

Targeting cancer stem cells and their niche by shRNA screening

Borot F¹, Jiang L², Churchill M¹, Austin A¹, Rivera S¹, Gu W² and Mukherjee S¹

¹Department of Medicine and Irving Cancer Research Center, Columbia University School of Medicine, New York 10032, USA.

²Institute for Cancer Genetics, and Department of Pathology, College of Physicians & Surgeons, Columbia University, 1130 St. Nicholas Ave., New York, NY 10032, USA.

ABSTRACT

Cancer cells are now known to co-opt several pathways that are required for the growth and self-renewal of normal stem cells. **Targeting cancer stem cells (CSC)**, while sparing closely-related normal stem cells, is crucial to the development of cancer-targeted therapy.

To identify genes that are lethal to CSC (but not to normal stem cells), we performed a **high throughput RNA-interference screen**. We screened a library of shRNAs for their capacity to **inhibit the growth of leukemia stem cells (LSC)** but not hematopoietic stem cells (HSC) (in the context of an osteoblast niche). We also performed a parallel screen for the capacity of the same shRNAs to inhibit the growth of MSCs knocked-out for p53(KO) or knocked-out for p53 and Rb1 (DKO), both of which are known to generate tumors in mice, compared to normal mesenchymal stem cells (MSC).

Screening description

• shRNA SCREENING STRATEGY

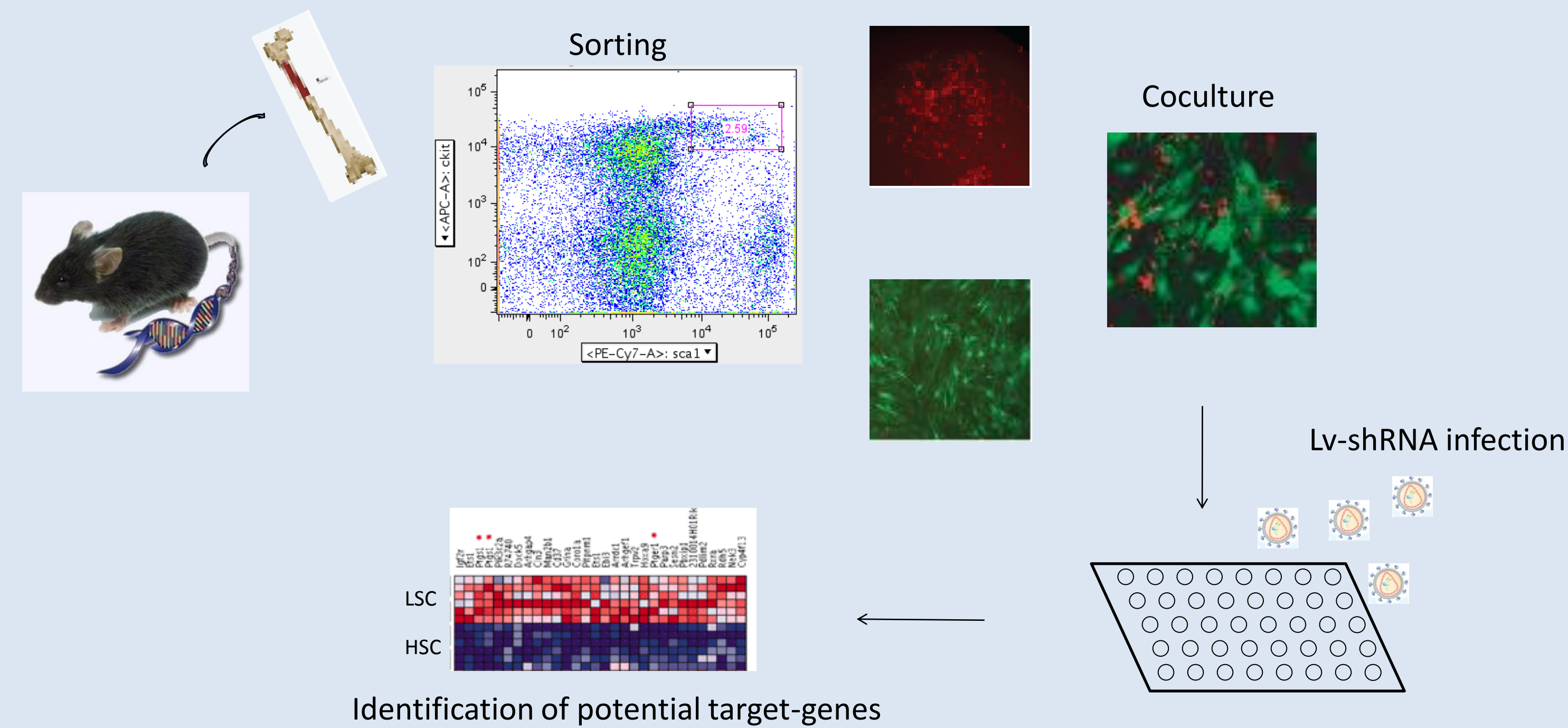


