100 machine, and median fluorescence intensity (MFI) values were acquired using StarStation software (ACS, Sheffield, U.K.).

**Bioinformatic analysis**

For MiR profiling of B cells across C57BL/6 and autoimmune strains of mice, well-to-well scaling was performed such that the total MFI of the postlabeling controls in each well was equal to the median value across all wells. Scale sampling was performed such that total MFI of the prelabeling controls in each sample was equal to the median value across all samples. A threshold of 1 was applied, and the data were log transformed. Filtering for “expressed” MiRs was performed using a cutoff value of 3 SDs above the median MFI of all readings. Differential gene expression between sample pairs was analyzed with one-way ANOVA for all combinations of sample pairs using GraphPad Prism software. Combinations with p < 0.05 were filtered to show only within-strain comparisons. Differential gene expression across all samples was analyzed using the GEPAS T-Rex multiclass comparison (ANOVA) tool (37), which provided p values adjusted for multiple testing, with control of family-wise error rate and false-discovery rate (38). All MiR expression data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE48186.

**mRNA extraction, microarray hybridization, and data analysis**

Total RNA was extracted using an RNeasy Mini kit (QiAGEN), of which 200 ng was labeled using a WT Sense Target labeling kit and hybridized to Mouse Gene 1.0 ST arrays (both from Affymetrix). Arrays were scanned using a GS 3000 scanner (Affymetrix), and raw data files were imported into R and subjected to variance stabilization normalization using the VSN package in BioConductor (39). Following normalization, differentially expressed genes were identified using the Limma package (40, 41). Differential expression was defined as fold changes ≥1.5 that were statistically significant following correction for multiple testing by setting the false-discovery rate to 5%. Gene set enrichment analysis was performed as previously described, with a false-discovery rate of q < 0.25 indicating significant enrichment (42). All mRNA expression data have been submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-1758.

**Results**

**MiR-210 is an activation-induced MiR regulated by Oct-2**

Splenic B cells from different mouse strains were stimulated with either anti-IgM + CD40L + IL-4 or LPS for 48 h, and MiR expression was determined using a bead-based microarray (36). In agreement with previous studies, most MiRs are downregulated upon B cell activation (Fig. 1A). Strikingly, MiR-210 was upregulated 15–20-fold in both BCR-dependent and BCR-independent conditions across all strains examined (Fig. 1A, 1B). Kinetic characterization revealed peak induction at 48 h, and high levels were maintained for ≥96 h after stimulation (Fig. 1B).

Because MiR-210 induction occurs relatively late in B cell activation, we were interested in its transcriptional regulation. Promoter analysis revealed the presence of putative binding sites for HIF-1α and Oct-2 upstream of the MiR-210 transcription start site. HIF-1α was shown to be a hypoxia-induced regulator of MiR-210 in cancer cells (20), and although deletion of the HIF-1α binding site resulted in a 50% reduction in reporter luciferase activity, it did not completely abolish MiR-210 induction in WEHI231 cells (Fig. 1C). In contrast, the role of Oct-2 in MiR-210 regulation has not been investigated. Oct-2 is expressed predominantly in lymphocytes (43), and its transcriptional activity is important in B cell activation (44). Deletion of the Oct-2 consensus octamer (ATTTCGAT) resulted in a 25% reduction in reporter luciferase activity (Fig. 1C). We demonstrated binding of Oct-2 to the MiR-210 promoter by chromatin precipitation analysis (5) in WEHI231 B lymphoma cells (Fig. 1D), and this same...
**FIGURE 1.** MiR-210 is an activation-induced MiR regulated by Oct-2. (A) B cells from 8–12-wk-old mice of various murine strains (top row) were stimulated with α-IgM + CD40L + IL-4 or LPS for 48 h, and their MiR profiles were compared with resting controls. Each row corresponds to an MiR gene, and each column corresponds to an individual mouse. Red indicates increased expression, and blue indicates reduced expression. MiRs are ranked according to the degree of differential expression across samples. MiR-210 expression, which is upregulated upon activation with both types of stimuli, is outlined in black. (B) Kinetics of MiR-210 induction in C57BL/6 B cells stimulated with α-IgM + CD40L + IL-4 (upper panel) or LPS (lower panel), quantified by RT-PCR and normalized to levels of U6snRNA. Error bars represent SEM of three biological replicates. (C) Site-directed mutagenesis of Oct-2 and HIF-1α binding sites in the MiR-210 promoter. Firefly luciferase activity of a reporter vector was assayed and normalized to control Renilla luciferase activity. (D) ChIP of endogenous Oct-2 bound to the MiR-210 or CD36 (positive control) promoters in WEHI231 cells. Data are normalized to regions 1 kb 5′ and 3′ of the MiR-210 promoter to demonstrate localized enrichment. (E) ChIP of H3K4Me3 (promoter-specific) and H3K9Ac (promoter/enhancer-enriched) histone modifications in WEHI231 cells, demonstrating enrichment of the Oct-2 binding site. Data are normalized to control IgG and input DNA. (F) Oct-2–deficient and WT B cells were stimulated with CpG for 48 h, and MiR-210 induction was quantified by RT-PCR, normalized to U6snRNA, and expressed as arbitrary units (AU). Data are representative of two to four independent experiments in (C–F), and error bars indicate SD of technical replicates.
region was also highly enriched for the promoter-specific histone modification H3K4Me3 and H3K9Ac, which indicates likely promoter or enhancer activity (Fig. 1E). In addition, we activated Oct-2–deficient and WT B cells with CpG for 48 h (6) and measured MiR-210 expression. Oct-2–deficient B cells exhibited a 40% reduction in MiR-210 levels compared with WT B cells (Fig. 1F), despite comparable levels of aicda induction (Y. Mok, unpublished observations). These findings identify MiR-210 as a novel regulatory target of Oct-2, with the latter being essential for the full induction of MiR-210 during B cell activation.

