Altering microRNA miR-15a/16 Levels as Potential Therapy in CLL: Extrapolating from the *de novo* NZB Mouse Model

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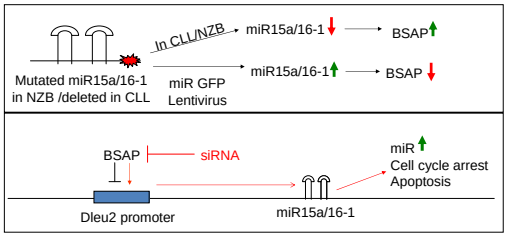
Abstract :

Abnormalities in miRNA mediated post-transcriptional regulation are found in CLL. Deletions/mutations in the *mir-15a/16-1* loci are present in many CLL and the NZB mouse model leading to decreased miR-15a/16. Increasing miR15a/16 in NZB by exogenous delivery of the microRNA by mimics (transitory) or lentiviral delivery(permanent) affected the ability of the B-1 malignant cells to grow *in vitro* and decreased the expression of BSAP (B-cell specific transcription factor), a negative regulator of DLEU2; the host gene for miR15a/16. Silencing BSAP served to increase miR15a/16 levels. For *in vivo* studies, diseased NZB were exposed systemically to lentiviral vectors expressing either GFP only or expressing both GFP and the wild-type *mir-15a/16-1*. In spleen, PWC and blood of NZB injected with mir15a/16 lentivirus, a decrease in proliferating malignant B-CLL clones followed by an overall reduction in malignant B cells when compared to NZB injected with control lentiviral vector occurred. Our data support the potential use of miR-15a/16 to ameliorate disease manifestations of CLL and point to decreased miR15a/16 levels as important contributors toward disease development.

Overall Scheme:



Materials and Methods

Lentivirus Production: Plasmids encoding the miR15a/16-1. GFP.puro or GFP.puro and the 3rd generation packaging plasmids were transfected into 293TN cells using Lipofectamine. The supernatant containing the infective lentivirus was collected 48hrs later and concentrated using PEG-It (System Biosciences, Inc).

Transduction and Sub-line Establishment: 1x10^5 LNC cells per well of a 24 well plate were transduced with 50µl of the respective lentivirus (2x10^7 IFU/ml) using 4µg/ml polybrene. 24hrs later the media was changed and the cells were sorted based on their GFP expression and the GFP+ cells were maintained in culture. 2x10^5 patient PBMC were transduced with 100ul of 4x10^6 IFU/ml lentivirus by spin-infection in retronectin coated plates.

In vivo Lentiviral Delivery: 100ul of lentiviral solution (10^8 TU) was injected i.v and i.p. Short term: 8-9 days. Long term: 2nd injection day 24 and sacrificed on day 28.

BSAP Knockdown: 0.2x10^6 cells per well of a 24 well plate were transfected with 1nM or 200nM of siRNA BSAP or negative control siRNA using HiPerFect Transfection reagent (Qiagen) following manufacturer's instructions. The cell cycle and BSAP expression was measured 72hrs post transduction. 2x10^6 patient PBMC were nucleofected with 2uM BSAP siRNA or negative control siRNA (Dharmacon) using Human B cell Nucleofection Kit (Lonza) according to manufacturer's instructions.

In vitro Lentiviral Delivery of miR15a/16-1 Leads to Reduced Proliferation of LNC

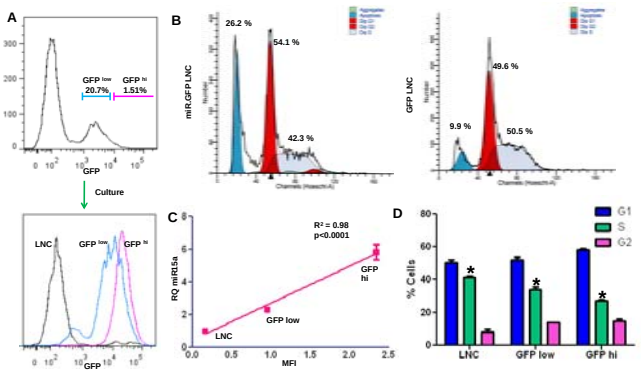


Figure 1: A) The murine CLL cell line LNC was transduced with miR15a/16-1 lentivirus and sorted based on GFP expression (top) and used to establish two LNC sub-lines – GFP^{hi} and GFP^{low} (bottom). B) LNC transduced with miR15a/16-1 lentivirus (left) exhibited a marked increase in apoptosis and a decrease in S phase, as compared to the empty GFP lentivirus (right). C) The LNC sub-lines exhibit increased expression of miR15a as compared to the original LNC. There is a strong co-relation between GFP intensity and miR15a expression. Thus, GFP can be used as a surrogate marker for miR15a levels in this system. D) The LNC sub-lines exhibit significantly reduced S phase as compared to original LNC.

