Identifying novel regulators of LIN28B through a genome-wide CRISPR/Cas9 screen

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ABSTRACT

LIN28 is a family of RNA-binding proteins that are well-conserved across species. It is dysregulated in a wide spectrum of cancer types, especially pediatric cancers such as hepatoblastoma and Wilms' tumor. Our laboratory previously showed that reactivation of *LIN28B* is sufficient to drive liver cancer, and that *LIN28B* deletion impairs tumor development. Nonetheless, the identity of factors that regulate *LIN28B* expression during normal development and cancer remains elusive. As *LIN28B* is a driver of oncogenesis, understanding its regulation in the process of oncogenesis will help uncover novel therapeutic targets.

Here, we show an original approach for identifying regulators of human *LIN28B* utilizing the CRISPR/Cas9 genome engineering system. Using CRISPR/Cas9, we knocked a GFP reporter into the endogenous locus of *LIN28B* in a human cancer cell line, engineering a fusion LIN28B-GFP protein. This approach is unique as GFP expression will be altered not only by changes in regulation at the coding sequence, mRNA and protein levels, but also changes at distal regulatory sequences.

To identify unknown regulators of *LIN28B*, we will perform a genome-wide CRISPR/Cas9-mediated knockout screen in human cells expressing the fusion LIN28B-GFP protein. By assessing sgRNAs that are enriched and depleted from the screen, we will discover novel regulators of *LIN28B* which can then be validated and further studied experimentally.

Through this screen, we hope to gain further insight into how *LIN28B* is regulated in normal development and cancer. Furthermore, identifying regulators of *LIN28B* can provide novel avenues for developing cancer therapeutics.

BACKGROUND

LIN28B is suppressed during embryonic development

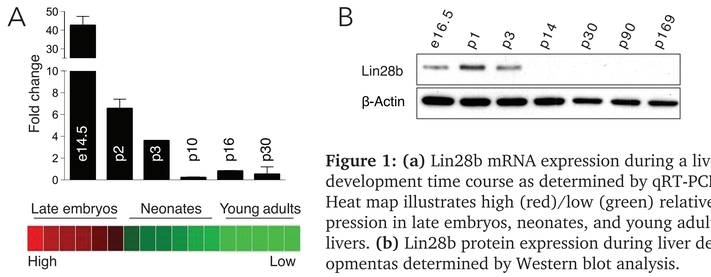

Figure 1: (a) Lin28b mRNA expression during a liver development time course as determined by qRT-PCR. Heat map illustrates high (red)/low (green) relative expression in late embryos, neonates, and young adult livers. **(b)** Lin28b protein expression during liver developmentas determined by Western blot analysis.

LIN28B is required for liver cancer development

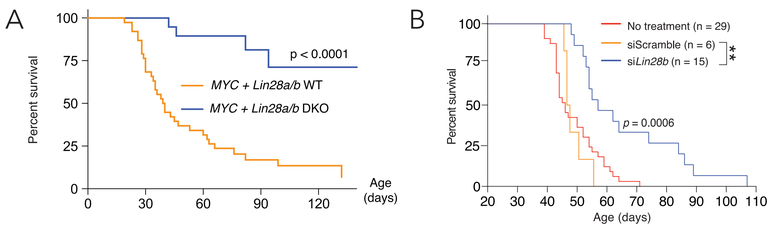

Figure 2: (a) Lin28a/b knockout improves survival of mice on MYC liver background. Survival curve of Lin28a/b WT mice (yellow, n=29) or Lin28a/b DKO mice (blue, n=13) with MYC induced on day of birth. **(b) Lin28b siRNA improves survival of mice on MYC liver background.** Survival curve of MYC-induced mice randomized to no treatment, siScramble, or siLin28b.

METHODS

Generate a template for HDR-mediated knock-in

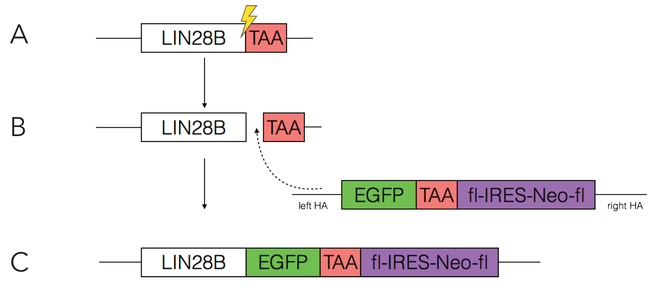

Figure 3: Design of GFP knock-in into endogenous locus of *LIN28B*. (a) A Cas9-induced double-strand break (DSB) is created near the end of the coding sequence of *LIN28B*. **(b)** EGFP-TAA-fl-IRES-Neo-fl casette surrounded by homology arms to corresponding regions of *LIN28B* is inserted before the endogenous stop codon through homology-directed repair (HDR) to engineer **(c)** a fusion LIN28B-GFP protein which cellular level can be measured by microscopy. IRES-Neo is an antibiotic resistance gene that enables selection for cells with the transgene. *loxP* sites surround the antibiotic resistance casette to enable its excision post-selection.

Screen design

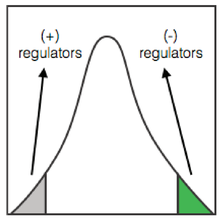

Figure 4: Using the Human Brunello CRISPR knockout pooled library containing 76,441 sgRNAs, we will knock out 19,114 genes individually in cells expressing LIN28B- GFP. Resulting changes in *LIN28B* expression are assessed by Fluorescence-activated cell sorting (FACS). Cells with significant change in GFP will be selected for sequencing. Enriched sgRNAs reads in the high GFP-expressing population suggest genes that normally function as inhibitors of *LIN28B*, while sgRNAs that are depleted suggest activators of *LIN28B*.