**MiR-210–deficient mice develop increased autoantibodies with age**

To investigate the physiological roles of MiR-210, we generated mice deficient in MiR-210 on a C57BL/6 background (26) (Supplemental Fig. 1a). MiR-210 KO mice were fertile, viable, and born at the expected Mendelian ratios to their littermates. These mice exhibited normal B, T, and myeloid cell subsets (Table I, Supplemental Table S1a), and there were no gross abnormalities in organ development.

Strikingly, however, MiR-210 KO mice developed spontaneous autoantibodies that were first detectable at 5 mo of age (Fig. 2A). MiR-210 KO mice had increased serum levels of anti-nuclear Abs, and, in particular, anti-dsDNA and anti-chromatin Abs classically associated with systemic lupus erythematosus, implying a role for MiR-210 in the maintenance of B cell tolerance (Fig. 2B). Baseline serum IgM, IgG1, IgG3, and IgA levels were normal, and the ability to mount an Ab response to T-dependent and T-independent Ags was largely intact (V. Schwierzeck, unpublished observations). Interestingly, germinal center B cells were increased in aged MiR-210 KO mice (Supplemental Fig. 2), suggesting that MiR-210 may be induced in the later stages of B cell activation to function as a negative-feedback regulator in the prevention of age-associated development of autoimmunity.

**Overexpression of MiR-210 in vivo results in B cell subset abnormalities**

To complement loss-of-function studies, we overexpressed MiR-210 in vivo under the control of the H chain (V_H) promoter, IgH intronic enhancer (E_H), and the Igκ 3′ enhancer (27) (Supplemental Fig. 2b). Three lines of MiR-210 TG mice were obtained in which expression of MiR-210 in B cells was moderately increased at 0.3-, 1.2-, and 12.3-fold that found in B cells activated for 48 h (Supplemental Fig. 2c). All three lines of MiR-210 TG mice were fertile and viable. Mice from the highest expressing line exhibited a complex lymphoid phenotype and were examined in more detail; henceforth, they are referred to as MiR-210 TG mice. Mice with low levels of expression did not exhibit obvious lymphoid abnormalities. MiR-210 TG mice were viable even when homozygous for the transgene, and karyotyping with fluorescence in situ hybridization revealed no obvious karyotypic abnormalities (Supplemental Fig. 3a). The expression of other MiRs (MiR-24, MiR-146b, let-7c, and MiR-342) in MiR-210 TG B cells was unperturbed, indicating that miR-processing pathways were not saturated (Supplemental Fig. 3b).