Fig.1 “Niche-based” screening of shRNAs to identify a library of genes relevant to the physiology of normal and cancer stem cells in the bone marrow microenvironment.

Primary murine HSCs and LSCs color-coded in red (from DS-Red expressing mice) and osteoblasts color-coded in green (from GFP-transgenic mice) are co-cultured in 384-well format and individual shRNAs delivered in arrayed format to each component. We have developed high content microscopy to quantify cell number, morphology and cobblestoning capacity (a measure of self-renewal ability) in these co-cultures. A parallel screen was done with primary murine MSC and KO/DKO cells (harvested from p53^{-/-} and p53^{-/-}, rb1^{-/-} mice).

• Lv-shRNA VECTORS CONSTRUCTION

Vector plasmid pLKO.1

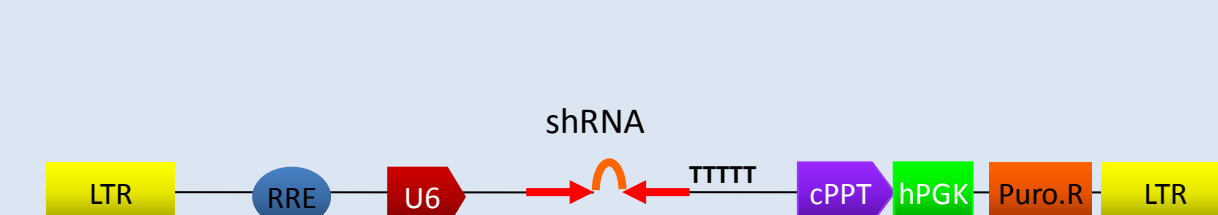


Fig.2 Lentiviral vectors expressing shRNA sequences directed against X mRNAs.

Stocks of viral particles were produced by transient cotransfection of phoenix cells with the vector plasmid (pLKO.1), the packaging vector pSPAX2 and the pVSV-G (Vesicular Stomatitis Virus pseudotype) pMD2.G envelope plasmid. The vector plasmid pLKO.1 is the backbone upon which the RNAi Consortium (TRC) has built a library of shRNAs which carries the shRNA sequence under the control of the human U6 RNA polymerase III promoter and the puromycin resistance gene under the control of the human phosphoglycerate kinase promoter (hPGK).

