Cytotoxic Therapy–Induced Effects on Both Hematopoietic and Marrow Stromal Cells Promotes Therapy-Related Myeloid Neoplasms

Angela Stoddart¹, Jianghong Wang¹, Anthony A. Fernald¹, Elizabeth M. Davis¹, Camille R. Johnson¹, Chunmei Hu¹, Jason X. Cheng²,³, Megan E. McNerney²,³,⁴, and Michelle M. Le Beau¹,³
ABSTRACT
Therapy-related myeloid neoplasms (t-MN) following treatment with alkylating agents are characterized by a del(5q), complex karyotypes, alterations of TP53, and a dismal prognosis. To decipher the molecular pathway(s) leading to the pathogenesis of del(5q) t-MN and the effect(s) of cytotoxic therapy on the marrow microenvironment, we developed a mouse model with loss of two key del(5q) genes, EGR1 and APC, in hematopoietic cells. We used the well-characterized drug, N-ethyl-N-nitrosurea (ENU), to demonstrate that alkylating agent exposure of stromal cells in the microenvironment increases the incidence of myeloid disease. In addition, loss of Trp53 with Egr1 and Apc was required to drive the development of a transplantable leukemia, and accompanied by the acquisition of somatic mutations in DNA damage response genes. ENU treatment of mesenchymal stromal cells induced cellular senescence and led to the acquisition of a senescence-associated secretory phenotype, which may be a critical microenvironmental alteration in the pathogenesis of myeloid neoplasms.

SIGNIFICANCE: This study challenges the historic view that prior cytotoxic therapy targets only hematopoietic cells and shows that chemotherapy-induced alterations to the microenvironment contribute to myeloid neoplasms in a model of del(5q) t-MN. The DNA damage response in hematopoietic cells and senescence of stromal cells are identified as potential therapeutic targets.

See related commentary by Shannon and Link, p. 10.

INTRODUCTION
Therapy-related myeloid neoplasms (t-MN) comprise therapy-related acute myeloid leukemia (t-AML) and myelodysplastic syndrome (t-MDS), and are a late complication of cytotoxic therapy, chemotherapy and/or radiotherapy, used in the treatment of both malignant and nonmalignant diseases (1, 2). The genetic profile of t-MN is markedly skewed toward high-risk cytogenetic and molecular abnormalities, and complex karyotypes with deletions of the long arm of chromosome 5, del(5q), and TP53 mutation/loss are profoundly over-represented in t-MNs as compared with de novo counterparts (3, 4). A proximal minimally deleted region (MDR) in 5q31.2 (containing EGR1) was previously identified in t-MN, de novo AML and high-risk MDS, and a distal MDR in 5q33.1 (containing mir145, RPS14, and CSNK1A1) was identified in MDS with an isolated del(5q), previously referred to as the 5q- syndrome (1, 5). However, the deletion of 5q in all but rare patients encompasses both MDRs, spanning 5q14-5q33, resulting in loss of one allele for hundreds of genes. Although challenging to identify involved genes, we previously chose to examine two well-characterized del(5q) tumor suppressor genes, early growth response 1 (EGR1; 5q31.2) and adenomatous polyposis coli (APC; 5q22.2), and showed that they cooperate with TP53 (p53) loss to induce acute myeloid leukemia in mice (6). The low frequency of AML and long latency observed in this model, however, suggests that additional stochastic events, and/or mutations arising from environmental exposures, may contribute to the etiology of disease.

Healthy individuals harbor clonal, somatic, gene mutations in white blood cells, and the frequency increases with age, leading to the expansion of some clones, a phenomenon known as “clonal hematopoiesis of indeterminant potential,” (CHIP) (CHIP; refs. 7, 8). Recent studies have revealed that CHIP increases the risk for t-MN (9, 10). The landmark finding of preexisting TP53 mutations in hematopoietic cells originating prior to cytotoxic treatment of a primary malignant disease and in the t-MNs that subsequently evolved, led to the proposal that chemotherapy and/or radiation may preferentially impair normal hematopoietic stem and progenitor cells (HSPC), while providing a selective advantage to clones with preexisting mutations (11). Follow-up studies showed that cytotoxic therapy results in the expansion of clones carrying mutations in DNA damage response genes, including TP53, PPM1D, ATM, BRCC3, SRCAP, and RAD21 (12); however, mutations in DNA repair genes, other than TP53 and PPM1D (11–13), are not present in leukemia samples isolated from patients with t-MN. Interestingly, the DNA double-strand break response is abnormal in myeloblasts from t-MNs, even though somatic mutations of DNA repair genes do not appear to be the dominant mechanism responsible for this phenotype (14).

In addition to alterations in hematopoietic cells, there is compelling data suggesting that alterations to the bone marrow microenvironment also play a role in the pathogenesis of myeloid neoplasms (15–18). These alterations may include establishment of an inflammatory microenvironment (19).
and/or age-associated changes of mesenchymal stromal cells (MSC; ref. 20). MSCs isolated from patients with MDS and AML display several characteristic features of senescence, including TP53 pathway activation (21, 22), increased senescence-associated β-galactosidase (SA-β-gal) expression (22–24), and upregulation of senescence-associated secretory phenotype (SASP) factors, such as IL6 (25, 26). Moreover, AMLs can induce a senescent phenotype in bone marrow (BM) stromal cells, and targeting these senescent cells improves the survival of mice with leukemia (25), illustrating the importance of a senescent microenvironment in the pathophysiology of leukemia.

