Knockdown of *Hnrnpa0*, a del(5q) gene, alters myeloid cell fate in murine cells through regulation of AU-rich transcripts

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ABSTRACT

The control of mRNA stability plays a central role in orchestrating gene-regulatory networks in hematopoietic cell growth, differentiation and tumorigenesis. *HNRNPA0*, which encodes an RNA-binding protein shown to regulate transcript stability via binding to the AU-rich elements of mRNAs, is located within the commonly deleted segment of 5q31.2 in myeloid neoplasms with a del(5q), and is expressed at haploinsufficient levels in these patients. We show that *HNRNPA0* is normally highly expressed in hematopoietic stem cells and exhibits dynamic changes in expression during the course of differentiation. To model *HNRNPA0* haploinsufficiency, we used RNAi interference in primary murine cells and an experimental cell system, and found that reduced Hnrnpa0 expression leads to a shift from monocytic towards granulocytic differentiation. Microarray-based global expression profiling revealed that *Hnrnpa0* knockdown disproportionally impacts AU-rich containing transcripts and alters expression of myeloid specification genes. In therapy-related myeloid neoplasms with a del(5q), AU-rich containing mRNAs are enriched in transcripts that encode proteins associated with increased growth and proliferation. Our findings implicate haploinsufficiency of *HNRNPA0* as one of the key initiating mutations in the pathogenesis of myeloid neoplasms with a del(5q), and suggest that therapies that target AU-rich elements warrant consideration in efforts to develop new mechanism-based treatment strategies.

Introduction

Therapy-related myeloid neoplasms (t-MNs) are a late complication of cytotoxic therapy typically for a primary malignant disease, and are characterized by a poor prognosis.[1-3] Notably, for patients treated with alkylating agents, deletions of the long arm of chromosome 5, del(5q), or unbalanced rearrangements leading to the loss of chromosomal material from 5q, occur in approximately 40% of cases.[3-5] Previously, we defined a 970 kb commonly deleted segment (CDS) at 5q31.2 that is lost in all t-MN and acute myeloid leukemia (AML) *de novo* patients with abnormalities of chromosome 5.[6] This region contains 19 genes and 1 micro-RNA sequence; however, none of the genes revealed inactivating mutations or silencing by DNA methylation of the remaining allele.[6,7] For this reason, we advanced the hypothesis that AML with a del(5q) results from haploinsufficiency of one or more genes on 5q. One del(5q) candidate of interest is the heterogeneous nuclear ribonucleoprotein A0 (*HNRNPA0*) gene encoding a RNA-binding protein that binds to adenylate-uridylate (AU)-rich elements (AREs) and is believed to be a positive regulator of transcript stability.[8-10] In t-MNs with a del(5q), *HNRNPA0* transcript levels are approximately 50% that of control subjects,[11] suggesting that a reduced or haploinsufficient dosage of *HNRNPA0* may be relevant to this genetic subgroup.

AU-rich RNA binding proteins (AUBPs) provide the cell with a rapid and precise mechanism to alter gene expression patterns in response to extracellular stimuli.[12] Although some AUBPs direct ARE mRNAs toward rapid decay, others increase stability of their mRNA ligands, and there is growing appreciation that many AUBPs serve both functions depending on the target gene and cellular context (e.g. HuR).[13] Moreover, it has been recognized that ARE-mediated decay and translational roles of AUBPs are influenced by miRNAs, the other well-known regulator of mRNA stability.[14] Several AUBP mRNA targets encode proteins that regulate cell growth and survival, such as cytokines, tumor suppressors and oncoproteins, and AUBPs have been identified as key regulators of both normal and malignant hematopoiesis, including AUF, HuR, KSRP/KHSRP, nucleolin, and members of the ZFP36 family.[13,15]

HNRNPA0 expression is up-regulated by p38-dependent signaling in response to LPS, mycobacterial proteins, heat shock and IL-3 stimulation, and the protein is phosphorylated at Ser84 by MAPKAP-K2 downstream of p38 signaling, enabling it to bind to AU-rich mRNA targets.[10,16] Modulation of mRNA stability by *HNRNPA0* has also been implicated in controlling cell cycle, especially with regard to DNA damage checkpoints.[9] Little is known of the role of *HNRNPA0* in hematopoiesis and leukemogenesis. Given its putative role of stabilizing ARE-containing transcripts, we hypothesized that reduced function of *HNRNPA0* would lead to a decrease in the stability and effective expression of target genes that may

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have a profound impact upon hematopoiesis, and consequently contribute to the changes seen in t-MN patients.

In this study, we now add *HNRNPA0* to the growing list of AUBPs that are recognized for their vital roles in hematopoiesis and leukemogenesis. Herein, we show that *HNRNPA0* is highly expressed in hematopoietic stem cells, and its expression exhibits dynamic changes during the course of differentiation. Deletion of a single allele of *HNRNPA0* is associated with haploinsufficient transcript levels in CD34$^+$ cells from t-MN patients with a del(5q). Modeling *HNRNPA0* haploinsufficiency in mouse cells alters myeloid lineage fate, in part through changes in the expression of ARE-containing genes, suggesting that a decreased dose of *HNRNPA0* in t-MN patients may contribute to leukemogenesis. Moreover, we determined that ARE mRNAs in t-MN patients with a del(5q) are enriched in transcripts that encode proteins associated with increased growth and proliferation.

Methods

Retroviral transduction and in vitro *differentiation of PUER and primary mouse hematopoietic cells*

A short interfering RNA hairpin (shRNA), designed to match bases 288 to 306 of the open reading frame of *Hnrnpa0* with a 9 nucleotide loop in the center, or a scrambled, irrelevant sequence,[17] was cloned into pBanshee-GFP (an MSCV-based construct with P_{CMV}-driven GFP). The PUER cell line or mouse bone marrow cells from BALB/cAnNTac mice (Taconic, Hudson, NY, USA) were transduced by spinoculation with viral supernatants from HEK-293T cells co-transfected with the shRNA-containing pBanshee and pCL-ECO packaging plasmids (Imgenex, San Diego, CA, USA) using Effectene (Qiagen, Germantown, MD, USA). Two days post infection, the cells were sorted on a FACSAria (BD Biosciences, San Jose, CA, USA) for GFP positivity. All animal studies were approved by the University of Chicago Institutional Animal Care and Use Committee and mice were housed in a fully-AALAC-accredited facility.

