Acute myeloid leukemias (AMLs) are a heterogeneous group of diseases that are sustained by relatively rare leukemia-initiating cells (LICs) that exhibit diverse genetic and phenotypic properties. AML heterogeneity presents a major challenge to development of targeted therapies, and effective treatment will require targeting of common molecular drivers of AML maintenance. The orphan nuclear receptors NR4A1 and NR4A3 are potent tumor suppressors of AML. They are silenced in all human AML LICs, irrespective of patient cytogenetics, and their deletion in mice leads to postnatal AML development. In the current report, we address the tumor-suppressive mechanisms and therapeutic potential of NR4As for AML intervention. We show that rescue of either NR4A1 or NR4A3 inhibits the leukemogenicity of AML cells in vivo and reprograms a subset of gene signatures that distinguish primary human LICs from normal hematopoietic stem cells (HSCs), irrespective of subtype. Central to NR4A reprogramming is the acute suppression of an LIC submodule that includes the transcriptional repression of MYC. Additionally, we show that upregulation of MYC is an acute preleukemic consequence of NR4A deletion and that MYC suppression functionally contributes to NR4A antileukemic effects. Collectively, these results identify NR4As as novel targets for AML therapeutic intervention and reveal molecular targets of NR4A tumor suppression, including the suppression of MYC.

INTRODUCTION
Acute myeloid leukemia (AML) is associated with a blockade in myeloid cell differentiation, which leads to accumulation of immature blasts and ultimately to the emergence of a transformed leukemia-initiating cell (LIC) population capable of sustaining leukemic expansion. AML architecture and LIC properties are highly variable between patients consequent of variable cytogenetics, stem/progenitor cell of origin, stage of disease progression and neoplastic evolution. Collectively, this heterogeneity represents a major challenge toward development of effective therapies for AML. Development of new molecular targeted therapies to specifically eradicate LICs and prevent AML recurrence will require a detailed understanding of the molecular features that distinguish LICs from normal stem and progenitor cells. To this end, recent genome-wide profiling of human LICs across distinct cytogenetic backgrounds has begun to identify common gene signatures that generally distinguish LICs and leukemogenic blasts from normal stem and progenitor cells. These gene signatures are likely to include common molecular drivers of LIC maintenance, in otherwise heterogeneous populations of LICs.

NR4As (NR4A1, NR4A2, NR4A3) represent a subfamily of nuclear receptor transcription factors that interact with overlapping target genes through conserved DNA-binding domains (DBDs). They are products of immediate early genes that are induced by diverse extracellular signals in a cell-specific manner. Upon activation, NR4As function in a stimulus and cell-context-dependent manner to activate or repress distinct molecular targets to regulate endocrine, metabolic, stress responses and cell fate. In addition to their transcriptional regulatory function, NR4As can regulate apoptosis through mitochondrial translocation and stimulation of cytochrome C release. Thus, both genomic and non-genomic mechanisms are likely to contribute to NR4A cellular functions.

NR4As are critical regulators of hematopoietic cell development, including regulation of hematopoietic stem cell (HSC) homeostasis, monocye differentiation and T-cell development. We previously demonstrated that NR4A1 and NR4A3 are also critical tumor suppressors of myeloid malignancies. Targeted deletion of NR4A1 or NR4A3 alone does not disrupt myeloid homeostasis. However, reduced expression of these genes below a critical level leads to chronic myelodysplastic/myeloproliferative disease, and their deletion leads to rapid early postnatal AML development due to abnormal proliferation and expansion of HSCs and myeloid progenitor cells. NR4A1 and NR4A3 expression is silenced in leukemic stem cells and blasts of human AML patients, regardless of their cytogenetic background or French-American-British (FAB) classification, and reduced expression is a commonly occurring feature of human myelodysplastic disease. Collectively, these data support an essential cooperative or redundant role for NR4A1 and NR4A3 in HSC regulation, and suggest that silencing of NR4A expression...
may be a critical event in AML etiology. NR4A molecular targets and whether NR4A expression exhibits antileukemic effects in AML cells are currently unknown.

In this study, we sought to determine the effects of NR4A rescue on the leukemogenicity and transcriptome of AML blasts by examining the consequences of NR4A rescue in a conditional NR4A1/3 knockout mouse model of AML and in human AML cell lines. We show that NR4A rescue eradicates LICs and reduces the leukemogenicity of AML cells in vivo. Through genome-wide expression profiling, we reveal a striking redundancy in NR4A1 and NR4A3 transcriptional regulation, and also identify a genomic LIC submodule that is acutely reprogrammed in response to NR4A rescue, including transcriptional repression of MYC. Additionally, we find that upregulation of MYC is an acute preleukemic response to NR4A1/3 deletion in normal hematopoietic progenitor cells. Together, these studies reveal molecular effectors of NR4A tumor suppression and identify NR4As as novel targets for AML therapeutic intervention.