The complete compendium of genetic alterations and selective pressures leading to t-MN with a del(5q) remain unknown, especially with regard to the effects of cytotoxic therapy on the microenvironment. In this study, we used a multifaceted approach to model del(5q) t-MN in mice, and to define genetic alterations in HSPCs by whole exome and RNA sequencing, while simultaneously probing hematopoietic stem cell (HSC)-intrinsic and cell-extrinsic effects of alkylating-agent therapy. Our results pinpoint chemotherapy-induced alterations to BM stromal cells and genetic alterations to DNA repair genes in HSPCs as two factors critical for the pathogenesis of del(5q) t-MN with aberrant TP53.

RESULTS

Alkylating Agent Exposure Increases the Incidence of Myeloid Disease

A striking 40% of patients with t-MN have a del(5q) (2), the majority (>95%) of which have haploinsufficient loss of both EGR1 (27) and APC (28). Moreover, approximately 75% of del(5q)t-MNs have loss and/or mutation of the TP53 gene (29). To model this, we previously showed that cell-intrinsic loss of both Egr1 and Apc, or individual loss of either Egr1 or Apc, together with Trp53 knock-down, was not sufficient to promote AML; however, concordant loss of the three genes, Egr1, Apc, and Trp53, modeled the development of del(5q) AML (ref. 6; Supplementary Fig. S1). In clinical settings, however, patients with t-MN receiving chemotherapy have exposure of the entire body, including the BM microenvironment and HSPCs. The relative contribution of drug treatment on the niche and/or HSPCs, and the interplay of genetics, the microenvironment, and chemotherapy, in subsequent t-MN development is unclear.

To isolate these variables, we employed our del(5q) mouse model, with Egr1 and Apc haploinsufficiency and loss of Trp53, which spontaneously develops leukemia at a low frequency (<20%; ref. 6). However, in this study, we treated HSPCs and the niche separately with N-ethyl-N-nitrosurea (ENU), a prototypical alkylating agent whose effects have been well-characterized in mice. We first assessed the outcome of ENU exposure of both the niche and hematopoietic cells. Egr1−/−, Apc−/−/+ donor and wild-type (WT) recipient mice were treated with ENU. Egr1−/−, Apc−/−/+ BM cells were isolated two weeks after ENU treatment, transduced with Trp53 shRNA to knockdown expression of Trp53 (Trp53 KD), and transplanted into ENU-treated, lethally irradiated WT recipient mice (Fig. 1A).

Compared with donor and recipient mice that did not receive ENU (no ENU), alkylating agent exposure significantly accelerated the onset of disease with a median survival of 200 days versus 508 days (P < 0.0001; Fig. 1B). In accordance with the Bethesda classification (30), the disease was classified as a myeloid leukemia when at least 20% blasts were observed in hematopoietic tissue, and as a MDS when anemia and morphologic dyspoiesis was observed. A significantly larger percentage of mice developed MDS (36% vs. 12%, P = 0.076) or AML (45% vs. 5%, P = 0.003) in the ENU-treated group as compared with untreated mice (Fig. 1C). Eighty percent of non-ENU–treated mice did not develop hematologic disease (non-Hem) and were sacrificed due to age and poor health. The myeloid malignancies that developed in ENU-treated mice resembled human disease ranging from MDS-refractory anemia with excess blasts-1 or -2 (RAEB-1 or -2) to AML with myelodysplasia-related changes (Fig. 1D; Supplementary Fig. S2A). Mice with myeloid neoplasms presented with splenomegaly and an effacement of the normal splenic architecture, as well as trilineage dysplasia similar to that observed in patients with t-MN with a del(5q) (Supplementary Figs. S2B and S3). Disease in mice with blast counts >20%, and classified as AML, was transplantable to secondary recipients.
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**A**

Donor

ENU

Egr1+; Apcdel

2 Weeks

BM + p53 shRNA

Recipient

ENU

WT

2–3 Weeks

Monitor

**B**

![Graph showing percent survival over time](image)

No ENU (n = 42)

ENU (n = 11)

200 d

508 d

P < 0.0001

**C**

![Bar chart showing percent disease](image)

Percent disease

P = 0.076

MDS

AML

T cell

Non-Hem

**D**

![Images of blood and bone marrow aspirate](image)

Blood

Bone marrow aspirate

Spleen

Spleen touch prep

**E**

Premalignant change with EA, Trp53 loss

- biocarta_toll_pathway
- biocarta_mapk_pathway
- reactome_activated_trl4_signaling
- kegg_mapk_signaling_pathway
- hallmark_oxidative phosphorylation
- kegg_oxidative phosphorylation
- reactome_respiratory electron transport
- reactome_nucleotide excision repair
- hallmark_dna repair
- reactome_dna repair
- kegg_nucleotide excision repair
- kegg_mismatch repair
- reactome_regulation of apoptosis
- reactome_cell cycle checkpoints
- reactome_G2_m checkpoints

Up in EA-Trp53 LSK

- hallmark_mtorc1_signaling
- hallmark_glycolysis
- hallmark_tnfa_signaling via nfkb
- hallmark_tgf beta_signaling

Down in EA-Trp53 LSK

- kegg_ribosome
- reactome_translation
- reactome_metabolism of proteins
- reactome_metabolism of mmr

NOS (NOM p/FDR q)