GFP$^+$ sorted PUER cells, expressing the *Hnrnpa0* or control shRNA, were expanded in IL-3 for four days and then treated with 1-5 nM 4-hydroxytamoxifen (4-OHT, >98% (TLC) Z-isomer, Sigma, St. Louis, MO, USA) to induce macrophage differentiation. For gene expression, flow cytometry and morphological analyses, cells were sampled at 24 h, 4 days, and 7days, respectively. GFP$^+$ bone marrow cells were plated in MethoCult GF M3434 (StemCell Technologies, Vancouver, BC, Canada). After 12 days of incubation, colonies of greater than 50 cells were scored for morphology by inverted light microscopy. Cells were then collected and stained with Mac-1 (CD11b) antibodies in combination with either F4/80 or Gr-1 (Ly-6G) antibodies, and analyzed on a FASCanto benchtop flow cytometric analyzer (BD Biosciences, San Jose, CA, USA).

Patient samples

All 38 t-MNs used for gene expression profiling analysis were obtained from patients treated with chemotherapy and/or radiation therapy at the University of Chicago who were diagnosed with t-MN between January 1984 and July 2007. Ten of the 38 samples had a del(5q) or unbalanced translocation leading to loss of 5q based on cytogenetic analysis. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki. Study approval was obtained from the St. Jude Children's Research Hospital and the University of Chicago Institutional Review Boards.

Gene expression profiling

GFP$^+$ sorted PUER cells, transduced with retroviral contructs containing either the *Hnrnpa0* shRNA or control vector, were expanded for four days and then harvested for total mRNA processing before 4-OHT induction (undifferentiated) and 24 h after treatment with 2nM 4-OHT (differentiated). Samples were hybridized to the 4x44K Whole Mouse Genome Oligo Microarray platform (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocols. Contingency analysis and gene set enrichment analysis (GSEA) were used to determine differential AU-rich gene expression. For human t-MN samples, total RNA was extracted from cryopreserved mononuclear cells from bone marrow or peripheral blood and gene expression profiling was performed using Affymetrix GeneChip HT HG-U133$^+$ PM microarrays according to the manufacturer's instructions. Gene expression data have been deposited in Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo/*. Accession numbers GSE39934 (PUER) and GSE39991 (t-MN)).

Results

Expression analysis of HNRNPA0 *in t-MNs with a del(5q) and during normal hematopoiesis*

In myeloid neoplasms with a del(5q), expression profiling of CD34$^+$ cells revealed that *HNRNPA0* transcript levels are on average 50% that of normal subjects and patients lacking abnormalities of chromosome 5 (Figure 1A), demonstrating that *HNRNPA0* is expressed at haploinsufficient levels in these patients. However, there is some variability, with some patients showing up to an 80% loss of *HNRNPA0* expression (Figure 1B and *Online Supplementary Table S1*). To determine whether *HNRNPA0* may also play a role in normal hematopoiesis, we analyzed expression in human hematopoietic cells, using previously published data[18] (GEO accession GSE24759). This analysis showed that *HNRNPA0* is dynamically expressed during hematopoiesis (Figure 1C). *HNRNPA0* is highly expressed in HSCs, CMPs and MEPs and suppressed as cells differentiate towards different hematopoietic lineages, most notable in the mature erythroid and lymphoid lineages. We found that *Hnrnpa0* expression was also dynamically expressed during mouse hematopoiesis, e.g. *Hnrnpa0* expression decreases as cells terminally differentiate (*Online Supplementary Figure S1*). Thus, *HNRNPA0* is highly expressed in multipotent hematopoietic progenitors, exhibits dynamic dosage changes during the course of differentiation, and is expressed at haploinsufficient levels in myeloid neoplasms with a del(5q).

Hnrnpa0 *knockdown shifts differentiation from monocytes to granulocytes*

To model *HNRPA0* loss in mouse cells, we developed a short-hairpin interfering RNA (shRNA) directed at the open reading frame of *Hnrnpa0*. RT-PCR analysis of total mouse bone marrow cells infected with the hairpin-expressing construct showed that *Hnrnpa0* transcript levels were only 15-25% of the level in cells expressing the control vector (Figure 2A). A colony forming assay showed that the number of BFU E, CFU GEMM and CFU GM colonies were not significantly different between the control vector and hairpin-expressing cells (P=0.3-1.0). However, hairpin-expressing cells showed a 38% increase in CFU G colonies (P=0.01) and a 35% decrease in CFU M colonies (P=0.0001) (Figure 2B). These results were con-

firmed with flow cytometric analysis. Hairpin-expressing cells showed a 38% increase in granulocytes (Mac-1^{lo}Gr-1^{hi}F4/80^{-}; P=0.0007) and a 27% decrease in monocytes (Mac-1^{hi}Gr-1^{lo}F4/80^{+}; P=0.07) (Figure 2C). Taken together, these results show that *Hnrnpa0* knockdown (KD) causes a shift in differentiation of myeloid progenitors that favors the granulocytic lineage over the monocytic lineage. The fact that t-MNs with a del(5q) are characterized by trilineage dysplasia (dysgranulopoiesis, dyserythropoiesis and dysmegakaryopoiesis) suggests that there is a defect in the function of the early myeloid progenitor cell. Our data raise the possibility that loss of *HNRNPA0* may be involved in deregulating normal myeloid differentiation, a key component in the changes that drive leukemic growth.

Hnrnpa0 *knockdown inhibits monocytic differentiation in PUER cells*

To test the hypothesis that a gene signature, that regulates myeloid cell fate, is altered by *Hnrnpa0* KD, we adopted the well-characterized PUER cell line as a model system for myeloid differentiation.[19,20] We chose PUER cells because this cell line was previously used in elegant studies to describe the gene regulatory network directing macrophage and granulocyte cell fate. The PUER cell line is a PU.1$^{-/-}$ (*Spi1*$^{-/-}$) fetal liver progenitor cell line engineered to express Spi1/PU.1 (when treated with 4 OHT). We established that our PUER cells differentiate in a dose-dependent manner into F4/80^{+} macrophages when treated with doses as low as 1-5 nM 4-OHT (*Online Supplementary Figure S2*). We and others (H. Singh *et al.,* personal communication, 2010) have noted that these drug concentrations are lower than the original publication,[19] suggesting that the PUER cell line has evolved and/or there were variations in drug purity. PUER cells were transduced with the control or *Hnrnpa0*-specific shRNA, and RT-PCR and Western analysis confirmed that *Hnrnpa0* expression was approximately 33-40% the level of control cells (Figure 3A and B). *Hnrnpa0* KD led to a significant reduction (P≤0.03)