Identification of potential hits affecting stem cell behavior

• Nup98 SILENCING BY LENTIVIRAL MEDIATED shRNA- INDUCES SPECIFIC AND EFFICIENT REDUCTION OF LSC AND DKO/KO CELL NUMBER

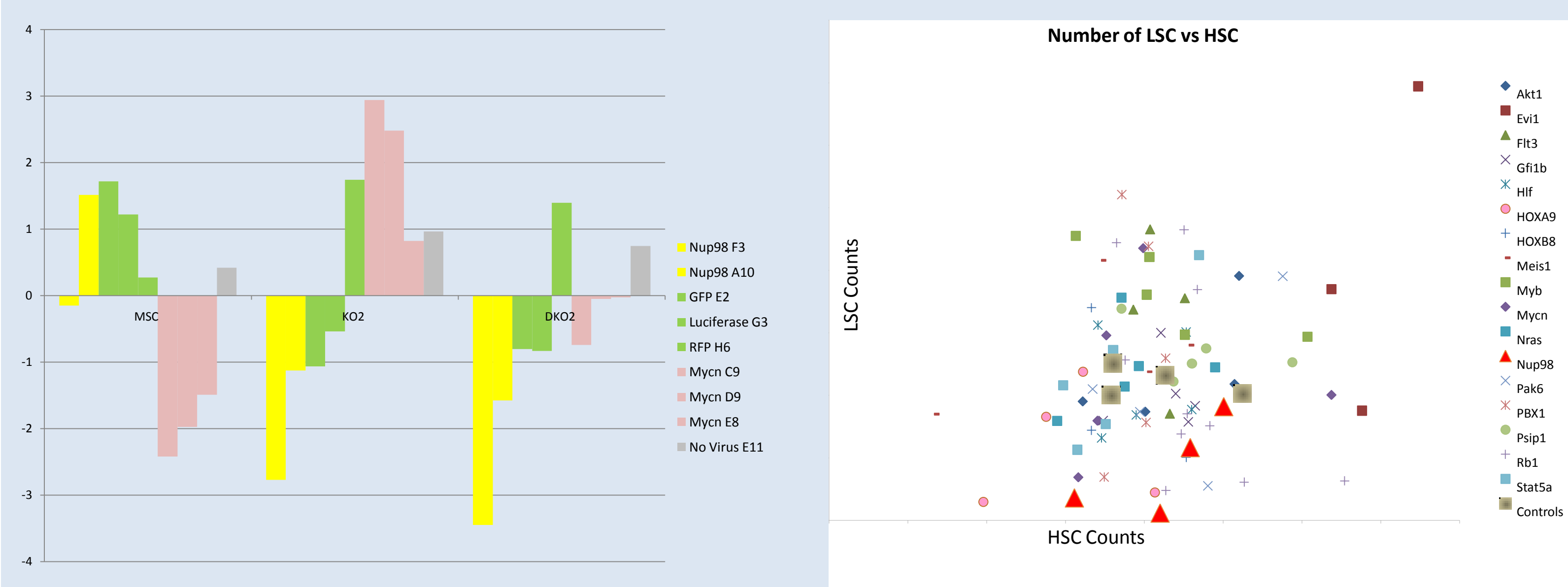


Fig.3 Example of potential “hits” hairpins that affect stem cell number. Stem cells were infected with shRNA-lentiviral particles. shRNA against GFP, RFP and Luciferase were used as negative controls and shRNA against Myc as positive control. Results are expressed as the number of SDev of the sample from the control (left) or as number of cells (right).