**F**

Premalignant change with ENU exposure

- kegg_ribosome
- reactome_translation
- reactome_metabolism of proteins
- reactome_metabolism of mmr
- hallmark_mtorc1_signaling
- hallmark_glycolysis
- hallmark_tnfa_signaling via nfkb
- hallmark_tgf beta_signaling

NOS (NOM p/FDR q)
As a control, three independent LSK posttransplant) and performed RNA sequencing (RNA-seq) 80 days ∼ n from both the no-ENU (WT control (Fig. 1E). Gene set enrichment analysis (GSEA; ref. 31) of the [includes both the no-ENU and ENU-treated groups; (n = 6); Fig. 1E). Gene set enrichment analysis (GSEA; ref. 31) of the top enriched curated pathways revealed that cell-intrinsic loss of Egr1, Apc, and Trp53 downregulates oxidative phosphorylation, DNA repair, apoptosis, and cell-cycle checkpoints. Similar results were observed when we compared only the three ENU-exposed EA-Trp53 LSK+ samples with WT controls (Supplementary Fig. S4).

To elucidate the effects of ENU, we compared EA-Trp53 cells with and without ENU exposure (Fig. 1F). ENU exposure increased the expression of genes involved in energy production, such as mTOR signaling and protein translation (Fig. 1F); however, we cannot rule out that this simply reflects cells that are at a more advanced stage along the continuum of malignant transformation with higher metabolic activity, rather than a direct effect of ENU exposure. Interestingly, similar changes in gene expression were observed in patients who subsequently developed t-MN (32). Together, these results are consistent with a role for loss of genome defense mechanisms in the development of myeloid neoplasms.

Incidence of AML Increases When Egr1, Apc, and Trp53-deficient HSPCs as well as the BM Microenvironment Are Exposed to Cytotoxic Therapy

We next separately tested the effects of alkylating agent therapy on HSPCs or the BM microenvironment. To test the effect on HSPCs, we treated only BM donors with ENU (2 weeks prior to BM isolation for Trp53 knockdown, Fig. 2A). To test the impact on the niche, we treated transplant recipients only (2–3 weeks prior to transplant).

Approximately 10% of Egr1+/−, Apc+/−, BM cells were transduced with the GFP-positive Trp53 shRNA (Trp53 shRNA-GFP+) prior to transplantation (Fig. 2B, % GFP+ black circles). This relatively low frequency allowed us to assess the effect of ENU on the competitive expansion of Trp53-deficient, Egr1+/−, Apc+/− BM cells. ENU treatment of recipient or donor mice led to a modest expansion of Trp53 shRNA-GFP+ cells 1.5 to 5 months after transplant. However, the greatest expansion of Trp53 shRNA-GFP+ cells was observed when both donor and recipient mice were treated with ENU, suggesting that ENU exposure of both HSPCs and the microenvironment contributes to clonal expansion.

Even though ENU treatment of donor or recipient mice increased the expansion of Trp53 shRNA-GFP+ cells, it did not accelerate disease development (Fig. 2C; 517 days or 533 days vs. 508 days without ENU treatment), nor did it increase the frequency of myeloid neoplasms beyond what is observed without ENU treatment (Fig. 2D; compare “none” to “donor” or “recipient”). Only 1 of 10 recipient-treated mice developed AML, and none of the donor-treated mice developed a myeloid neoplasm [two died early from T lymphomas; the remainder were sacrificed (>500 days) due to age-related causes]. In contrast, 82% of mice, in which both donor and recipient mice were exposed to ENU, developed MDS or AML. Thus, whereas exposure of HSPCs or the microenvironment alone to ENU promotes expansion of Trp53-shRNA+ cells, t-MN development is driven by the synergistic effects of chemotherapy exposure of premalignant hematopoietic cells, together with deleterious effects of cytotoxic therapy on the supporting microenvironment. Historically, it has been assumed that the effect of prior therapy in patients who develop t-MN was primarily on the HSPCs. To our knowledge, this is the first mouse model of del(5q)-t-MN to directly show that chemotherapy-induced alterations to the BM microenvironment contribute to disease development.

Alkylating Agent Exposure Induces Senescence of Mesenchymal Stromal Cells

In solid tumors, there is evidence that senescence of non-tumor cells promotes tumor growth and metastasis (33). Furthermore, cytotoxic treatment induces an acute inflammatory response in the BM microenvironment. We hypothesized that ENU induces a sustained effect via senescence of MSCs within the microenvironment and, thereby, alters the function of HSPCs. To evaluate this hypothesis, MSCs were treated for 24 hours with increasing doses of ENU, and senescence was assessed 7 days later. MSCs treated with ENU showed an increase in SA-β-gal activity and a decline in DNA synthesis as determined by EdU incorporation (Fig. 3A and B). Senescence was further confirmed by elevated levels of mRNA encoding p21 (Cdkn1a), p16INK4a (Cdkn2a), and IL6 (Fig. 3C).

We next examined MSCs isolated from mice treated with one dose of ENU, which showed a decreased number of colony-forming unit-fibroblasts (CFU-F; Fig. 3D and E) and an increase in SA-β-gal activity (Fig. 3F). Consistent with the finding that damage-initiated senescence strongly depends on TP53 (34), GSEA analysis of RNA-seq data revealed activation of the p53 pathway in MSCs isolated from ENU-treated mice, compared with mock-treated mice (Fig. 3G). Senescent cells express and secrete a variety of extracellular modulators collectively referred to as the senescence-associated secretory phenotype (SASP; ref. 35). Enrichment of a SASP GSEA signature was observed in MSCs isolated from ENU-treated mice (Fig. 3H), providing a potential mechanism by which they contribute to the pathogenesis of leukemia.

