AD______________

Award Number: W81XWH-F€ËFË€HÏ J

TITLE: Ô[{]|^{ ^}cæeã[}Á[-ÁT ˆ^|[åˆ•] |æ• cã&ÁÙˆ}å¦[{ ^ÁÔ|[}^•Á¸ ãc@ÁŠ^}cãçã ˇ•ÁÒ¢] ¦^••ã[}ÁŠãã¦æ¦ã^•

PRINCIPAL INVESTIGATOR: Öæ}ã^|ÁŠã}å}^¦

CONTRACTING ORGANIZATION: V@^ÁÔ|^ç^|æ}åÁÔ|ã}ã&ÁØ[ˇ}åæã[}
Ô|^ç^|æ}åÉÁUPÁ IFJÍ Á

REPORT DATE: Ju|ˆÁG€FG

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2012	Annual	1 JUL 2011 - 30 Jun 2012

4. TITLE AND SUBTITLE
Complementation of Myelodysplastic Syndrome Clones with Lentivirus Expression Libraries

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0379

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Daniel Lindner

E-Mail: lindned@ccf.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Cleveland Clinic Foundation
Cleveland, OH 44195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have successfully prepared sense expression libraries, characterized the size of their inserts which are fused to a green fluorescent protein tag, and packaged these libraries into lentivirus. Four different human MDS bone marrow isolates have been transfected with lentivirus at multiplicity of infection of 0.1. A collection of 252 puromycin resistant clones were isolated, genomic DNA was prepared, and clones were screened for ability to undergo myeloid differentiation in response to GM-CSF. Only ~30% of the puromycin resistant clones (78) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 78 clones. DNA sequencing has identified the cDNA inserts. These inserts were expressed in naïve MDS cell pools. Eight of these cDNAs were validated; they induced myeloid colonies in vitro and engrafted in the marrow of SG3, but not NSG mice.

15. SUBJECT TERMS
Myelodysplastic syndrome, lentivirus, cDNA libraries, complementation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

Introduction……………………………………………………………….………..….. 4

Body………………………………………………………………………….………..….. 4-8

Key Research Accomplishments………………………………………….…….. 8

Reportable Outcomes……………………………………………………………… 8

Conclusion…………………………………………………………………………… 9

References……………………………………………………………………………. 10

Introduction

Rationale: The focus of our proposal is the identification of normal human genes, that when overexpressed by transduction of a lentiviral vector, complement the underlying genetic defect in MDS cells. Our alternate approach was to downregulate gene expression using shRNA libraries, and identify genes whose overexpression promote the MDS phenotype. Our ultimate endpoint is the detection of terminal differentiation and colony formation resulting in normal myeloid cells.

Hypothesis: Expression of a normal human cDNA library in MDS cells will correct the aberrant phenotype and permit normal proliferation and myeloid differentiation in selected clones.

Body

In this study we test the assumption that over-expression of a wild type gene, or suppression of an abnormally expressed gene by shRNA, in a transfected MDS clone will interrupt the pathogenic signaling that gives rise to the MDS phenotype. Therefore, the transfected gene will permit normal differentiation of the MDS cell into a normal myeloid lineage. The first objective (months 1 – 6 in SOW) was to prepare and characterize the lentivirus libraries.

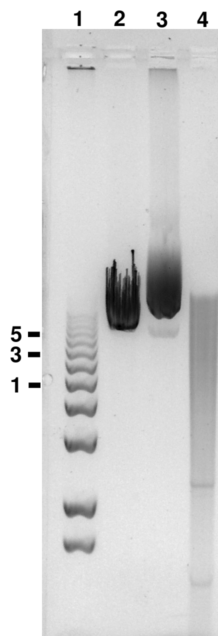


Fig. 1. Construction of human liver cDNA expression library. Library contains ~7 x 10^7 individual clones, and the average insert size is >2 kb. Lane 1: marker, size indicated in kb; Lane 2: linearized plasmid; Lane 3: plasmid and ligated insert; Lane 4: human liver cDNA insert prior to ligation.