• Nup98 IS DIFFERENTIALLY EXPRESSED AT THE mRNA AND PROTEIN LEVEL IN CSC

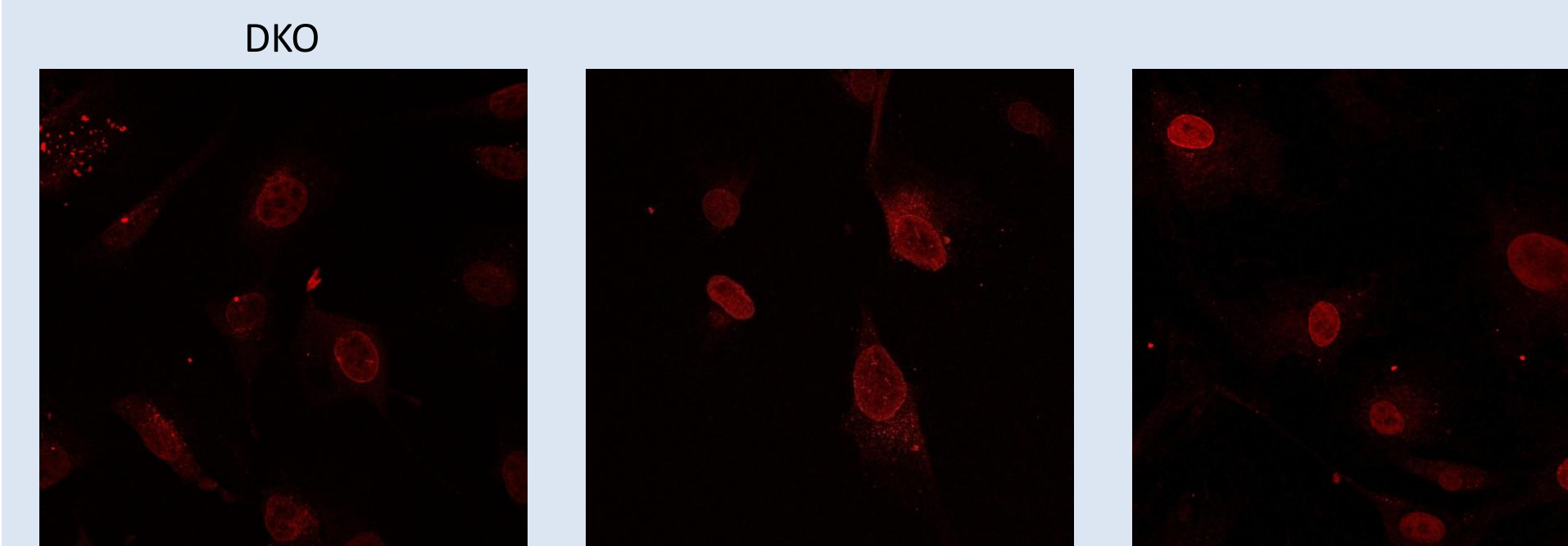


Fig.4 Immunocytochemistry of Nup98 in normal and tumor stem cells. Immunostaining was performed on fixed cells after permeabilization with Triton 0.2%.

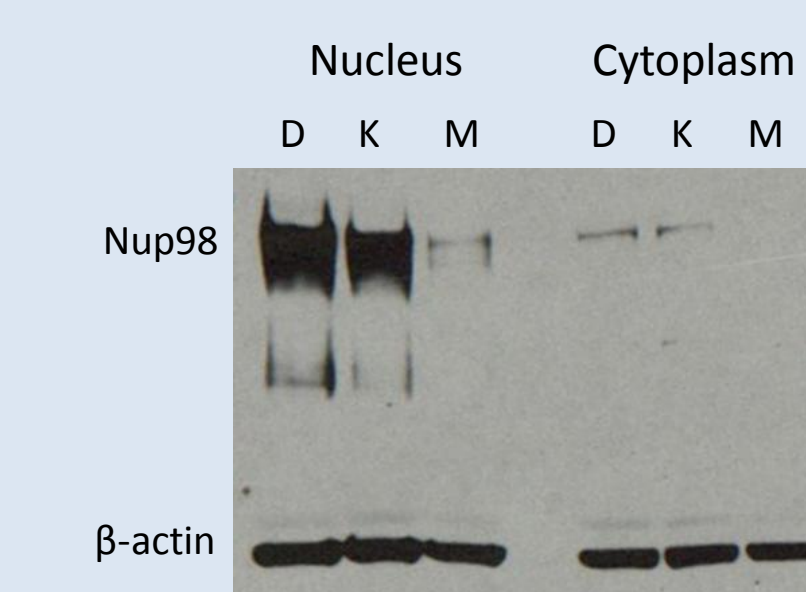


Fig.5 Expression of Nup98 in nuclear extract Cytoplasmic and nuclear extract were obtained with the NE-PER extraction reagents and 20 µg of protein were loaded after quantification with BCA assay.