To assess whether an ENU-treated microenvironment affects stem cell fitness in vivo, a competitive BM transplant assay was performed as outlined in Fig. 3I. We used Egr1+/− donor cells, because Egr1 has been documented to play a role in stem cell quiescence (36). The repopulation ratio of CD45.2+ (Egr1+/−) to CD45.1+ short-term-HSC (ST-HSC) cells was normalized to 100% (mock conditions). In mock conditions, Egr1+/− ST-HSCs displayed a reduced normalized repopulation ratio of 31%. Although not statistically different (P = 0.31), this trend may reflect stem cell exhaustion due to Egr1’s role in maintaining stem cell quiescence. With ENU treatment (red bars), Egr1+/− ST-HSCs showed...
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thes exposed to ENU, survival time is significantly decreased (200 days). There was no statistical difference in survival between ENU-donor versus no ENU treatment at 1.5 months are shown. At 5 months, only “donor” and “both” conditions showed a significant expansion of GFP mice received one injection of ENU (100 mg/kg) 2 weeks before BM harvest. Recipient mice received one injection of ENU (100 mg/kg) 3 weeks before transplantation. There was a significantly greater percentage of GFP+ cells in the BM at the time of transplantation, and in the blood of mice at 1.5 and 5 months posttransplantation. There was a significantly greater percentage of GFP+ cells in the blood of ENU-treated mice at 1.5 month and 5 months. The P values from a two-tailed Student t test at 1.5 months are shown. At 5 months, only “donor” and “both” conditions showed a significant expansion of GFP+ cells (P < 0.0001). ENU exposure of both donor and recipient creates a more optimal environment for the expansion of Trp53 shRNA BM cells transduced with Egr1+/−. Apcdel/+ BM cells transduced with Trp53 shRNA. When both donor and recipient mice are exposed to ENU, survival time is significantly decreased (200 days). There was no statistical difference in survival between ENU-donor versus no ENU (P = 0.831) or ENU-recipient versus no ENU (P = 0.737), or ENU-donor versus ENU recipient cohorts (P = 0.910).

Figure 2. Exposure of the BM microenvironment to ENU plays a critical role in the development of MDS and AML. A, Schematic of the transplantation schemes used to elucidate the effects of alkylating agents on HSPCs, with Trp53 knockdown (donors), and BM microenvironment (WT recipients). Donor mice received one injection of ENU (100 mg/kg) 2 weeks before BM harvest. Recipient mice received one injection of ENU (100 mg/kg) 3 weeks before lethal irradiation and transplantation. B, Percentage of GFP+ cells in the BM at the time of transplantation, and in the blood of mice at 1.5 and 5 months posttransplantation. There was a significantly greater percentage of GFP+ cells in the blood of ENU-treated mice at 1.5 month and 5 months. The P values from a two-tailed Student t test at 1.5 months are shown. At 5 months, only “donor” and “both” conditions showed a significant expansion of GFP+ cells (P < 0.0001). ENU exposure of both donor and recipient creates a more optimal environment for the expansion of Trp53 shRNA, GFP+ cells. C, Kaplan–Meier survival curves of WT recipients transplanted with Egr1+/−; Apcdel/+ BM cells transduced with Trp53 shRNA. When both donor and recipient mice are exposed to ENU, survival time is significantly decreased (200 days). There was no statistical difference in survival between ENU-donor versus no ENU (P = 0.831) or ENU-recipient versus no ENU (P = 0.737), or ENU-donor versus ENU recipient cohorts (P = 0.910). B, Histologic classification of diseases arising in the mice.

an increased repopulation of 95% (P = 0.05) suggesting that an ENU-treated microenvironment may effect Egr1+/− stem cell fitness. Thus, alterations to the microenvironment may induce senescence of MSCs and trigger SASP, thereby creating a permissive environment that impacts stem cell fitness, and contributes to the pathogenesis of myeloid neoplasms.

Loss of Trp53, Concurrent with Two Del(5q) Genes, Egr1 and Apc, Models High-risk MDS/AML

TP53 mutations are associated with complex karyotypes, high-risk MDS, transformation to AML, and poor overall survival (3, 4, 37). We next compared disease development in mice transplanted with Egr1+/−; Apcdel/+ BM transduced with luciferase control shRNA cells (EA-Luc, black) versus Trp53 shRNA (EA-Trp53, red; Fig. 4A). Both the donor and the recipient mice were treated with ENU. EA-Luc mice survived significantly longer (370 days vs. 200 days, P = 0.0014; Fig. 4A), and none developed AML (Fig. 4B). Most (93%, 13/14) developed MDS with marked proliferation of dysplastic erythroblasts, and some mild granulocytic and megakaryocytic dysplasia (Supplementary Fig. S5). In contrast, the addition of Trp53 knockdown led to leukemia development; 45% (5/11) of EA-Trp53 mice (red) developed AML versus none (0/14) of the EA-Luc mice (P = 0.009; Fig. 4B). Together, our data show that cell-intrinsic loss of Egr1 and Apc is not sufficient to promote the development of overt leukemia, even...
when HSPCs and the microenvironment are exposed to ENU. However, concordant loss of Trp53 leads to the development of AML in almost half of the mice when both the HSPCs and microenvironment are exposed to ENU. The severity of disease appears to worsen with the additional loss of Apc, because E-Trp53 mice did not succumb to disease as quickly as did EA-Trp53 mice (369 days vs. 200 days, \( P = 0.0177 \); Supplementary Fig. S6A), and showed a trend towards more MDS and less AML compared with EA-Trp53 mice (Supplementary Fig. S6B). Thus, the EA-Trp53 mouse model mimics del(5q) MDS patients, where TP53 mutation and/or loss confers the highest risk for disease progression and death in patients.