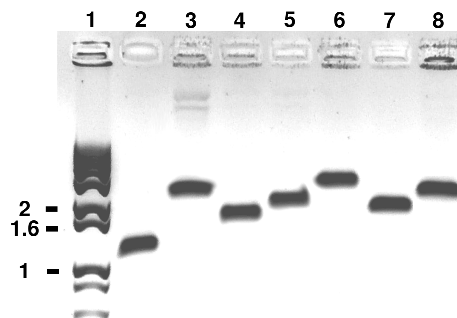


Fig. 2. Characterization of lentivirus cDNA expression library. Following packaging and titering of the lentivirus preparation, 293T cells were infected with library at multiplicity of infection of 0.1 and selected with puromycin x 1 wk. Individual surviving clones were expanded over 10 days and screened by PCR using LTR-specific primers. Lane 1: marker, size indicated in kb; Lanes 2 - 8: PCR product from individual clones.

Our next goal was to transfect human MDS cells with lentivirus, select for survivors with puromycin, and test for expression of cDNAs contained in the library. Surviving colonies were dissociated, plated as replicas, and aliquots were cryopreserved for future murine studies. Replated colonies were tested for their proliferative and differentiative response to GM-CSF (months 3 – 9 in SOW).

Fig. 3. Transfection of human MDS cells with lentivirus cDNA expression library. MDS bone marrow cells from 5q- (pictured at right), mono 7 / 7q-, trisomy 8, and del 20q were transfected at an moi of 0.1 with the lentivirus library described above. After 14 days growth in soft agar surviving colonies were harvested, dissociated, replated as replicates, and aliquots were cryopreserved. Cells transfected with empty lentivirus particles did not generate proliferating colonies. 200x

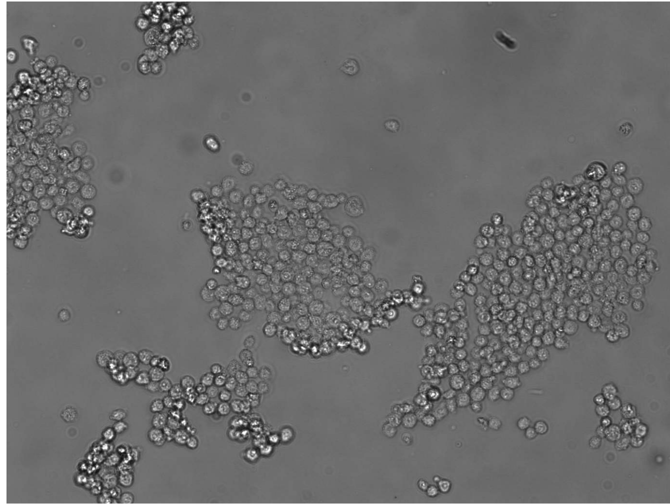


Fig. 4. Detection of lentivirus-encoded GFP in transfected human MDS cells. cDNA inserts encode GFP fusion proteins. Fluorescence microscopy demonstrated strong expression of GFP in puromycin-resistant surviving colonies. 200x

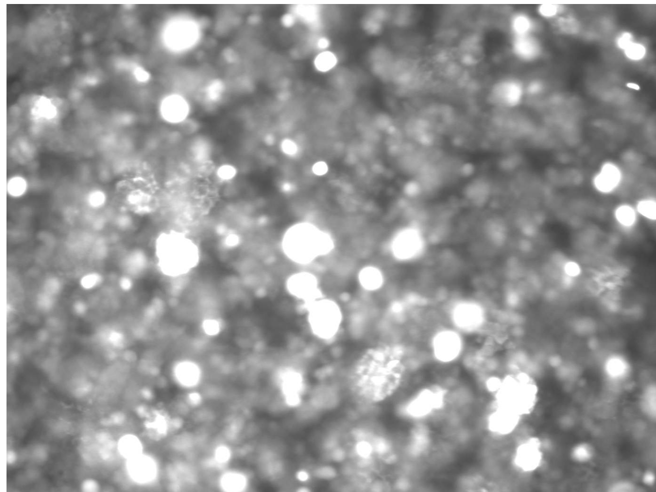
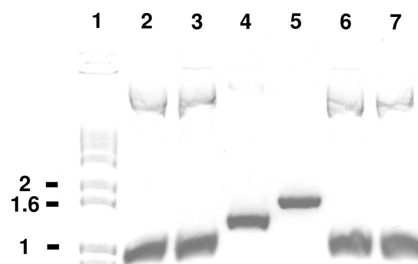


Fig. 5. Expression of lentivirus-encoded cDNA-GFP fusion proteins in transfected human MDS cells. Seven GFP-positive colonies were isolated and screened by PCR using LTR-specific primers to demonstrate that fluorescent MDS cells also retained expression of the encoded library-derived cDNA.