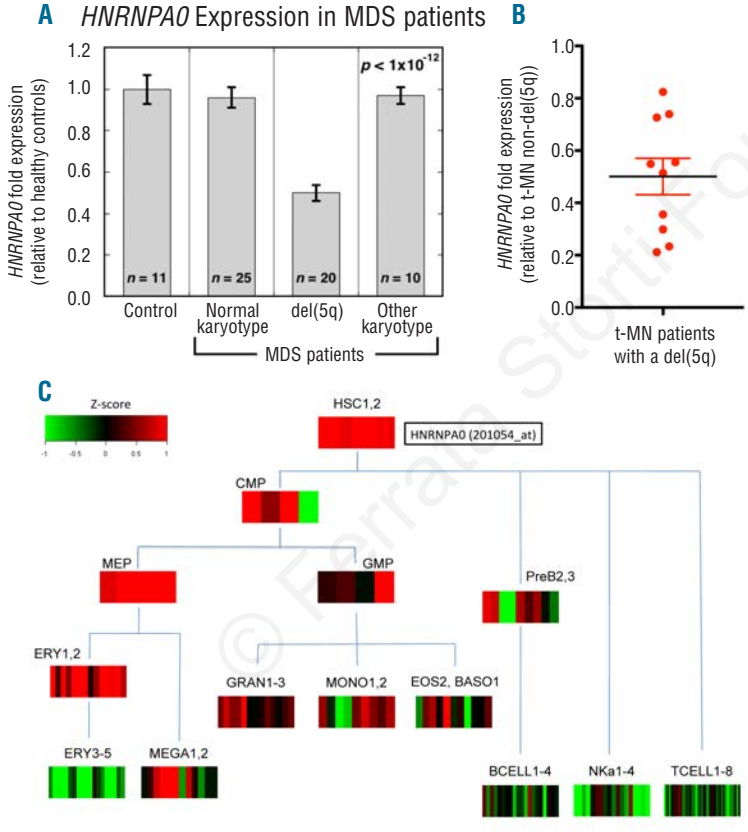

Figure 1. Expression of *HNRNPA0* in MDS and during normal hematopoiesis. **A.** Microarray expression analysis of CD34^{+} cells from healthy individuals and patients with MDS (data generated from GEO accession: GSE4619). The number of subjects in each group, and standard error bars are indicated. Patients with a del(5q) had significantly lower (P<1x10^{-12}) expression than each of the other groups of patients, or control CD34^{+} cells. **B.** Microarray expression of mononuclear cells from t-MN patients with or without a del(5q) (data generated from GEO accession: GSE39991). *HNRNPA0* expression in 10 t-MN patients with a del(5q) were expressed relative to the average *HNRNPA0* expression in t-MN patients without a del(5q). This analysis revealed that, on average, *HNRNPA0* is expressed at haploinsufficient levels, but some variability exists among patients. **C.** Gene expression analysis in human hematopoietic cells using previously published data (GEO accession GSE24759) from Novershtern *et al.*[18] Cell populations (defined in *Online Supplementary Methods*) representing hematopoietic stem and progenitor cells, terminally differentiated cells and intermediate states were purified from 4-7 independent donors. For easier visualization, populations within the same lineage are aligned. Expression signals were normalized by RMA and batch-corrected by the ComBat method. The resulting probe expression signals were z-transformed for visualization.

Table 1. KEGG pathway enrichment analysis of genes showing a >1.5 fold difference between Hnrnpa0 hairpin-expressing and control PUER cells.

PUER cells[1]	Pathway term[2]	P[3]	Genes
Undifferentiated up-regulated	Cytokine-cytokine receptor interaction	0.004	Ccl22, Il4, Il2, Il12b, Pdgfb, Tnfrsf10b
	Jak-STAT signaling pathway	0.004	Il4, Il2, Il12b, Pias3, Pik3cd
	Pathways in cancer	0.055	Fgfr2, Hhip, Pdgfb, Pias3, Pik3cd
Undifferentiated down-regulated	Cytokine-cytokine receptor interaction	0.096	Csf2, Il6ST, Il20, Tnfsf11
Differentiated up-regulated	Glycosphingolipid biosynthesis	0.073	B3galt2, B3gnt5
Differentiated down-regulated	Cytokine-cytokine receptor interaction	0.003	Bmpr1b, Ccl3, Ccl4, Cxcl15, Il1r1, Il1b, Il5, Lep
	MAPK signaling pathway	0.005	Cacnb4, Cacna2d, Dusp1, Fgf12, Il1r1, Il1b, Kras, Ntrk2
	Chemokine signaling pathway	0.057	Ccl3, Ccl4, Cxcl15, Gng2, Kras

[1]PUER cells were transduced with Hnrnpa0 shRNA or empty constructs. After expansion, GFP+ cells were harvested (undifferentiated) or treated with 2nM 4-OHT for 24 hours (differentiated). [2]The KEGG pathway analysis tool within DAVID was used to identify biologically relevant pathways. [3]Enriched pathways with a P value <0.05 are discussed in the text.

in monocytic (F4/80+) differentiation at all doses of 4 OHT (Figure 3C); with decreasing doses of 4 OHT, the reduction in differentiation was more pronounced (Figure 3D) (P=0.002-0.03). There was no obvious change in PUER cell morphology upon *Hnrnpa0* KD at 24 h; however, as anticipated, there were fewer macrophages observed upon *Hnrnpa0* KD, most apparent 4 days after differentiation with 2 nM 4-OHT (approx. 40% *vs.* approx. 60% macrophages). For subsequent experiments, we examined the biological consequences of reduced *Hnrnpa0* expression after treatment with 2 nM 4 OHT, since the effects of *Hnrnpa0* KD were best seen at this concentration. At lower doses, a very small percentage of cells undergo differentiation, potentially masking the effects of *Hnrnpa0* KD in subsequent assays. At higher doses, there is such a strong pro-differentiation signal that the phenotype resulting from *Hnrnpa0* KD becomes overwhelmed. For example, reduced differentiation following *Hnrnpa0* KD was confirmed by the reduced induction of *Emr1, Il1r2*, and *Csf1r*, after 24 h of 2nM 4-OHT treatment (P<0.01) (*Online Supplementary Figure S3*). Spi1 transcript levels (encoding PU.1) were comparable in control and *Hnrnpa0*