Several lines of evidence suggest that the EA-Trp53 AMLs model the progression to high-risk disease. Gene expression profiling of EA-Trp53 AML versus EA-Luc MDS samples revealed a positive enrichment of the hematopoiesis early progenitor signature, and a negative enrichment of the hematopoiesis mature cell and the myeloid cell maturation signatures in AML samples (Fig. 4C and D), as well as an elevated (>20%) myeloblast count, modeling the transition from MDS to AML. A strong upregulation of MYC target genes and WNT/β-catenin signaling in EA-Trp53 AML samples (Fig. 4E and F) modeled our previous expression profiling data of del(5q) t-MN patients, which showed a distinctive upregulation of the MYC oncogene, and deregulated WNT signaling (38, 39). In EA-Trp53 samples, compared with EA-Luc MDS, there were a greater number of mice with a clonal chromosomal aberration [75% (6/8) vs. 28% (2/7), \( P = 0.13 \)], and on average a greater number of aberrations (7 vs. 1.6, \( P = 0.22 \); Supplementary Table S2); however, the sample size was small and these differences did not reach statistical significance. Overall, our mouse model provides a tractable system to understand the molecular events defining del(5q) t-MNs with TP53 deficiency (through 17p loss or TP53 mutation) and, potentially, to test novel therapies.

Although AML samples were transplantable to secondary recipients, MDS samples (with or without Trp53 shRNA) were not (Supplementary Table S1). The inability to transplant MDS is similar to the observation that transplantation of CD34+ cells from patients with MDS does not result in engraftment or propagation in murine xenograft models, unless MDS-derived MSCs, but not MSCs from age-matched healthy controls, are injected (16), supporting a contribution of the BM microenvironment to the pathogenesis of MDS. In our EA-luc model, we noted that development of MDS was particularly sensitive to ENU’s effect on the microenvironment (Fig. 5A). ENU treatment of donor Egr1, Apcdel/+ mice exposing the HSPCs to ENU was not sufficient to promote the development of MDS (Fig. 5B). However, when WT recipient mice were treated with ENU, EA-luc mice developed MDS at the same penetrance and latency as when both donor and recipient were treated with ENU (80% MDS vs. 93% MDS, \( P = 0.55 \); 390 days vs. 374 days median MDS survival, \( P = 0.271 \); ENU-recipient vs. ENU-both, respectively; Fig. 5B and C). Because an expansion of erythroblasts in conjunction with morphologic dyspoeisis was observed, these data are consistent with a neoplastic rather than reactive process. These data suggests that ENU treatment of the stromal microenvironment influences disease progression of Egr1, Apcdel/+ HSPCs resulting in MDS. Thus, chemotherapy may, in some instances, be a cell-extrinsic factor driving emergence of MDS clones.

**DNA Damage Response Genes Are Somatically Mutated in AMLs Modeling Del(5q) t-MN**

To identify whether loss of Egr1, Apc, and Trp53 was sufficient to drive AML transformation, or whether additional mutations were required, we performed whole-exome sequencing (WES) on seven EA-Luc MDS and eight EA-Trp53 AML samples. All seven MDS and five of eight AML samples were derived from ENU-both conditions; two AMLs were derived without ENU (1586 and 4525) and one AML (7914) after ENU treatment of the recipient only. The median number of deleterious mutations per mouse was eight (range: 0–24) for the EA-Luc MDS samples, and 12.5 (range: 1–40) for the EA-Trp53 AML samples (Fig. 6A). Indel mutations were detected only in the EA-Trp53 AML group and ranged from 0 to 3 per sample. The median variant allele frequency (VAF) was similar for the EA-Luc MDS and EA, Trp53-AML groups (27%, range 20–55%; and 30%, range 20–64%, respectively). A complete list of mutated genes, with a Genomic Evolutionary Rate Profiling (GERP) score >2 and considered to be deleterious, is found in Supplementary Table S3.

Mutated genes were categorized into the 10 functional categories of genes that are recurrently mutated in MDS and AML: genes encoding chromatin modifiers, DNA methylation regulators, DNA repair proteins, ribosomal proteins, signal transducers, transcription factors, tumor suppressors, mutation signatures in AML samples (Fig. 4C and D), as well as an
Figure 4. Following exposure to an alkylating agent, haploinsufficiency of both Egr1 and Apc promotes development of MDS; loss of these genes together with Trp53 promotes AML development. A, Kaplan–Meier survival curves of WT recipients transplanted with Egr1+/−, Apcdel/+ BM cells transduced with luc shRNA (EA-luc: black) or Trp53 shRNA (EA-Trp53: red). Both donor and recipient mice were treated with ENU. Disease development is significantly faster in EA-Trp53 mice compared with EA-luc mice (200 days vs. 370 days, \( P = 0.0014 \)).