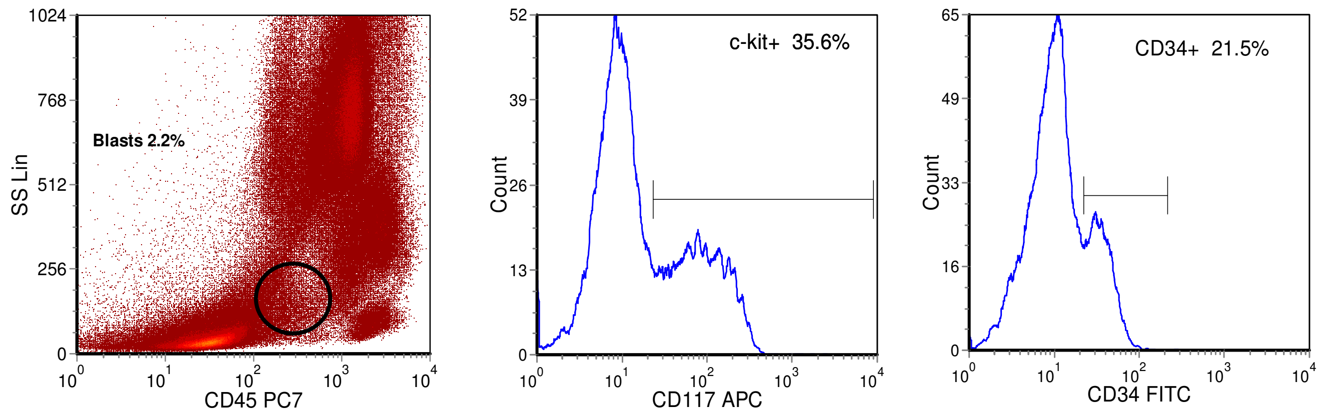


A total of 252 puromycin-resistant 7q- clones were isolated, genomic DNA was prepared, and clones were screened for ability to undergo myeloid differentiation in response to GM-CSF in vitro. Only ~30% of the puromycin resistant clones (78) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 78 clones. These inserts were PCR amplified from isolated colonies, packaged into lentivirus and used to infect naïve MDS cell pools. Eight of these cDNAs were validated; that is, they induced myeloid colonies in transfected MDS cells vitro. Sequencing identified the cDNA inserts (Table 1).

Table 1	
Symbol	Description
HRAS	Homo sapiens v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), transcript 1
CDC25C	Homo sapiens cell division cycle 25 homolog C (CDC25C), transcript variant 1
MYC	Homo sapiens v-myc myeloctomatosis viral oncogene homolog (avian) (MYC)
MAP3K7	Homo sapiens mitogen-activated protein kinase kinase kinase 7 (MAP3K7)
MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8)
SF3B1	Homo sapiens splicing factor 3b, subunit 1, 155kDa (SF3B1), transcript variant 1
SIK1	Homo sapiens salt-inducible kinase 1 (SIK1)
TET2	Homo sapiens tet oncogene family member 2 (TET2), transcript variant 2

The next objective was to characterize surface markers and phenotype the successful transfectants (months 6 – 12 in SOW). Normal bone marrow cells have a low percentage of blasts (~2-3%), represented by gated population (Fig. 7, black circle). This gated population has low expression of c-kit and CD34 in normal marrow. MDS marrow with elevated blasts (lower panels) have high expression of c-kit and CD34. By flow cytometry, the expression of c-kit and CD34 was unchanged in our collection of eight lentivirus-transfected MDS isolates when compared to mock-transfected or empty virus transfected cells.