Establish cell line suitable for screen

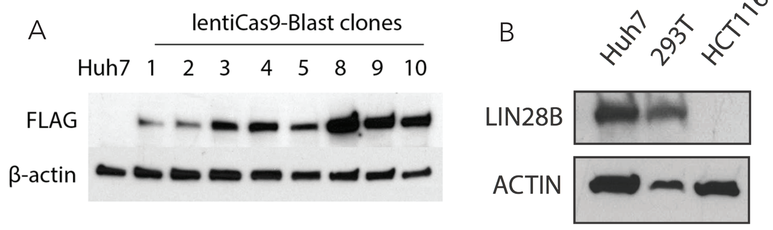

Figure 5: (a) A Huh7 human liver cell line stably expressing Cas9 was established. Stable expression of Cas9 allows efficient subsequent knockout with the sgRNA lentiviral library. **(b)** Several cell lines were evaluated for expression of the LIN28B protein. Huh7 was selected for its high baseline *LIN28B* expression.

Test efficacy of sgRNAs

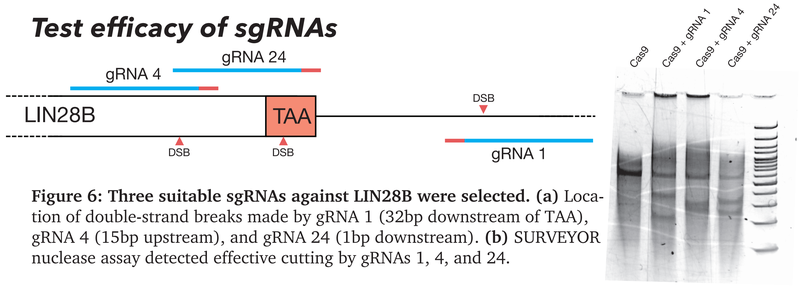

Figure 6: Three suitable sgRNAs against LIN28B were selected. (a) Location of double-strand breaks made by gRNA 1 (32bp downstream of TAA), gRNA 4 (15bp upstream), and gRNA 24 (1bp downstream). **(b)** SURVEYOR nuclease assay detected effective cutting by gRNAs 1, 4, and 24.

Establish cell line expressing fusion LIN28B-GFP protein

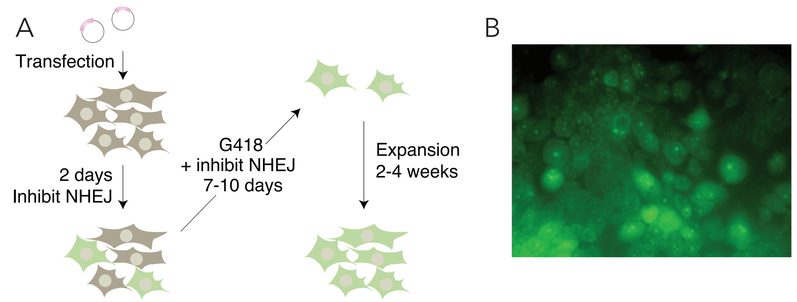

Figure 7: (a) Knock-in of GFP to establish a Huh7 Cas9-LIN28B-GFP cell line. *Transfection*: Cells are transfected with a plasmid containing gRNA targeting *LIN28B* and another containing EGFP-TAA-fl-IRES-Neo-fl surrounded by LIN28B-specific homology arms. *Inhibit NHEJ*: To promote HDR, SCR7 is added for 10 days to inhibit non-homologous end joining, the nonspecific repair of DSBs. *G418*: Cells are subjected to selection with G418 to eliminate cells without the *Neo* casette. *Expansion*: Cells are allowed to grow; single colonies expressing GFP are isolated and expanded. **(b)** A GFP-expressing colony after a week of expansion.

FUTURE DIRECTION

Verify LIN28B-GFP expression

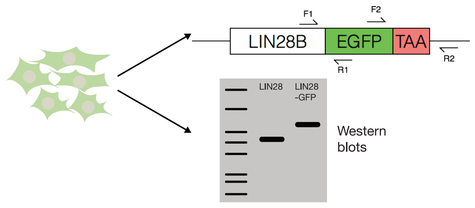

Figure 8: Once a sufficient number of cells have grown, we will perform genotyping PCRs followed by Sanger sequencing to verify in-frame knock-ins. We will also perform Western blots to verify the expression and size of the fusion LIN28B-GFP protein.

Perform screen and validate hits

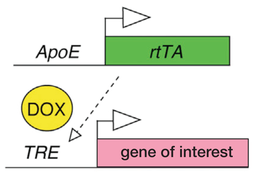

Figure 9: After verifying the cell line and performing the screen, we will validate top hits individually through single gRNA knockout, and validating its effect on *LIN28B* expression. We can then move to a mouse model using our laboratory's ApoE-rtTA mouse system, which allows liver-specific, inducible expression of genes of interest when crossed to mice with a TRE-controlled gene of interest.

Apply genome-wide screen to other genes

Templates for HDR-mediated knock-in of two important genes in hepatocellular carcinoma, and more broadly, liver development, albumin and alpha-fetoprotein (AFP) had been concurrently engineered. Once the screen is established to work, we will also perform our screens on these genes. We will also be able to generalize this powerful framework to investigate gene networks involving any gene of interest.

REFERENCES

Doench JG, Fusi N, Sullender M, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol*. 2016;34(2):184-91.

Nguyen LH, Robinton DA, Seligson MT, et al. Lin28b is sufficient to drive liver cancer and necessary for its maintenance in murine models. *Cancer Cell*. 2014;26(2):248-61