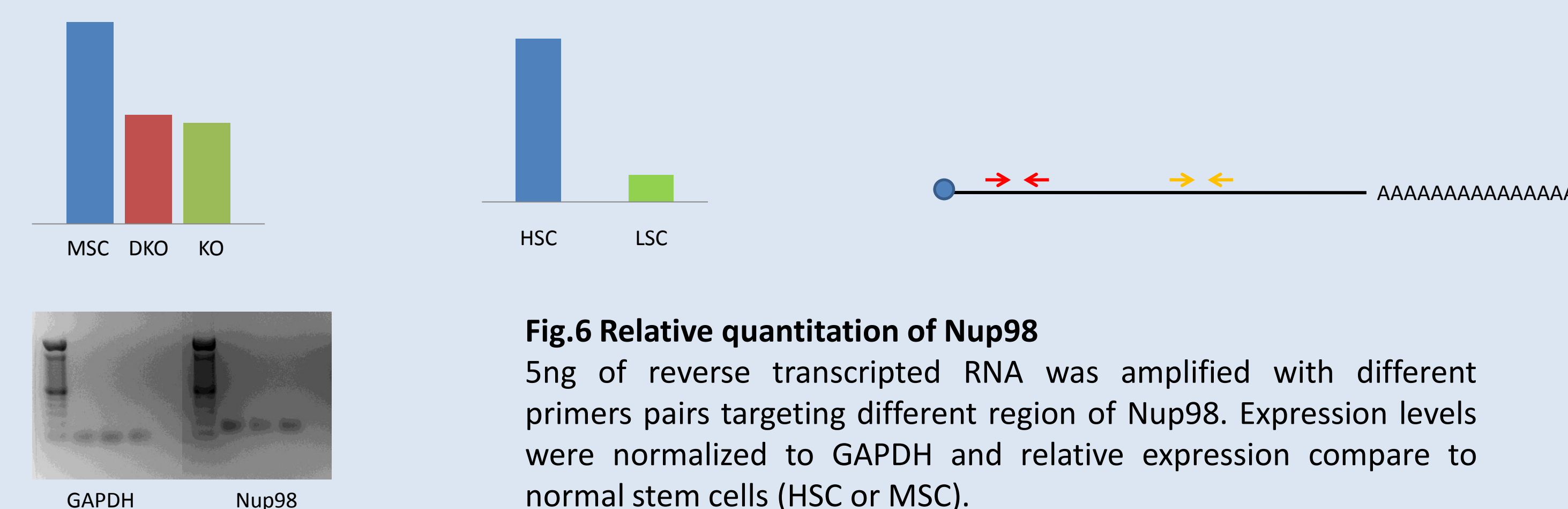
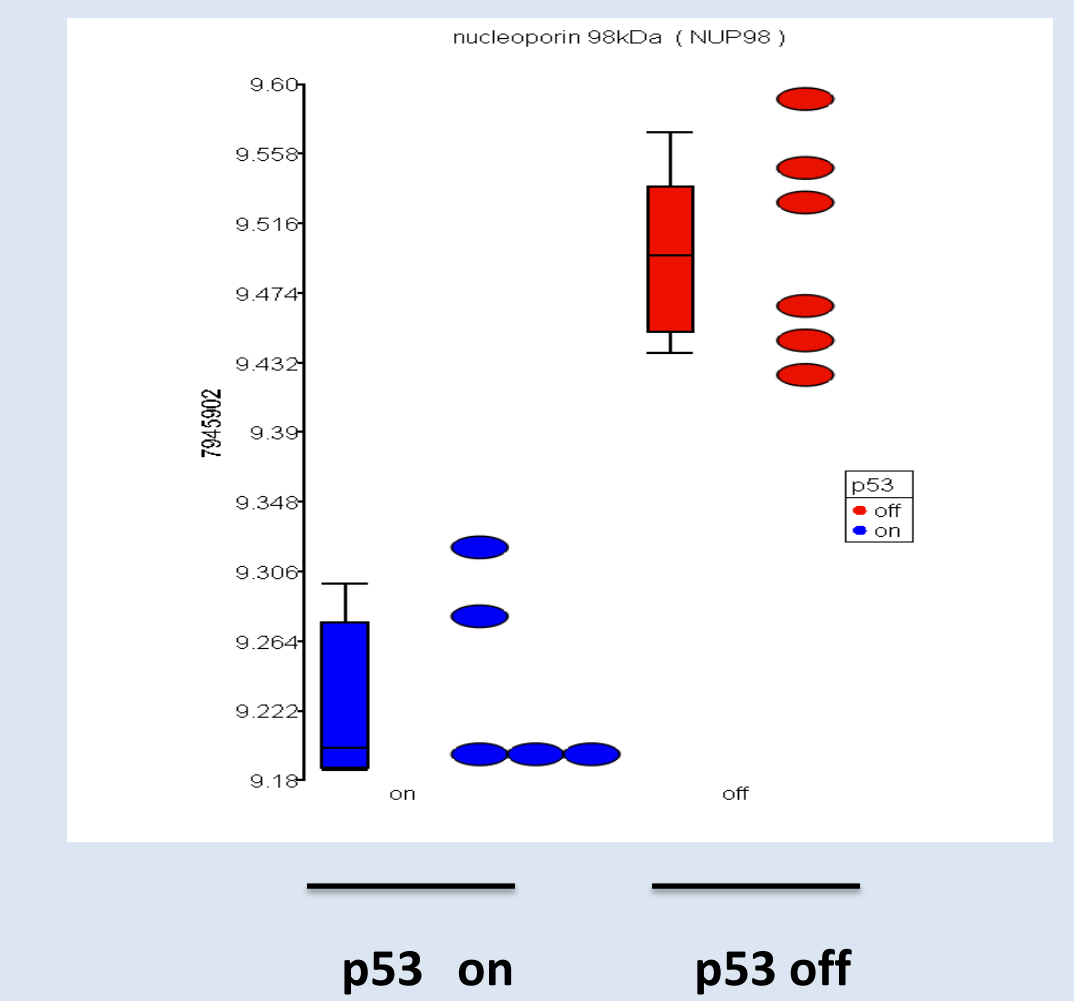


Fig.6 Relative quantification of Nup98 5ng of reverse transcribed RNA was amplified with different primers pairs targeting different region of Nup98. Expression levels were normalized to GAPDH and relative expression compare to normal stem cells (HSC or MSC).