Normal Bone Marrow



MDS with blasts Bone Marrow

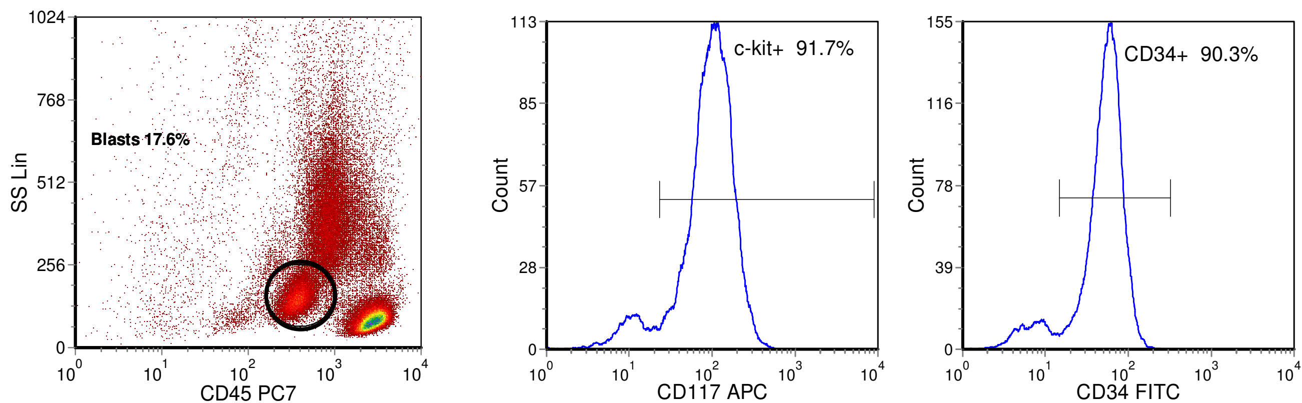


Fig. 7. Characterization of MDS surface markers. Normal human bone marrow (upper panels), and 7q- MDS marrow (lower panels) was stained for CD45 (leukocyte common antigen), CD34 (stem cell marker), and CD117 (c-kit).

We inoculated NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ mice (NSG mice) (1-8) with 7q- transfectants expressing the nine candidate genes. Our objective was to test ability of transfectants to engraft NSG mice and generate chimeric bone marrow (months 12 – 18). NSG mice did not engraft well with transfected human normal or MDS marrow. Engraftment was defined as presence of >5% human CD45 cells in murine marrow sixty days after inoculation. Therefore, we utilized SG3 mice (Jackson Laboratories), in which NSG mice express three human trans genes: stem cell factor, GM-CSF, and IL-3, all of which support myeloid growth and differentiation (9). Mice were sacrificed at 3 and 6 months following inoculation with transfected MDS cells to evaluate the extent of engraftment. The transfected clones were all able to engraft SG3 mice to varying degrees and persisted in marrow up to 6 months (Table 2). Empty lentivirus-transfected MDS cells (Null) were unable to engraft murine marrow. In no case were human CD45 cells detectable in peripheral blood.

Table 2			
Symbol	% engraftment (human CD45 vs. murine)	Presence of human CD45 in peripheral blood	Duration of marrow engraftment (mo)
Null	0	-	0
HRAS	35	-	6
CDC25C	27	-	3
MYC	41	-	3
MAP3K7	14	-	6
MAP3K8	11	-	3
SF3B1	19	-	6
SIK1	13	-	3
TET2	20	-	6

Key Research Accomplishments

1. Creation of cDNA lentivirus libraries
2. Transfection of human MDS marrow with lentivirus libraries
3. Expression of cDNA-GFP fusion proteins in MDS cells
4. Identification of eight cDNAs that drive myeloid differentiation in MDS cells
5. Characterization of markers associated with MDS blasts

Reportable Outcomes

This data was reported at the 2011 Case Comprehensive Cancer Center Retreat, Corporate College East, July 8, 2011.

This data will be presented at the 2012 Case Comprehensive Cancer Center NCI P30 Site Visit, Corporate College East, September, 2012.

Conclusion

We have identified eight genes that when overexpressed, conferred proliferative and differentiative capabilities to MDS isolates. Some of these were found to be cellular oncogenes such as Ras, Myc, and Tet2. Their identification in this genetic screen give support to the validity of the method. Another candidate, CDC25C is a tyrosine phosphatase that drives cellular entry into mitosis. The mitogen-activated protein kinase kinase kinase 7 (MAP3K7) controls transcriptional regulation, whereas MAP3K8 may function as an oncogene. SIK1 is a kinase that associates with the Na+K+ATPase responsible for sodium transport and maintaining cell volume. Function or overexpression of SIK1 has not previously been associated with growth promotion. Hence, in the context of MDS, this may represent a novel gene that modulates cell growth control and differentiation. SF3B1 and TET2 are particularly exciting candidates to emerge from this screen, since mutation of both of these genes has been observed in a significant fraction of MDS patients. Thus, in both of these cases, complementation with the wild type cDNA had the effect of permitting colony formation in vitro and chimeric engraftment in vivo.