B, Histologic classification of diseases shows that most EA-luc mice developed MDS, and none developed AML. C–F, Mouse genes were collapsed to human gene names; differentially expressed genes in EA-Trp53 AML samples (n = 6) versus EA-luc MDS samples (n = 6) were analyzed using the GSEA software. Features, such as a block in myeloid differentiation and upregulation of MYC target genes and WNT/β-catenin signaling recapitulate human t-MN.
cohesin and splicesome components, and NPM1 (refs. 40, 41; Fig. 6B). There were two nonsense mutations (Recql4, Aff2) and two indel mutations (Fer1l5, Herc5); the remaining missense mutations were considered to be deleterious by the GERP, Sorting Tolerant from Intolerant (SIFT) and/or Polymorphism Phenotyping v2 (PolyPhen-2) algorithms (Fig. 6B; Supplementary Table S4). Interestingly, six similar missense gene mutations (Idh1, Atm, Lrba, Pdgfra, Pik3r3, and Dicer) were identified in human patients using the Clinvar database.

Within the ten categories listed above, there was a significantly greater number of genes mutated in the EA, Trp53-AML versus EA-Luc MDS group (26 vs. 12, \( P = 0.0026 \)) (Fig. 6B). Ribosomal and signaling genes were mutated in both groups, with signaling gene mutations occurring more frequently in EA-Trp53 AML samples (\( P = 0.0089 \)). Genes encoding transcription factors were mutated more frequently in EA-Luc MDS samples (\( P = 0.057 \)). The chromatin modifying (Crebbp, Kdm5a, and Vprbp) and DNA methylation (Idh1) genes were mutated in EA, Trp53-AMLs. Mutations in genes encoding cohesin or splicesome components or Npm1, which are not enriched in the del(5q) subgroup of t-MN (1), were not detected.

Investigation of the mutated genes with the Molecular Signatures Database revealed that there was a significant enrichment of genes within the Gene Ontology (GO) DNA repair (\( P = 1.18 \times 10^{-8} \)) collection and include Atm, Fanca, Fancc2, Mre11a, Ppp3r, Recql4, Sirt1, Top3a, Usp3, and Usp47 (Supplementary Table S5). Remarkably, a significantly larger number of DNA damage response (DDR) gene mutations were in the EA-Trp53 AML versus the EA-Luc MDS group (\( P = 0.0003 \), Fisher exact test). In addition, Idh1 mutations have been linked to altered DNA repair (42), and mouse 7914 had a somatic Idh1 mutation that corresponded with the IDH1 mutation involving arginine 132 (R132 “hotspot”).

Figure 5. ENU exposure of the BM microenvironment is a major force driving MDS development in mice transplanted with Egr1 and Apc haploinsufficient HSPCs. A, Schematic of the transplantation schemes used to elucidate the effects of alkylating agents on HSPCs (donor) versus the microenvironment (recipient) in the absence of Trp53 knockdown. WT recipients were transplanted with Egr1\textsuperscript{+/-}, Apc\textsuperscript{del+} BM cells transduced with Luc shRNA (control) under 4 different ENU conditions. Donor mice received one injection of ENU (100 mg/kg) 2 weeks before bone marrow harvest. Recipient mice received one injection of ENU (100 mg/kg) 3 weeks before lethal irradiation and transplantation. B, Kaplan–Meier survival curves of mice developing myeloid disease. Survival time is similar when only recipient mice are exposed (390 days) versus when both donor and recipient mice are exposed to ENU (374 days; \( P = 0.271 \)). As a control, WT BM was transplanted into WT recipients, and then treated with ENU; median survival was not reached as only 2 of 15 (13%) mice developed MDS. C, Histologic classification of diseases arising in the mice. The percent of mice that developed MDS in ENU-recipient (80%) and ENU-both (83%) conditions was similar (\( P = 0.55 \)) by Fisher exact test.
frequently observed in human malignancies (Fig. 6C). Mutations were confirmed by Sanger sequencing (Supplementary Fig. 7). Figure 6B illustrates that each EA-Trip53 AML had at least one mutation in a DNA repair gene (with the exception of mouse 7101). Somatic mutations in IDH1 and ATM, and germline mutations in Fanconi anemia genes have been observed in patients with t-MN (refs. 29, 43; Supplementary Table S6). In AML samples from patients, curated by the Catalogue Of Somatic Mutations In Cancer (COSMIC) database, there are codon-altering mutations in IDH1, ATM, FANCA, MRE11, RECQ4L3, USP3, and USP47; FANCD2, PPINS, TOP3A are mutated only in lymphoid neoplasms (Supplementary Table S6). These findings support the hypothesis that mutation of the DDR genes identified in EA-Trip53 AMLs contributes to the development of myeloid disease.

To test for a defect in the DNA damage response, we measured the induction and resolution of phosphorylated histone H2AX (γH2AX), a well-established marker for DSBs, after 2 Gy of irradiation, and showed that cells from 4 of 5 EA-Trip53 AMLs tested have an aberrant double-strand break response (as measured by resolution of γH2AX fluorescence), similar to our previously published results (ref. 6; Fig. 6D). Cells from one leukemia mouse (7103) had a normal γH2AX response even though they had a mutation in the Ppp5c gene, which encodes a serine/threonine phosphatase involved in multiple cellular processes, including the DNA damage response. At 6 hours postirradiation, the mean fluorescence intensity of γH2AX was approximately 2.4-fold higher in EA-Trip53 AML samples suggesting that AML cells are unable to resolve DSBs to the same extent as WT cells (Fig. 6E). Thus, haploinsufficiency of two critical del(5q) genes, Egr1 and Apc, with concurrent Trip53 loss is sufficient to initiate disease; however, acquisition of a DDR gene mutation, and ensuing abnormal DNA repair response, appears to be critical for transformation to AML.