MiR-210 TG mice exhibited a 20-fold expanded splenic B1a compartment, and the proportion of B1a cells in the lymphocyte pool in the peritoneal cavity was increased by a factor of 2.5 (Fig. 3A). The B1a cells in both spleen and peritoneal cavity expressed markers consistent with the conventional phenotypic definition of B1a cells (CD19·B220·CD5·CD43·IgMhiIgDlo CD23·) (45), and V_H gene analysis (46) showed that they were polyclonal (Y. Mok, unpublished observations). Although there was no difference in splenic B2 cell numbers, immunohistochemistry revealed the absence of a well-defined marginal zone in MiR-210 TG mice, and a reduced MZB cell population was verified by flow cytometry (Fig. 3B, 3C). In the bone marrow, there was a decrease in the numbers of pro-B, pre-B, and mature B cells (Fig. 3D), indicating impaired production, proliferation, or survival of MiR-210 TG B cell progenitors.

**Impaired competitive fitness of MiR-210 TG B2 cells**

To elucidate the cellular basis of the increased B1a/B2 ratio in MiR-210 TG mice, we investigated the behavior of MiR-210 TG B2 cells in a competitive environment, using the allotopic markers IgH* (WT) and IgHβ (TG/NTG). Mixed chimeras were generated by transferring MiR-210 NTG or TG fetal liver cells mixed with WT B cells in a 1:1 ratio into sublethally irradiated mice doubly deficient in Rag2

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**Table I. Cell populations in MiR-210 KO mice**

<table>
<thead>
<tr>
<th>Cell Populations</th>
<th>Cell Surface Markers</th>
<th>MiR-210 WT*</th>
<th>MiR-210 KOν</th>
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<tr>
<td><strong>Spleen</strong></td>
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<tr>
<td>B cells (× 10⁶)</td>
<td></td>
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<tr>
<td>B1 cells</td>
<td>CD19·CD5·CD43·</td>
<td>2.35 (0.94)</td>
<td>1.94 (0.44)</td>
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<tr>
<td>B2 cells</td>
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<tr>
<td>Transitional B, T1</td>
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<td>2.94 (0.49)</td>
<td>2.32 (0.88)</td>
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<td>MZB cells</td>
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<td>90.5 (19.4)</td>
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<td><strong>T cells (× 10⁶)</strong></td>
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<tr>
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<td>CD3·CD4·</td>
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<td>3.70 (0.96)</td>
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<td>CD3·CD8·</td>
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<td><strong>Myeloid cells (× 10⁵)</strong></td>
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<td><strong>Peritoneal cavity (× 10⁵)</strong></td>
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<tr>
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<td>0.58 (0.35)</td>
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<td>Bone marrow (× 10⁵)</td>
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<td>Pro-B cells</td>
<td>B220·c-kit·</td>
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<td>25.83 (4.90)</td>
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<td>117.8 (41.5)</td>
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<td>108.3 (37.2)</td>
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<td>Mature B cells</td>
<td>IgM·B220·</td>
<td>85.0 (32.5)</td>
<td>101.5 (21.2)</td>
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</table>

Cell counts were derived from n = 4–6 mice (6–8 wk old). Data are mean (SEM).
and the common cytokine receptor γ-chain (Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup>) (Fig. 3D). Preservation of the 1:1 ratio in reconstituted B cell compartments reflects a similar competitive fitness between IgH<sup>A</sup> (WT) and IgH<sup>B</sup> (TG/NTG) B cells. Surprisingly, splenic B2 cells in MiR-210 TG/WT chimeras were derived mainly from WT progenitors, with an IgH<sup>B</sup>/IgH<sup>A</sup> ratio of 4:1. In contrast, the B1a compartment had an approximately equal contribution from TG and WT cells (Fig. 3E, left panel). A similar pattern was observed in the peritoneal cavity (Fig. 3E, middle panel). In addition, 80% of IgM<sup>+</sup>B220<sup>+</sup> cells in the bone marrow of MiR-210 TG/WT chimeras were derived from IgH<sup>B</sup> progenitors (Fig. 3E, right panel), consistent with the decreased numbers of B2 cell progenitors in the bone marrow of nonchimeric mice (Fig. 3D). Altogether, the data indicate that, although the developmental fitness of MiR-210 TG B1 cells is relatively unaffected, the ability of B2 cells to proliferate or survive is impaired in the presence of competing WT cells.