We have identified eight cDNAs that when expressed singly in human MDS bone marrow cells have been able to confer a phenotype in which transfected MDS clones exhibited gain of function and regained the ability to differentiate into functional myeloid cells and supported successful engraftment of SG3 mice. Identification of some of these cDNAs that can successfully complement the MDS phenotype can be potentially investigated in future human gene therapy trials. Isolation of SIK1 suggests that there are alternate pathways that when activated, can confer a normal differentiation phenotype in myeloid cells. Due to the unbiased, completely random nature of this complementation technique, and the underlying abnormal biochemical pathways at work in the MDS clones, it is unlikely that simple complementation with a single cDNA will correct a majority of MDS defects and allow normal differentiation to proceed in patients. We never observed circulating human CD45 cells in peripheral blood, suggesting that SG3 mice, while and improvement over the NSG strain, is still not an ideal model system for study of MDS. However, the successful long term engraftment (6 mo) of transfected MDS cells, compared to the inability of untransfected cells to engraft, suggest that expression of these cDNAs in trans is sufficient to enhance cell survival and differentiation in the marrow compartment.

References

1. Agliano, A., I. Martin-Padura, P. Mancuso, P. Marighetti, C. Rabascio, G. Pruneri, L. D. Shultz, and F. Bertolini. 2008. Human acute leukemia cells injected in NOD/LtSz-scid/IL-2Rgamma null mice generate a faster and more efficient disease compared to other NOD/scid-related strains. Int J Cancer 123:2222-7.
2. Ishikawa, F., S. Yoshida, Y. Saito, A. Hijikata, H. Kitamura, S. Tanaka, R. Nakamura, T. Tanaka, H. Tomiyama, N. Saito, M. Fukata, T. Miyamoto, B. Lyons, K. Ohshima, N. Uchida, S. Taniguchi, O. Ohara, K. Akashi, M. Harada, and L. D. Shultz. 2007. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol 25:1315-21.
3. Ito, M., H. Hiramatsu, K. Kobayashi, K. Suzue, M. Kawahata, K. Hioki, Y. Ueyama, Y. Koyanagi, K. Sugamura, K. Tsuji, T. Heike, and T. Nakahata. 2002. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 100:3175-82.
4. King, M., T. Pearson, L. D. Shultz, J. Leif, R. Bottino, M. Trucco, M. A. Atkinson, C. Wasserfall, K. C. Herold, R. T. Woodland, M. R. Schmidt, B. A. Woda, M. J. Thompson, A. A. Rossini, and D. L. Greiner. 2008. A new Hu-PBL model for the study of human islet alloreactivity based on NOD-scid mice bearing a targeted mutation in the IL-2 receptor gamma chain gene. Clin Immunol 126:303-14.
5. Majeti, R., C. Y. Park, and I. L. Weissman. 2007. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1:635-45.
6. Shultz, L. D., F. Ishikawa, and D. L. Greiner. 2007. Humanized mice in translational biomedical research. Nat Rev Immunol 7:118-30.
7. Shultz, L. D., B. L. Lyons, L. M. Burzenski, B. Gott, X. Chen, S. Chaleff, M. Kotb, S. D. Gillies, M. King, J. Mangada, D. L. Greiner, and R. Handgretinger. 2005. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 174:6477-89.
8. Simpson-Abelson, M. R., G. F. Sonnenberg, H. Takita, S. J. Yokota, T. F. Conway, Jr., R. J. Kelleher, Jr., L. D. Shultz, M. Barcos, and R. B. Bankert. 2008. Long-term engraftment and expansion of tumor-derived memory T cells following the implantation of non-disrupted pieces of human lung tumor into NOD-scid IL2Rgamma(null) mice. J Immunol 180:7009-18.
9. Wunderlich, M., F. S. Chou, K. A. Link, B. Mizukawa, R. L. Perry, M. Carroll, and J. C. Mulloy. 2011. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia 24:1785-8.