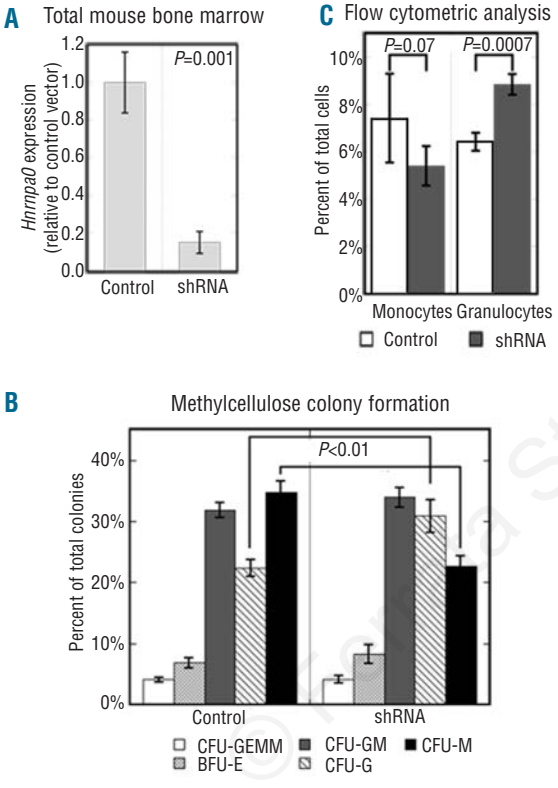

Figure 2. ***Hnrnpa0*** **knockdown shifts colony formation away from monocytic towards granulocytic lineage in primary murine cells. Total mouse bone marrow cells were retrovirally-transduced with an empty vector (Control) or an *Hnrnpa0* shRNA construct, and sorted for GFP positivity. A. RT-PCR analysis of *Hnrnpa0* expression in total mouse bone marrow cells infected with control or shRNA-expressing vectors. Expression was normalized against Gapdh expression and reported relative to expression in controls. B. Cells were plated in methylcellulose media with IL-3, IL-6, SCF and Epo for 10-14 days, and colonies were scored by morphology. Each colony type is reported as percent of total colonies scored. CFU-GEMM: granulocyte-monocyte-erythroid-megakaryocyte colony-forming unit; BFU-E: erythroid burst-forming unit; CFU-GM: granulocyte-monocyte colony-forming unit; CFU-G: granulocyte colony-forming unit; CFU M: monocyte colony-forming unit. Significant differences from the control are indicated. C. Colonies were harvested and cells were classified by flow cytometry, as either monocytes (Mac-1^{hi} Gr-1^{lo} F4/80^{+}) or granulocytes (Mac-1^{lo} Gr-1^{hi} F4/80^{-}). For all experiments, the average of 3 independent experiments is shown. Bars represent the standard error of the mean.**

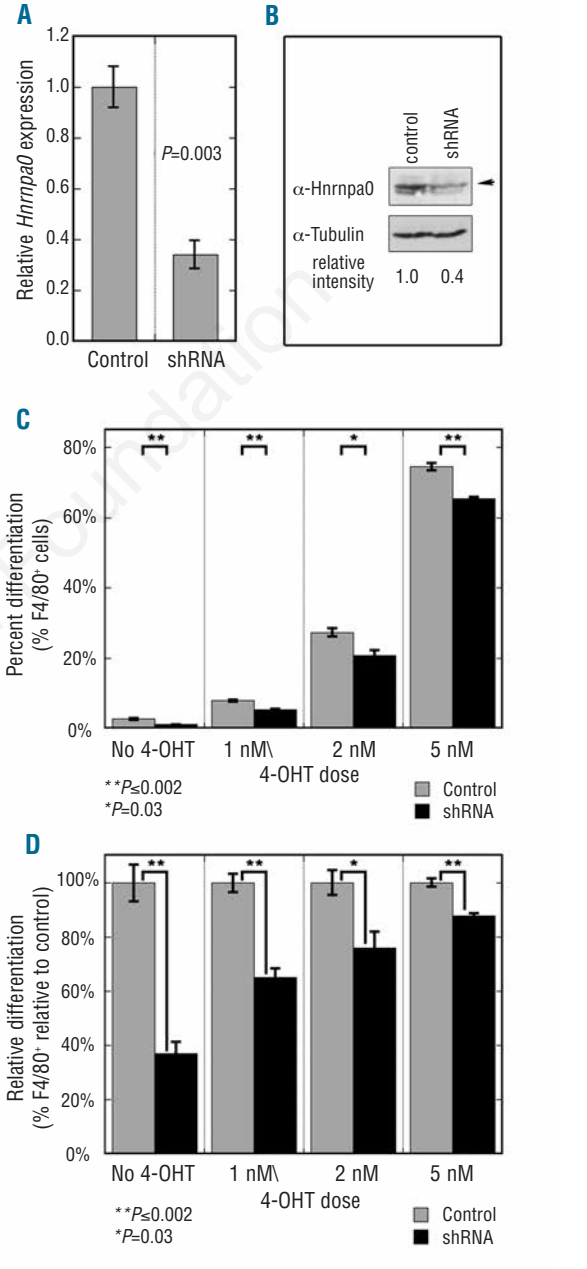

Figure 3. **Knockdown of *Hnrnpa0* in PUER cells reduces monocytic differentiation. PUER cells were transduced with either empty (Control) or Hnrnpa0 hairpin-expressing (shRNA) vector, and sorted for GFP positivity. Relative *Hnrnpa0* expression was determined by RT-PCR (A) and Western (B) analysis. Expression was normalized against Gapdh or Tubulin expression and reported relative to expression in controls. A scrambled shRNA was used as the control. C and D. PUER cells were induced to differentiate using different doses of 4 OHT as indicated. After four days of induction, the cells were labeled to detect F4/80 expression (marker of monocytes) and analyzed by flow cytometry. The histogram depicts the percent (C) or fraction (D) of F4/80^{+} cells after shRNA treatment, relative to controls.**

shRNA-expressing cells, confirming that changes in gene expression were not due to variations in *Spi1* induction.