**Overexpression of MiR-210 results in impaired production of class-switched Abs in a B cell–autonomous manner**

MiR-210 TG mice exhibited a marked decrease in serum levels of class-switched Abs, with a >10-fold decrease in IgG1, IgG2a, IgG2b, IgG3, and IgA isotypes (Fig. 4A). Class-switched IgG1 Ab responses to the T-dependent Ag NP-KLH in MiR-210 TG mice were also decreased, whereas the IgM response was intact (Fig. 4B). This was also consistent with an ~8-fold decrease in the numbers of anti-NP IgG1 Ab-secreting cells in the spleen and bone marrow 14 d postimmunization (Fig. 4C). Similarly, when immunized with the T-independent Ag NP-Ficoll, IgM responses were preserved, with a nonsignificant trend toward lower IgG3 anti-NP Abs 7 d postimmunization (Y. Mok, unpublished observations).

We investigated the cell autonomy of these B cell abnormalities using µMT fetal liver chimeras to exclude the effects of transgene expression in other lymphopoietic lineages (47). Mixed chimeras were created by transferring 80% µMT-deficient fetal liver cells/20% MiR-210 TG or NTG fetal liver cells into Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup> mice, as previously described (15). Because µMT-deficient fetal liver cells are unable to generate B cells, the 80:20 ratio favors the reconstitution of non–B lineage hematopoietic cells from WT precursors, whereas the B cell compartment is reconstituted solely from MiR-210 TG or NTG precursors. Consistent with our findings in germline mice, MiR-210 TG chimeras exhibited a 8-fold increase in their splenic B1a population, as well as the absence of a well-defined MZB cell population in the spleen (Y. Mok and D.R. Withers, unpublished observations). Serum levels of class-switched Abs were also reduced, with a less pronounced reduction in IgM (Fig. 4D). When immunized with the T-dependent Ag NP-KLH, µMT–MiR-210 chimeras exhibited markedly decreased serum levels of anti-NP IgG1 Abs, with a slight reduction in anti-NP IgM (Fig. 4E). IgG1, but not IgM, Ab-forming cells were reduced in the spleen 7 d postimmunization (Fig. 4F). Consistent with the above findings, immunohistochemical analysis revealed the absence of anti-NP IgG1 cells in the extrafollicular region of the µMT-TG spleen, whereas anti-NP IgM cells were present (Fig. 4G). To further validate these observations in an independent system, given that only our highest expressing strain demonstrated this phenotype, we transfected activated WT B cells with MiR-210 mimics; consistent with our in vivo findings, we found impaired class switching to IgG1 upon activation with LPS + IL-4 for 96 h (Fig. 4H).

**MiR-210 overexpression results in impaired B2 cellular proliferation, cell cycle entry, and class switching in vitro**

To investigate the underlying cause of the impaired production of class-switched Abs and reduced “fitness” of MiR-210 TG B2 cells observed in the IgH<sup>B</sup>/IgH<sup>A</sup> chimeras, we examined cellular proliferation and cell cycle entry in vitro. Splenic B2 cells were labeled with the intracellular dye CFSE and stimulated with dGm + CD40L + IL-4, in addition to activatory stimuli that induce class switching to IgG1 (48). MiR-210 TG B2 cells exhibited a marked defect in proliferation in response to different activatory conditions, and cell cycle analysis revealed a defect in G<sub>1</sub> to S phase transition in MiR-210 TG B2 cells (Fig. 5A), indicating that a defect in cell cycle regulation contributes to the impaired proliferation of MiR-210 TG B2 cells.