**DISCUSSION**

In this study, we addressed the long-standing question of the role of cytotoxic therapy in the pathogenesis of t-MN, and why prior cytotoxic therapy strongly selects for Trip53 loss and/or mutation in conjunction with a del(5q). We showed that loss of Trip53 in HSPCs in conjunction with two key tumor suppressor del(5q) genes, Egr1 and Apc, was required for the development of AML; the development of AML was associated with the acquisition of deleterious mutations in DNA repair genes. Modeling the consequence of a cytotoxic-exposed repair gene environment, our data suggest that chemotherapy-induced alterations to the microenvironment should be considered one of the predisposing risk factors for the development of t-MN. Although the seminal study by Wong and colleagues demonstrated that selection of cells with Trip53 mutations/loss occurs after exposure to DNA-damaging chemotherapeutics (11), this study did not distinguish the effect of chemotherapy on HSPCs versus the microenvironment. Our in vivo model shows the unexpected finding that a chemotherapy-exposed microenvironment promotes this expansion. Moreover, we introduce the concept that senescent phenotype within the BM microenvironment, induced by prior cytotoxic therapy, may contribute to the pathogenesis of t-MN.

Jacoby and colleagues previously demonstrated that myeloblasts from the majority of patients with t-MN are characterized by deregulation of the DNA damage response, even though few somatically mutated DDR genes were identified (14). It is striking that in our model of del(5q) t-MN, leukemia development in mice selected for mutations of DDR genes, impairing the DDR, similar to patients with t-MN. The results of our studies suggest that Trip53 loss, on its own, does not lead to an impaired DNA damage response in t-MN, because nearly every mouse AML with Trip53 loss had acquired a mutation of a DDR gene. Although patients with t-MN do not generally exhibit somatically mutated DDR genes (1), inherited and acquired mutations, epigenetic modifications, and/or chromosomal loss of DNA repair gene(s) may all contribute to an aberrant DNA damage response in patients. The finding that the AML developing in mice selected for DDR gene mutations in a model of t-MN lends support to the idea that therapeutic approaches exploiting a dysfunctional double-strand break response should be considered for t-MN.

A new paradigm in leukemogenesis is that alterations of the microenvironment contribute to the pathogenesis of myeloid malignancies (15, 17, 18, 44). This may be due to genetic
alterations, as is the case for the Shwachman–Bodian–Diamond syndrome gene, remodeling of the niche by malignant myeloid cells (16, 25), or environmental exposures such as cytotoxic therapy, as suggested by this study. We hypothesize that MSCs in the BM microenvironment are key players, because recipient mice were lethally irradiated, leading to cell death of immune cells but not the MSCs, which are typically radioresistant. Although chemotherapy is known to transiently increase proinflammatory cytokines (45), we waited two weeks after ENU administration before transplanting mice. Because ENU treatment of recipient mice appeared to “set the stage” for malignant transformation, only observed months later, we hypothesized that chemotherapy must have long-term consequences on BM stromal cells, such as establishing a senescent phenotype. In support of this, senescence of nontumor cells, following doxorubicin treatment, was shown to play a key role in cancer relapse and spread to other tissues in a mouse model of breast cancer (33). Our study expands on this idea and suggests that cytotoxic therapy for a prior malignancy may have long-term effects on the microenvironment, possibly through the induction of senescence, creating an inflammatory environment and favorable conditions for the development of t-MN. Consistent with our hypothesis, it was recently shown that MSCs from t-MN patients had a higher senescent rate as compared with MSCs isolated from healthy controls, patients with primary MDS, and patients with two unrelated cancers, without prior cytotoxic exposure (46).

In Fig. 7, we propose that there are several pathways that lead to t-MN. Various predisposing factors may influence whether a patient develops t-MN (left; ref. 1). The presence of a germline mutation in genes involved in DNA repair and/or DNA damage-sensing pathways, such as BRCA1, BRCA2, CHEK2, TP53, and Fanconi anemia genes, has been identified in some patients with t-MN, and may lead to ineffective DNA repair following cytotoxic therapy and the acquisition of somatic mutations (43, 47–49). Individuals who have CHIP, or small preexisting, age-related resilient clones, may be at increased risk of developing t-MN following cytotoxic therapy (9–13). Finally, it is also possible that the bone marrow microenvironment is damaged prior to cytotoxic therapy, due to aging, infection, chronic inflammation, or inherited mutations (50). No recurrent somatic mutations or karyotypic abnormalities have been found in MDS-derived MSCs (51). Rather, it has been proposed that malignant stromal alterations may be of an epigenetic nature (52); however, at present, it is not known if alterations precede disease or are induced by malignancy, and how chemotherapy may impact these epigenetic changes.