Nup98 is a potential p53 direct repressive target

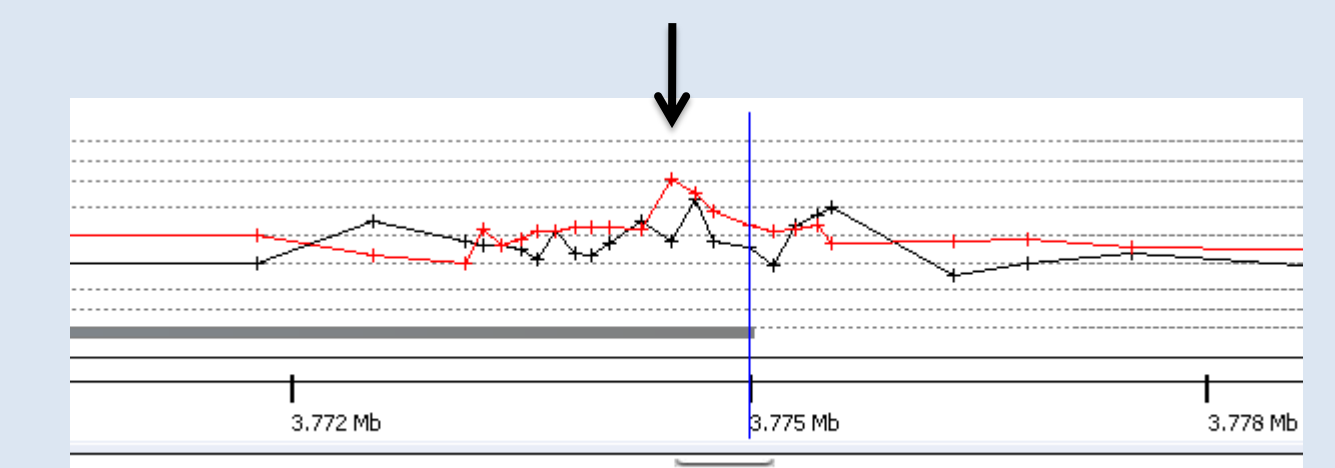
• THE p53 OVEREXPRESSION IN HUMAN H1299 CELLS REPRESSES THE Nup98 mRNA LEVEL

Fig.7 Dot plot of Nup98 mRNA expression from microarray data (y-axis shows log2 transformed value). Total RNA was extracted from a tet-off p53 stable line established in H1299 cells (p53 was activated by removing doxycycline from the media and cells were harvested 24 hours later). The total RNA was processed and hybridized to Affymetrix human gene ST 1.0 chips and data was analyzed by Partek software.



• POTENTIAL p53 BINDING SITES EXISTS IN Nup98 GENE

Fig.8 Snap shot from Agilent DNA analytics software showing potential binding location of p53 in Nup98 gene. p53 was over-expressed in H1299 cells and chromatin precipitated by two individual p53 antibodies. ChIP DNA was amplified and labeled before hybridized to Agilent Human promoter ChIP-on-chip.



CONCLUSION

shRNA against nucleoporin 98 selectively inhibits growth of the malignant stem cells and not their normal counterpart.

Nup98 could represent a specific target in CSC (in two heterologous systems) with limited effect on normal stem cells.

These results also raise the possibility that Nup98 may be a novel target of p53, thereby connecting nuclear transport to the p53 pathway.

Understanding specific modulators of CSC in their microenvironment and CSC-specific pathway will provide paradigms of the biology of cancer stem cells and open new perspectives in therapeutics

Further studies will investigate (a) the mechanism of the specificity of Nup98 knockdown in normal stem cells and cancer cells (b) relationship between p53 deletion and Nup98 expression.