To rule out off-target effects, we compared the *Hnrnpa0* shRNA to an irrelevant, scrambled shRNA (*Online Supplementary Figure S4*). The level of *Hnrnpa0* KD in BM and PUER cells is comparable to the decrease seen in some t-MN patients with a del(5q), since many have a greater than 50% decrease in *HNRNPA0* expression (Figure 1B). However, on average, most patients express haploinsufficient *HNRNPA0* levels. Thus, we used a second *Hnrnpa0*-specific shRNA that resulted in 50% KD in undifferentiated PUER cells to model *HNRNPA0* haploinsufficiency. This shRNA showed similar effects on monocyte differentiation, as confirmed by the suppression of *Hnrnpa0, Emr1*, and *Egr2* (*Online Supplementary Figure S5*), suggesting that our system effectively tests the effects of *Hnrnpa0* haploinsufficiency on myeloid differentiation. Moreover, it emphasizes that deregulated *HNRNPA0* expression, be it a decrease of 50% or 80%, likely has similar effects on myelopoiesis.

Hnrnpa0 *knockdown alters gene expression consistent with a shift towards a pro-granulocytic program in PUER cells*

To examine how a decrease in *Hnrnpa0* transcript levels translates into a deregulation of the normal myeloid program, we used microarray-based expression profiling. PUER cells expressing the *Hnrnpa0* shRNA were sorted for GFP-positivity, before (undifferentiated) and 24 h after differentiation with 2 nM 4-OHT. The network of genes involved in monocytic and granulocytic specification is very well characterized and has been mapped extensively.[19,21,22] We grouped these genes broadly into two categories: genes that regulate differentiation in a pro-monocytic (*Egr1, Egr2, Nab2, Spi1/PU.1, Csf1r, Maf, Irf8*) or pro-granulocytic (*Cebpa, Gfi1, Csf3r*) manner, and genes that serve as markers of monocytic (*Emr1, Mmp12, Itgam, Tlr2*) or granulocytic (*Ltf, Ncoa3, Prtn3, Il1r2*) differentiation. It should be noted that many of these 'marker' genes play critical roles in mediating the function of mature myeloid cells. We also examined several genes that block myeloid

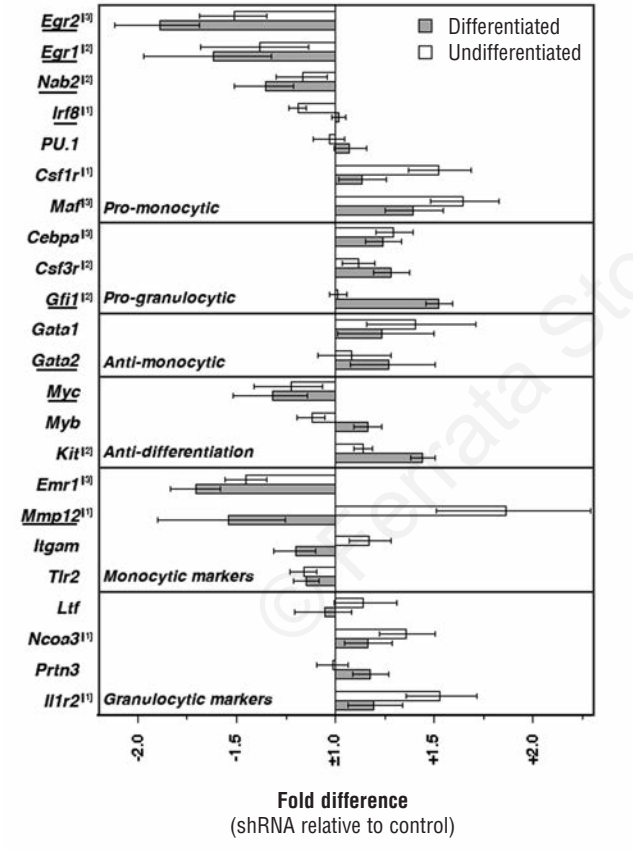

Figure 4. The effect of *Hnrnpa0* knockdown upon the expression of select myeloid genes during PUER differentiation. Microarray expression analysis was performed prior to differentiation and 24 h after induction by 2nM 4-OHT. Expression is reported as the fold difference in expression in the shRNA (n=3) and control samples (n=3). The genes are grouped broadly according to their effects upon myeloid lineage specification. Underlined gene symbols indicate transcripts known to contain AU-rich 3' UTR elements. Significant differences between hairpin-expressing and control cells (*P* 0.05, corrected for multiple comparisons) are indicated: [1] undifferentiated only, [2] differentiated only, [3] both undifferentiated and differentiated. Bars indicate the estimated standard error of the fold difference as determined by linear modeling.

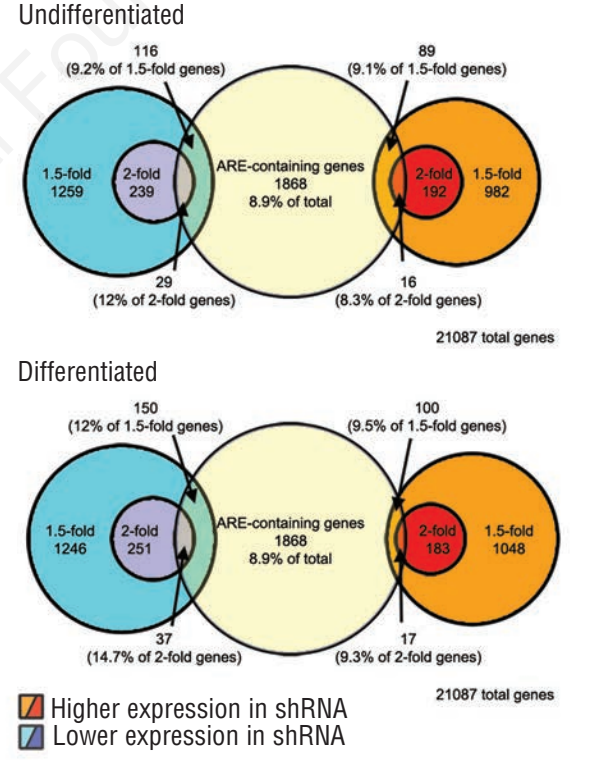

Figure 5. Venn diagram of differentially expressed genes with and without AU-rich elements. All transcripts that were differentially expressed 1.5 or 2- fold between the Hnrnpa0 shRNA-expressing cells and control PUER cells were classified as either up-regulated (orange/red) or down-regulated (blue/blue-gray), in both the undifferentiated and differentiated cells. On the Agilent microarray platform, 1868 transcripts contain AREs (8.9% of 21,087 unique transcripts). The overlap of these ARE-containing genes (yellow) and the genes differentially expressed upon Hnrnpa0 KD can be visualized by means of Venn diagrams. These classifications show that genes containing AREs are over-represented in the group of genes that are down-regulated upon Hnrnpa0 KD, especially in differentiated cells (lower panel). This conclusion is supported by contingency table analysis (*Online Supplementary Table S2*).