We further investigated the contribution of impaired B2 proliferation to reduced class switching observed in vivo and in vitro. Because class-switch recombination is a division-linked process (48, 49), the percentage of class-switched B2 cells was assessed in a specific subset of cells that had undergone at least four divisions (Fig. 5B). There was a significant decrease in the proportion of IgG1 class-switched B cells within this subset, suggesting a defect in class switching that is independent of the proliferative defect. Quantification of aicda expression did not reveal significant...
FIGURE 3. B cell subset abnormalities in MiR-210 TG mice and impaired developmental fitness of B2 cells. (A) Representative FACS of B1a cell expansion in spleen and peritoneal cavity of 8–12-wk-old MiR-210 TG mice, with quantification shown (right panel). Each symbol represents one NTG (○) or TG (●) mouse. (B) The MZB cell population, seen as IgMhiIgDlo MZB cells surrounding MAdCAM-1–expressing marginal zone macrophages, is not well-defined in the MiR-210 TG spleen (original magnification ×10). Data are representative of n = 3 mice/group. (C) Quantification of follicular and MZB cells by flow cytometry. CD19+CD93hi splenic B cells are divided into follicular and marginal zone subsets based on higher expression of IgM and CD21 in the latter. (D) Quantification of B cell progenitors in the bone marrow of MiR-210 TG mice by flow cytometry—pro-B (B220+cdkitt+), pre-B (B220+CD25+), immature B (IgM+IgM+B220+), and mature B (IgM+B220hi)—with six to eight biological replicates in each group, at 6–8 wk of age. Each symbol represents one NTG (○) or TG (●) mouse. p = 0.0655 for immature B cells. (E) Impaired developmental fitness of MiR-210 TG B2 cells in the presence of WT competitors. Recipient mice were reconstituted with mixtures of WT IgH+ and NTG or TG IgH+ fetal liver cells. Bar graphs indicate the mean relative contribution of IgH+ B cells to each B cell compartment. Striped gray columns represent the contribution of IgH+ TG mice, and solid gray columns represent the contribution of IgH+ NTG mice. **p < 0.01, Student t test.
changes between TG and NTG B2 cells (Y. Mok, unpublished observations), indicating that other components of the class-switch recombination machinery are responsible for the impaired class switching observed in MiR-210 TG B2 cells.

Influence of MiR-210 on the B cell transcriptome

Because MiR-210 is an activation-induced MiR, we investigated its effect on the B cell transcriptome by performing gene-expression arrays of MiR-210 mimic–transfected, MiR-210 TG, and MiR-210 KO–activated B cells. Overall, transcriptome changes with MiR-210 overexpression were more striking than the effects of MiR-210 deletion. Genes that were differentially expressed in mimic-transfected and MiR-210 TG B cells overlapped significantly (hypergeometric test, \( p < 2.2 \times 10^{-16} \)), with 1458 downregulated genes and 115 upregulated genes in common. Compared with a list of MiR-210 targets predicted by five algorithms (31–35),

**FIGURE 4.** Overexpression of MiR-210 results in impaired production of class-switched Abs in a cell-autonomous manner. (A) Levels of total serum Ig in MiR-210 NTG (○) and TG (●) mice. (B) MiR-210 TG mice were immunized with the T-dependent Ag NP-KLH, and anti-NP IgM (upper panel) and IgG1 (lower panel) titers were measured at days 0, 7, and 14 after immunization with NP12-BSA. (C) ELISPOT analysis of anti-NP IgG1 Ab-secreting cells in the spleen and bone marrow of MiR-210 TG mice 14 d postimmunization with NP-KLH. (D) Levels of total serum Ig in μMT-NTG (○) and μMT-TG (●) chimeras. (E) Measurement of anti-NP Ab titers in μMT chimeras following immunization with the T-dependent Ag NP-KLH. (F) Anti-NP IgG1 Ab-secreting cells were quantified by ELISPOT in μMT-NTG (○) or μMT-TG (●) chimeras 7 d postimmunization with NP-KLH. (G) Immunohistochemistry of NP-specific B cells in the spleen 7 d postimmunization with NP-KLH (original magnification \( \times 10 \)). Data are representative of \( n = 3 \) mice/group. (H) C57BL/6 B cells transfected with MiR-210 mimics exhibit increased class switching to IgG1 upon activation with LPS (10 \( \mu \)g/ml) + IL-4 (40 ng/ml) for 96 h. Data are pooled from two independent experiments. A one-tailed Mann–Whitney U test was performed, and the \( p \) value is shown. *\( p < 0.05 \), **\( p < 0.01 \), Student t test.
both the MiR-210 mimic and MiR-210 TG data sets showed significant overrepresentation of MiR-210 target genes in their lists of downregulated genes (hypergeometric $p < 0.00021$ and $p < 0.00021$, respectively), with no such enrichment in the lists of upregulated genes. Genes that were upregulated in KO B cells also overlapped with genes downregulated by MiR-210 mimics with gene set enrichment analysis (gene set enrichment analysis, $q \geq 0.22$) (42). Although no single gene was strikingly deregulated in MiR-210 KO versus WT cells, there was a further overlap between genes upregulated in KO B cells and MiR-210–predicted targets ($q \geq 0.25$). Taken together, these data suggest that MiR-210 functions by exerting a relatively subtle effect on a number of target genes, and this effect is amplified by overexpression.