Our model suggests that multiple cycles of cytotoxic therapy may create conditions that promote clonal expansion of cells with preexisting and/or newly acquired mutations and/or chromosomal aberrations. Cytotoxic therapy may also alter the BM microenvironment, possibly through therapy-induced senescence, to help promote malignant transformation in t-MN. Consistent with this idea, senescence-associated changes have been observed in MSCs isolated from patients with MDS and AML (20, 25). The pathways illustrated in Fig. 7 are not mutually exclusive, and one or multiple pathways may ultimately lead to decreased DDR, deficient checkpoint responses, and alterations to metabolism, collectively contributing to increased survival and expansion of the t-MN clone. Our results suggest that niche-based therapeutic approaches, most likely in conjunction with approaches targeting the hematopoietic cells, should be considered. Thus, elucidating the effects of cytotoxic therapy on the BM microenvironment and on the HSPCs, as well as the contribution of an aberrant DNA damage response, may provide insights relevant to the development of targeted therapies for t-MN.
METHODS

Mice
All mouse studies were approved by The University of Chicago's Institutional Animal Care and Use Committee (IACUC), and mice were housed in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Egr1<sup>+/−</sup>, Mx1-Cre Apclk<sup>−/+</sup> (Egr1<sup>+/−</sup>) and Egr1<sup>−/−</sup>, Mx1-Cre Apclk<sup>−/−</sup> (Egr1<sup>−/−</sup>, Apclk<sup>−/−</sup>), mice were generated by crossing Mx1-Cre transgenic mice with Egr1<sup>+/−</sup>, Apclk<sup>−/−</sup> mice (C57BL/6 genetic background) as described previously (6).

Mouse Model of MDS/AML
Total BM cells, isolated from 10- to 12-week-old Egr1<sup>+/−</sup> or Egr1<sup>−/−</sup>, Apclk<sup>−/+</sup> mice, were transduced with a Lec (control) or Trp53-specific shRNA (p53.1224; ref. 6) and transplanted, via retro-orbital injection, into lethally irradiated (8.6 Gy) WT mice (B6.SJL/Cd45.1). Where indicated, donor mice were injected once with ENU (100 mg/kg) 2 weeks after polysinomic-polyacrylic acid (pl-pc) injections to induce the deletion of the floxed Apc allele, and recipient mice were injected once with ENU (100 mg/kg) two weeks before transplantation. In ENU, “both” conditions, Mx1-Cre Apclk<sup>−/+</sup> mice were used as WT recipient mice, instead of B6.SJL/Cd45.1 mice. Peripheral blood, histology, spectral karyotyping, and flow cytometric analyses were performed as described previously (6). To ensure that we were not biasing the disease phenotype, animals were euthanized when they displayed at least four of the following symptoms: poor grooming, abnormal hunched posture, decreased activity, listlessness or lethargy, a body conditioning score of 2 (lean), anemia (Hb < 8 g/dL), leukocytosis (> 25 K/μL), or thrombocytopenia (< 500 K/μL), and enlarged spleen, thymus, or masses (by palpation). Survival times (time to sacrifice) were estimated by the Kaplan–Meier method and compared between groups via log-rank test.

WES and RNA-seq
Exome libraries were generated using the SureSelectXT Mouse All Exon kit (Agilent, Santa Clara, CA) following the manufacturer’s directions. 100 bp paired-end sequencing was performed on a HiSeq 4000 sequencing system. Reads were assessed for quality (Short Read and FastQC), overlapping reads were merged using FLASH (ver 1.2.11) and aligned to mm9 using BWA (ver 0.7.12.ref. 53). SAMtools v0.1.18 (53) was used to remove duplicates, and GATK was used for realignment and score recalibration (54). Mutation calling was performed with reads with mapping qualities ≥30, requiring ≥2x coverage in both tumor and normal DNA (Egr1<sup>+/−</sup>, Apclk<sup>−/+</sup>) requiring a variant read on each strand, and a minimum VAF of 20%. Indels identified using VarScan v2.3.4 (55), and single nucleotide mutations identified using both MuTect v1.1.4 (56) and VarScan were retained. Known single-nucleotide polymorphisms (SNP) for the C57BL/6 strain were removed. Only mutations with a GERP score (57) >2 predicted to have deleterious consequences were included. All gene mutations listed in Fig. 5B were validated by Sanger sequencing.

RNA-seq analysis was performed as described, with the exception that reads were aligned to mm10 and the MSC dataset was pseudoaligned with Kallisto (58). Sequencing data are available on the Gene Expression Omnibus database (GSE135866) and Sequence Read Archive (PRJNA597332).

MSC Senescence Assays
To isolate MSCs, femur, tibia, and humerus bones were flushed to remove hematopoietic cells, treated with collagenase, and cultured using MesenCult (Mouse) with MesenPure in 5% O2 according to the manufacturer’s recommendations (Stemcell Technologies). To measure SA-β-gal that is detectable at pH 6 (due to increased lysosomal biogenesis in senescent cells), the internal pH of lysosomes was increased to approximately pH 6 using bafilomycin A1. Cells were then incubated with 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG; Cayman Chemicals), a β-galactosidase substrate that becomes fluorescent after cleavage by the enzyme. SA-β-gal activity was estimated using flow cytometry. Cell proliferation was assayed with the EZClick EdU proliferation kit (BioVision, Inc.). For colony-forming units-fibroblastic (CFU-F) assays, either 2 × 10<sup>4</sup> directly isolated MSCs (postcollagenase) or 2 × 10<sup>4</sup> first-passage MSCs were plated per well of a 6-well plate, and stained with 0.5% crystal violet after 14 days to enumerate colonies. Quantitative PCR was performed as described previously (59). Primer sequences are provided in Supplementary Table S7. For RNA-seq analysis, MSCs were isolated from mock- or ENU-treated mice, approximately 1 month posttreatment, and expanded in vitro to passage 1 or 2.

Disclosure of Potential Conflicts of Interest
M.M. Le Beau is a member, board of directors at Varian Medical Systems and is a consultant/advisory board member for American Cancer Society and Leukemia and Lymphoma Society. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Angela Stoddart, Jianghong Wang, Anthony A. Fernald, et al.


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