differentiation either directly (*Myc, Myb, Kit*), or by promoting differentiation along other lineages at the expense of myeloid differentiation (*Gata1, Gata2*). Among these genes, *Egr1, Egr2, Gata2, Gfi1, Irf8, Myc, Mmp12*, and *Nab2* transcripts contain AREs and, as such, represent potential direct targets of *Hnrnpa0*.[23-26]

Analysis of the expression of these genes by microarray (Figure 4) and subsequent validation by RT-PCR (*Online Supplementary Figure S6*) revealed a shift away from genes that regulate monocytic differentiation. For example, the microarray data confirmed significant reductions of the monocytic marker, *Emr1* (F4/80), and pro-monocytic gene, *Egr2*, in *Hnrnpa0* hairpin-expressing cells, both before and after differentiation. The pro-monocytic genes encoding Nab2, an Egr1/Egr2 binding partner, and Irf8 (Icsbp) were suppressed in differentiated and undifferentiated cells, respectively. Analysis of the pro-granulocytic genes showed changes consistent with a shift towards a pro-granulocytic program. Upregulation of the Gfi1 and Cebpa transcription factors induce granulocytic differentiation in a complementary manner.[19] Cebpa, an antagonist of PU.1 activity, has been shown to promote granulocytic differentiation, and Gfi1 is a direct antagonist of the pro-monocytic activity of Egr2 and Nab2. Consistent with a shift towards a granulocytic program, the expression of the *Cebpa*, *Csf3r*, and *Gfi1* genes was elevated in hairpin-expressing cells relative to control. Together, these data suggest that *Hnrnpa0* is involved in myelopoiesis by suppression of pro-monocytic genes (*Emr1, Egr2, Nab2, Irf8*) and induction of pro-granulocytic genes (*Gfi1, Cebpa, Csf3r*), some of which contain AREs (underlined genes in Figure 4).

Hnrnpa0 *knockdown preferentially alters the expression of AU-rich genes in PUER cells*

To determine whether *Hnrnpa0* KD leads preferentially to differential expression of AU-rich genes, we used the AU-rich Element Database (ARED 3.0) to identify a signature of transcripts known to contain AREs[26] and classified them by the fold difference between the *Hnrnpa0* hairpin-expressing cells *versus* the controls, for both the undifferentiated and differentiated PUER samples. The overlap between all differentially expressed genes and those containing AREs was visualized using Venn diagrams (Figure 5). From these classifications, it becomes apparent that genes containing AREs are over-represented in the group of genes whose expression is lower in the hairpin-expressing cells. Although this overrepresentation is observed in both undifferentiated and differentiated sample, the difference is most striking in differentiated samples, where ARE-containing genes make up 12-14.7% of the genes that show a 1.5-2 or more fold decreased expression, but only 9.3-9.5% of the genes that

Table 2. KEGG pathway enrichment analysis of AU-rich genes differentially expressed in t-MN del(5q) patients *versus* non-del(5q) patients.

Pathway term[1]	*P*	Genes[2]
Cell cycle	0.008	*CCNA1*, ***CCNA2***, ***CCNE2***, *CDC23*, ***CDK2***, ***CHEK1***, ***E2F1***, *GSK3B*, ***ORC1L***, ***RAD21***, ***STAG1***
MAPK signaling pathway	0.030	*ATF2*, ***CACNA2D1***, *DUSP10*, ***DUSP16***, ***FGFR3***, ***IL1R1***, *KRAS*, ***MAPK1*** (ERK2), ***MAP2K1*** (MEK1), *NLK*, *NF1*, ***NTRK2***, ***PLA2G12A***, *PPP3CB*, ***RAP1A***, *RPS6KA5*
Chemokine signaling pathway	0.044	***CCL2***, ***CCL7***, ***CXCL5***, ***CXCL6***, *GNAI1*, *GSK3B*, *KRAS*, ***MAPK1*** (ERK2), ***MAP2K1*** (MEK1), ***PTK2*** (FAK), ***RAP1A***, ***TIAM1***

[1]*The KEGG pathway analysis tool within DAVID was used to identify biologically relevant pathways.* [2]*Genes up-regulated are in bold; genes down-regulated are in regular font.*

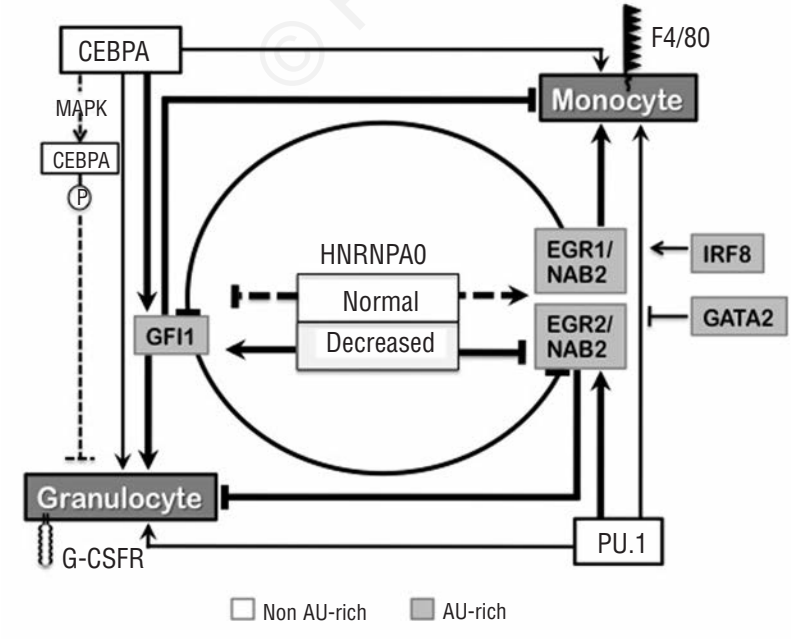

Figure 6. Model of the impact of 5q deletion upon myeloid cell fate. A schematic diagram showing some of the factors that regulate myeloid cell fate (adapted from P. Laslo *et al.*, 2006[19]). PU.1 normally induces EGR2 and NAB2 to induce monocytic differentiation. CEBPA and GFI1 promote granulocytic differentiation, partially through suppression of EGR1, EGR2 and NAB2. Knockdown of Hnrnpa0 in murine cells leads to the suppression of pro-monocytic transcripts (Egr2, Nab2, Irf8, and Emr1 (F4/80)), many of which contain AREs (light gray boxes), and induction of pro-granulocytic genes (Cebpa, Gfi1, Csf3r (G-CSFR)). MAPK-induced phosphorylation of CEBPA has been shown to inhibit granulopoeisis[37]. KD of Hnrnpa0 leads to a decreased expression of many genes within the MAPK signaling pathway, consistent with the observed shift towards granulocytic differentiation. Loss of EGR1 (also mapped to human 5q) also favors granulocytic over monocytic differentiation[36]. Egr1 and Egr2 function in a redundant manner. In del(5q) patients, haploinsufficiency for both EGR1 and HNRNPA0 may synergistically disrupt myeloid differentiation.