Of the 169 predicted targets downregulated in both MiR-210 mimic and MiR-210 TG B cells (Fig. 6A, Supplemental Table 1b), gene ontology overrepresentation analysis demonstrated significant enrichment of genes involved in cell division, cell cycle, and B cell activation (Fig. 6B), consistent with the impaired cellular proliferation observed in MiR-210–overexpressing B cells. Because MiRs are known to exert subtle effects on a large number of genes (50, 51), it is likely that the observed cellular phenotype is a consequence of the collective downregulation of several of these genes rather than a single target. Nevertheless, our array approach was validated by CD23 (FcεRII), which acts both as a low-affinity IgE receptor and as an adhesion molecule that interacts with CD21 to regulate IgE production, survival of germinal center B cells, and the presentation of soluble protein Ag by B cells to T cells (52). MiR-210 TG B2 cells exhibited lower surface expression of CD23 compared with controls (Fig. 6C), and a closer examination of the CD23 mRNA sequence revealed the presence of a 7-mer MiR-210 seed sequence target site CGCA-CAG located in exon 9 of the coding sequence. The ability of this site to mediate functional repression by MiR-210 was confirmed using luciferase assays (Fig. 6D).

**Discussion**

In this article, we demonstrate a role for MiR-210 in B cells and describe the effects of germline deletion of MiR-210 in mice. Because MiR-210 was markedly upregulated after B cell activation, we investigated what controlled its expression and demonstrated that MiR-210 is a novel regulatory target of Oct-2. The peak of MiR-210 induction occurs late in B cell activation in comparison with other inducible MiRs, such as MiR-155 (14), which is consistent with the kinetics of Oct-2 induction because maximal levels occur 8–12 h poststimulation with LPS (44). Oct-2 is essential in B cells for progression through the G1 phase of the cell cycle upon stimulation in vitro and at the same time contributes to the induction of MiR-210, which has the opposite effect, suggesting a built-in regulatory mechanism to limit the activatory effects of Oct-2. Consistent with this, MiR-210–deficient mice spontaneously develop autoantibodies with age, implying loss of an inhibitory mechanism to balance the immune response. Interestingly, mice with Dicer-deficient CD19<sup>+</sup> B cells also develop spontaneous autoantibodies, which was attributed to lower amounts of MiR-185, resulting in accumulation of Btk and skewing of the B cell repertoire toward more self-reactive MZB cells (11). In contrast, MiR-210–deficient mice displayed normal peripheral B cell compartments, including follicular and MZB cells, suggesting a distinct mechanism for MiR-mediated maintenance of tolerance, which is likely to contribute to the phenotype observed in the Dicer-deficient mice.

MiR-210 is expressed at low levels in bone marrow precursors and naive peripheral B cell subsets (53), but it was reported to be overexpressed in B cell malignancies (24, 25). In this study, we show that ectopic overexpression of MiR-210 in vivo can result in B cell–autonomous subset abnormalities, including more B1a cells, a population thought to be of fetal origin that undergoes autorenewal in the periphery (54–56). Our studies with IgH<sup>H</sup>2IgH<sup>H</sup> chimeras suggest that their expansion in MiR-210 TG mice may...
reflect a homeostatic response to the failure of B2 cells to divide, because B1a cells were derived equally from WT and TG B cell precursors, whereas the B2 cells were derived predominantly from WT precursors. B2 cells of MiR-210 TG mice also had other abnormalities, including a decreased MZB cell population and dysregulated expression of cell surface markers, including CD23.

Overexpression of MiR-210 also resulted in cell-autonomous B cell functional abnormalities, in particular reduced class-switched Abs, both at baseline and in response to immunization with a T-dependent Ag. Impaired isotype switching to IgG1 was confirmed in WT B cells transfected with a MiR-210 mimic. Our in vitro experiments demonstrate that the underlying basis of this defect is due, at least in part, to reduced proliferation and cell cycle entry of MiR-210 TG B2 cells, consistent with gene-expression studies demonstrating the downregulation of genes involved in these processes.