miR15a/16-1 & BSAP Negatively Regulate Each Other

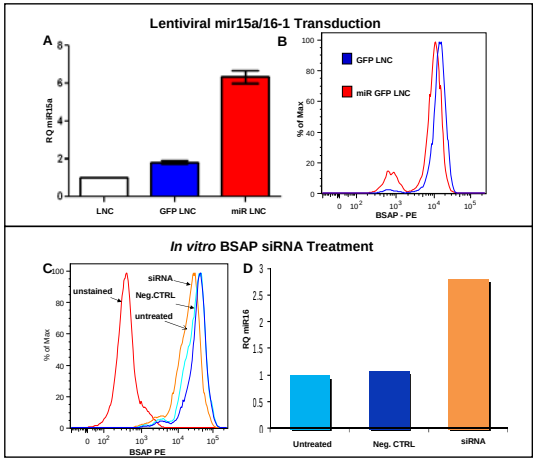


Figure 3: A) LNC transduced with miR15a/16-1 lentivirus shows higher levels of miR15a as compared to empty lentivirus or untreated LNC (TaqMan Assay). B) Higher levels of miR15a correspond with lower MFI of BSAP (intracellular flow cytometry). C) 72hrs post transfection with siRNA BSAP (orange), the amount of BSAP protein is reduced as compared to the untreated (light blue) and the negative control siRNA (dark blue) (intracellular flow cytometry). D) BSAP knockdown shown in (C) translates into increased miR16-1 level (TaqMan Assay).

Acknowledgements

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2.NJMS Flow Cytometry Core.
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Systemic In vivo Lentiviral Delivery of miR15a/16-1 Leads to Reduced Malignancy

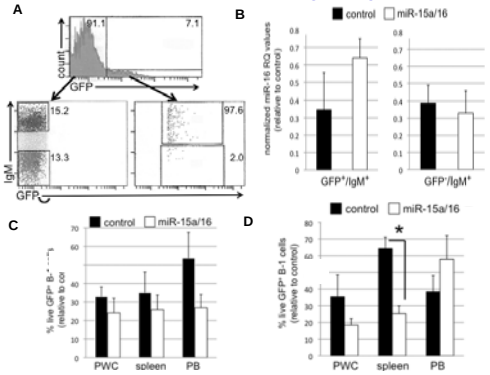

Figure 2: A) Representative sorting strategy for spleens of day 8-9 post-lentiviral injection. B) miR16 levels in GFP+/IgM+ sorted cells compared to GFP-/IgM+ cells. C) Live GFP+ B-1 cells are reduced in animals treated with miR lentivirus as compared to control (Short Term). D) Live GFP+ B-1 cells are significantly reduced in the speen of mice injected with miR Lentivirus as compared to the control (long term).

Extrapolating to CLL Patients

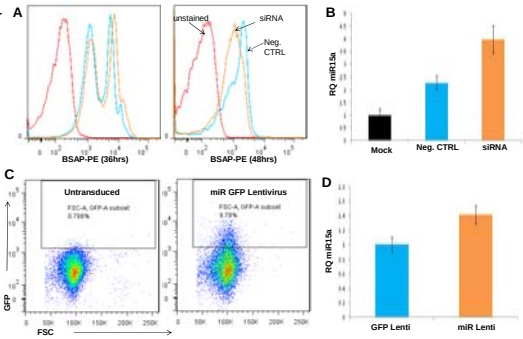

Figure 4: A) Ex-vivo treatment of patient PBMC with BSAP siRNA leads to decreased expression of BSAP (orange) as compared to a negative control siRNA (blue). B) Decreased BSAP expression co-relates with increased level of miR15a (TaqMan Assay). C) Representative plots showing GFP expression 48hrs after transduction, untransduced (left) or miR GFP lentivirus (right). D) Transduction with miR GFP lentivirus leads to increased expression of miR15a relative to GFP transduced cells.

Conclusion:

Using the NZB mouse model of CLL, we successfully employed lentiviral delivery of miR15a/16-1 both in vitro (Fig.1) and in vivo (Fig.2) and demonstrated the anti-neoplastic effect of upregulating these microRNAs. In addition,miR15a/16-1 and BSAP form a negative feed-back loop in CLL (Fig.3). This loop was exploited to increase the miR15a/16-1 levels in CLL patient samples (Fig.4). These findings have potential clinical implications for increasing the levels of the pro-apoptotic miR15a/16-1 either by directly increasing the miRNAs or decreasing BSAP, the negative regulator.