show a 1.5-2 or more fold increased expression. Contingency table analysis also confirmed that there are significantly more down-regulated ARE-containing genes upon *Hnrnpa0* KD than expected for differentiated cells (*Online Supplementary Table S2*). These data suggest that KD of *Hnrnpa0* results in a preferential decrease in the transcript levels of ARE-containing transcripts.

GSEA analysis reveals a set of potential Hnrnpa0 *target transcripts*

To study the possible enrichment of AU-rich genes at the extremes of the differentially expressed genes, we implemented a simplified GSEA algorithm.[27] GSEA demonstrated a significant ($P<0.001$) enrichment of AU-rich genes at both extremes of differential expression, with a greater enrichment of down-regulated genes (*Online Supplementary Figure S7*). An analysis of genes showing at least a 1.5-fold difference between the hairpin-expressing cells and control cells revealed 172 genes in undifferentiated cells (72 up-regulated, 100 down-regulated) and 222 genes in differentiated cells (80 up-regulated, 142 down-regulated) (*Online Supplementary Table S3*). The genes that showed altered expression in both undifferentiated and differentiated cells (11 were up-regulated and 30 were down-regulated) represent potential *Hnrnpa0* targets. Of particular interest are *Cbfb* and *Sp3* given their roles in hematopoiesis,[28,29] as well as *Creb5, Egr2*, and *Tcfap2e*, given their role in positive regulation of transcription. Thus, upon acute decrease in *Hnrnpa0*, AU-rich genes made up a disproportionately large fraction of the under-expressed genes and, as a group, were found to be more dysregulated than was seen globally for all genes interrogated.

To infer biological meaning from the list of differentially expressed ARE genes, we employed the Database for Annotation, Visualization and Integrated Discovery (DAVID).[30,31] Of significance, the JAK-STAT signaling pathway ($P=0.004$) was enriched in the up-regulated genes in undifferentiated cells; the MAPK pathway ($P=0.005$) was enriched, and the chemokine signaling ($P=0.057$) pathway showed a trend toward enrichment in the down-regulated genes in differentiated cells. Moreover, the cytokine-cytokine receptor interaction path was enriched in both of these gene lists ($P=0.004$ and $P=0.003$) (Table 1). The control of mRNA stability is a key means of regulating cytokine production,[13] suggesting that *Hnrnpa0* may be a positive or negative regulator of cytokine transcript stability, depending on the gene and cellular context. Alternatively, it remains possible that *Hnrnpa0* KD may have had indirect effects on cytokine expression, independent of transcript stabilization. To determine if any of these pathways could predict functional changes, we focused on the MAPK signaling pathway, given its importance in myeloid differentiation.[32] A comparison of phospho-p42/44 MAPK (Erk1/2) in differentiated *versus* undifferentiated PUER cells revealed that Erk1/2 was less activated upon differentiation in *Hnrnpa0* shRNA cells, compared to the scrambled controls (*Online Supplementary Figure S8*), consistent with our model for how *HNRNPA0* contributes to the regulation of myeloid differentiation (*see* Discussion section and Figure 6).

Expression of the AU-rich transcriptome in t-MN del(5q) patients

The dysregulation of regulatory proteins affecting ARE mRNA stability can lead to abnormalities in many critical cellular processes. In addition to *Hnrnpa0*, which is haploinsufficient in del(5q) t-MN patients, several other genes encoding AUBPs were differentially expressed in t-MN patients with a del(5q) *versus* non-del(5q) t-MN patients (*Online Supplementary Table S1*). Since AUBPs can increase and/or decrease mRNA stability, depending on the cellular context,[13] their differential expression may indicate an intricate regulation of mRNA stability. Given the importance of AREs in disease,[12,26] we examined ARE-mRNAs that are differentially expressed in del(5q) *versus* other t-MN cases. Using the ARED 3.0 database, we identified 1674 unique ARE transcripts in the Affymetrix t-MN array. Among these, 515 genes showed a significant differential association, with 237 down-regulated and 278 up-regulated (FDR <0.3) (*Online Supplementary Table S4* and *Figure S9*). Using the KEGG pathway analysis tool within DAVID, we identified the cell cycle pathway as being enriched ($P=0.008$) along with the MAPK ($P=0.030$) and chemokine ($P=0.044$) signaling pathways (Table 2). Extending this analysis of biological significance with the Ingenuity software, we found that del(5q) patients have gene expression changes consistent with an increase in cellular growth and proliferation, as compared to non-del(5q) t-MN patients (*Online Supplementary Table S5*). This analysis raises the intriguing possibility that stabilization of transcripts, through regulation of the AU-rich transcriptome, may be a contributing factor to the dysregulation of critical growth pathways in t-MN del(5q) patients.

Discussion

To date, none of the individual 5q genes show homozygous loss-of-function, or recapitulate all of the features of myelodysplastic syndrome (MDS) or AML when expressed at haploinsufficient levels in hematopoietic cells in mouse models. Rather, current studies support a haploinsufficiency model, in which loss of a single allele of more than one gene on 5q contributes to the pathogenesis of t-MN.[33] The current challenge is identifying which of the numerous genes in the deleted chromosome 5 segment are involved in the pathogenesis of human t-MN. In this paper, we show that decreased *HNRNPA0* expression is characteristic of leukemias with a del(5q), and that recapitulating loss of *Hnrnpa0* expression in mouse hematopoietic cells alters myeloid differentiation. Our work suggests that KD of *Hnrnpa0*, a gene encoding an AUBP, alters myeloid fate by shifting differentiation away from the monocytic in favor of the granulocytic lineage. This shift is accompanied by the suppression of pro-monocytic and induction of pro-granulocytic transcriptional regulators, several of which contain AREs, e.g. *Irf8, Nab2*, and *Egr2*.