It is not unexpected that the effects of MiR-210 overexpression and deletion do not result in exactly inversely correlated phenotypes; this was observed for other MiRs (57–59) and is consistent with the finding that MiRs rarely regulate a single target, but instead are thought to exert modest effects on multiple genes in specific cellular contexts (50, 51). In our studies, the subtlety of these interactions is reflected at the transcriptome level; although there were no striking abnormalities in the expression of individual genes, genes upregulated in KO B cells overlapped with genes that were downregulated by MiR-210 mimics, as well as with MiR-210–predicted target genes. Thus, in physiological contexts, the likely role of MiR-210 is in fine-tuning cellular responses rather than acting as a “master regulator,” achieving the balance between pathogen clearance and autoimmunity. In contrast, overexpression of MiR-210, linked with malignancy and hypoxia, can clearly result in abnormal B cell behavior and impaired Ab responses. Our findings in both mouse models are consistent with MiR-210 acting as an overall negative regulator of the B cell immune response, which is reminiscent of a hypoxia-induced increase in MiR-210 resulting in downregulation of proliferation-related genes in xenografted cancer cell lines (20). Thus, the late induction of MiR-210 may provide a more subtle, linked mechanism for control and inhibition of B cell activation that accompanies the robust early activatory effects of Oct-2–driven transcription.

We demonstrated that MiR-210 is an Oct-2–regulated MiR induced upon B cell activation. Mice deficient in MiR-210 develop increased spontaneous autoantibodies with age. In addition, overexpression of MiR-210 causes abnormalities in B cell subsets and function, in particular reducing B2 cell proliferation and isotype switching, as well as the downregulation of genes involved in these processes. These results indicate that MiR-210 provides a novel inhibitory mechanism that controls B cells to prevent autoimmunity.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.
References


Supplemental Data

Figure S1. Generation of miR-210 KO and miR-210 TG mice.
(a) MiR-210 was deleted by homologous recombination in JM8.F6 ES cells. Correct targeting was established by long range PCR across the targeting arms. The selection cassette was removed in tissue culture by transient transfection of a Cre recombinase expression plasmid followed by selection with FIAU.
(b) Construct used to generate miR-210 TG mice.
(c) MiR-210 expression in B cells of three lines of transgenic mice, relative to resting and activated NTG B cells, as quantified by RT-PCR. Error bars indicate the SEM of three biological replicates. MiR-210 expression is normalised to levels of U6snRNA and shown in arbitrary units (AU).
Figure S2. Increased germinal centre B-cells in aged miR-210 KO mice.
(a) Germinal centre (GC) B cells, T-follicular helper (Tfh) and T-follicular regulatory (Tfr) cells were analysed in unimmunised, aged 10-11 month old miR-210 KO and littermate controls by flow cytometry. Cellular subsets were quantified by flow cytometry using the following phenotypic markers: GC B cells (B220⁺ GL7⁺ Fas⁺), Tfh (CD3⁺ CD4⁺ Bcl6⁺ CXCR5⁺) and Tfr (Foxp3⁺ CD3⁺ CD4⁺ Bcl6⁺ CXCR5⁺).
(b) Analysis of germinal centre B-cells (B220⁺Ki67⁺Bcl6⁺) in a replicate cohort of 10-12 month old miR-210 KO mice.

For (a) and (b), each spot represents an individual wild-type (open) or KO (filled) mouse, and Mann-Whitney test was performed with p values indicated where significant. If both cohorts are analysed together, the difference in numbers of GC B-cells between KO and WT is significant with $p = 0.0006$. 
Figure S3. Karyotype analysis and microRNA expression in miR-210 TG B cells.

(a) FISH karyotyping of metaphase spreads prepared from LPS stimulated splenocytes showed no obvious karyotypic abnormalities. Both 1.8kb and 3.9 kb transgene probes hybridised to chromosome 2C1, as indicated by the yellow arrow in the top right panel. No hybridisation was observed in NTG metaphase spreads (bottom panel) and pictures are representative of n=2 biological replicates.

(b) RT-PCR quantification of miR-24, miR-146b, let-7c and miR-342 in resting CD19+ B cells isolated from the spleens of 6-8 wk old miR-210 NTG and TG mice. Error bars represent SEM of 3 biological replicates and miRNA expression is normalised to levels of U6snRNA. Student’s t-test was performed, with no significant differences observed.
### Table S1(a). Cell populations in aged miR-210 KO mice.
Cell counts derived from n= 4-5 mice (10-12 mths old); Data shown are mean (SEM). Germinal centre data is shown in Figure S2.

<table>
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**Table S1(b). Gene Symbols of 169 predicted targets downregulated in both activated miR-210 mimic and miR-210 TG B cells.**