One interesting phenomena that this work, as well as our previous work on *EGR1*, raises is that decreasing expression of del(5q) genes by half (i.e. haploinsufficiency) may be as detrimental as complete loss of the gene when placed in an environment conducive to malignant transformation. For example, we previously showed that reducing *Egr1* expression in mice by 50% ($Egr1^{+/-}$) or knocking it down completely ($Egr1^{-/-}$) was equally effective at cooperating with secondary mutations induced by the alkylating agent ENU to induce a myeloproliferative disease with ineffective erythropoiesis. Herein, we show that, on average, t-MN patients with a del(5q) express half of the levels of *HNRNPA0*, but there is some variability with some

patients showing greater loss. Moreover, loss of *Hnrnpa0* expression, ranging from 50-80%, appears to have similar effects on myeloid differentiation in primary and PUER mouse cells.

Numerous studies have established that a core set of genes act as 'master regulators' of myeloid differentiation.[19,21,22,34,35] Monocytic differentiation is promoted by the PU.1-induced transcription factors EGR2 and NAB2, whereas granulocytic differentiation is promoted by GFI1 and CEBPA (Figure 6). Our data show that loss of *Hnrnpa0* leads to the suppression of pro-monocytic genes (*Egr2, Nab2, Irf8, Emr1*) and induction of pro-granulocytic genes (*Cebpa, Gfi1, Csfr3*), within this network. *EGR1*, like *HNRNPA*, is located within the CDS of 5q31.2, and is expressed at reduced levels in CD34$^+$ cells from patients with a del(5q). Similar to *HNRNPA0*, loss of *EGR1* expression favors granulocytic over monocytic differentiation.[36] Thus, loss of a single allele of *EGR1* and *HNRNPA0*, as a result of a del(5q), may lead to a synergistic disruption of EGR1, and the functionally redundant family member, EGR2, leading to aberrant myeloid differentiation, a hallmark of t-MN. Any disruption to the gene program that regulates the transition from the CMP to the GMP and beyond can uncouple lineage commitment and proliferation control during myeloid differentiation and lead to malignant transformation. Although, t-MN is thought to be due to the simultaneous loss of multiple 5q genes together with secondary cooperating mutations, the observed shift towards granulocytic differentiation and ensuing change in gene expression, following *Hnrnpa0* KD, may be one piece of the puzzle explaining the dysgranulopoiesis observed in patients.

The ability of CEBPA to induce granulocytic differentiation is negatively regulated by ERK1/2- and p38MAPK-mediated phosphorylation.[32,37] Since many genes in the MAPK signaling pathway were down-regulated upon *Hnrnpa0* KD in PUER cells (Table 1), these results raise the possibility that *HNRNPA0* haploinsufficiency may shift differentiation towards granulopoiesis, not only through a deregulation in the expression of the myeloid specification genes, but also through an inhibition of MAPK activity (Figure 6). Consistent with this, Erk1/2 was less activated upon differentiation in PUER cells expressing the *Hnrnpa0* shRNA as compared to scrambled shRNA (*Online Supplementary Figure S8*). Together, our data raise the possibility that *HNRNPA0* haploinsufficiency may lead to numerous gene changes that may be small in magnitude, yet coordinately de-regulate myeloid differentiation, possibly together with a reduced dose of *EGR1*.

Consistent with the proposed role of *Hnrnpa0* as a positive regulator of transcript stability, this work determined that AU-rich genes made up a disproportionately large fraction of the under-expressed genes in cells expressing an *Hnrnpa0* shRNA. However, like many other AUBPs, its regulatory ability may be more complex and regulated in a cell-type and activation-dependent manner.[38-40] The observed changes in transcript profiles upon *Hnrnpa0* KD were numerous, yet small in magnitude for AU-rich genes, but also for some non-AU rich genes, suggesting that *HNRPNA0* likely 'fine-tunes' gene expression, and that some of its effects on the pathogenesis of disease may be secondary to its post-transcriptional regulation. We attempted to explore the effects of *Hnrpa0* loss *in vivo*; however, suppression of *Hnrpa0* was not maintained past 12 weeks in the bone marrow of mice (*data not shown*). This may suggest that without a clonal advantage, such as TP53 mutations which are frequent in del(5q) patients, hematopoietic cells may not tolerate low levels of *Hnrnpa0*. An analysis of ARE genes revealed that the MAPK, cell cycle and chemokine signaling pathways were enriched in t-MN with del(5q) *versus* non-del(5q) patients. Most of these changes are consistent with pathway activation and increased cell cycling and growth (Table 2 and *Online Supplementary Table S5*). This is not entirely surprising given that activation of these pathways has been observed in the spectrum of myeloid malignancies. However, it is of interest that deletion of chromosome 5 alters gene expression of ARE-containing genes in a direction that predicts enhanced cellular growth, since cells from non-del(5q) myeloid neoplasms would also be predicted to be actively proliferating. Whereas, *Hnrnpa0* led to the immediate decreased expression of some MAPK genes in PUER cells, in t-MN patients with a del(5q) there was an upregulation of similar MAPK genes. This is likely due to the complex genetic alterations in del(5q) patients and emphasizes that the use of genetic knockdown in the PUER cell line provided an informative model to pinpoint gene changes resulting from deregulated *Hnrnpa0* expression. We recognize that this is a preliminary investigation into the relationship between dysregulation of AU-rich transcript stability and growth pathways in t-MNs; however, it is interesting to speculate that the post-transcriptional control of ARE-mRNA stability may be a critical factor influencing leukemia cell proliferation in t-MNs with a del(5q).

Our study shows that *HNRNPA0*, normally highly expressed in multipotent hematopoietic progenitors, is expressed at reduced levels in CD34$^+$ cells from patients with a del(5q), suggesting that it is a candidate gene in malignant myeloid disorders with abnormalities of chromosome 5. We modeled *HNRNPA0* loss in murine cells and showed that a decreased dose leads to a dysregulation of myeloid cell differentiation, through suppression of pro-monocytic genes and induction of pro-granulocytic genes, mediated in part by alterations in the expression of AU-rich genes. Moreover, our analysis of differentially expressed AU-rich genes in t-MN patients reveals that post transcriptional control of mRNA stability warrants consideration especially with regard to *HNRNPA0*'s role in the dysregulation of myeloid differentiation in the pathogenesis of t-MNs with a del(5q).

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