RESULTS

Sustained suppression of NR4A1/3 is required for AML maintenance and NR4A rescue is sufficient to deplete AML LICs. We previously showed that germline deletion of both NR4A1 and NR4A3 (NR4A1/3 DKO) in mice leads to rapid AML development and pup death within 3 weeks after birth.17 The rapid lethality of NR4A1/3 DKO mice prevents us from properly addressing disease rescue, as limited numbers of bone marrow cells are isolated from young pups. To overcome this challenge, we conditionally controlled NR4A deletion in adult mice using a tamoxifen-regulated CRE–LoxP strategy to inactivate NR4A1 in a NR4A3 homozygous null background (NR4A3/C0/C0) (Supplementary Figure 1a). These mice were intercrossed with mice carrying a CRE recombinase–estrogen receptor T2 (Cre-ERT2) fusion gene targeted to the Rosa 26 locus to generate (rosa26) CreERT2NR4A3/C0/C0 Nr3a1fl/fl (conditional-DKO) mice. At 6 weeks, conditional-DKO and appropriate control mice were treated with 1 mg of tamoxifen per day for 5 days to activate Cre-ERT2. All conditional-DKO mice developed AML with an average latency of 15 weeks (Figure 1a) after tamoxifen treatment, as evidenced by immature myeloid blast accumulation (>20%) in peripheral blood, bone marrow and spleen (Figure 1b). Histological analysis of spleens, livers and lungs revealed disrupted tissue architecture and perivascular myeloid infiltrates, indicative of extramedullary hematopoiesis (Supplementary Figure 1c).

To address the consequences of NR4A rescue on established AML in this model, we isolated leukemic bone marrow cells from...
Transcriptional repression of MYC
SP Boudreaux et al

Efficient expression of NR4A1 and NR4A3 in human AML cells using direct IVT-RNA electroporation

Next, we asked if NR4A tumor suppression extended to human AML cells. In general, we found DNA-based transfection methods to yield low efficiency, high lethality and overall poor NR4A rescue in human AML cell lines. Attempts to express NR4A coding sequences in AML cells under the control of a heterologous promoter (cytomegalovirus, CMV) and 3′-untranslated region (bovine growth hormone polyadenylation sequence, BGH pA) failed to generate measurable NR4A expression (Supplementary Figure 3a). These observations suggested that NR4A silencing in AML cells likely involves mechanisms operating within the coding region of the NR4A genes. To overcome these limitations, we utilized a novel electroporation system to directly electroporate pure polyadenylated and capped \textit{in-vitro}-transcribed RNAs (IVT-RNA) into AML cells. Using EGFP RNA as control for electroporation efficiency, we found this method extremely effective and robust, yielding \textit{>87}\% EGFP transfection efficiency with little cell death in multiple leukemic cell lines (Figures 2a and b and Supplementary Figures 3b and c). More importantly, this system led to successful expression of NR4A1 and NR4A3 in AML cell lines (Figure 2c and Supplementary Figure 3d and e). The NR4A-RNA electroporation system also has several major advantages including, (1) rapid and high transfection efficiency, (2) control of input RNA levels to minimize overexpression, and (3) it permits the evaluation of early cellular responses to \textit{acute} gene expression under relatively innocuous conditions. RNA transfections have previously been used to generate antigen-presenting dendritic cells and induce pluripotent stem cells.\textsuperscript{25,26} Our work extends the utility of RNA transfection to AML cells and reveals this system as a promising tool for investigating effects of gene rescue in AML cells.

Acute expression of NR4A1 or NR4A3 suppresses growth and survival of human AML cells

Of the cell lines examined, we achieved the best NR4A expression in Kasumi-1 cells, with detection of NR4A protein as early as 1 h and up to 24 h post-electroporation, and with NR4A3 protein exhibiting greater stability than NR4A1 (Figure 2c, Supplementary Figure 3d–f). We found that expression of either NR4A1 or NR4A3 greatly suppressed the viability of Kasumi-1 cells over a 96-h time course (Figure 3a). Reduced viability was found to be non-synergistic between NR4A1 and NR4A3, and dependent on DNA binding, as mutation of two amino acid residues essential for DNA binding within the NR4A DBD\textsuperscript{27} in either receptor abolished their growth-suppressive activity (Figure 3b). Expression of either NR4A1 or NR4A3 also reduced the colony-forming capacity of Kasumi-1 cells in a cytokine-independent manner, demonstrating potent long-term suppression of Kasumi-1 growth despite transient NR4A expression (Figure 3c). Decreased viability of Kasumi-1 cells after NR4A rescue was associated with an increase in cell death (Figure 3d, Supplementary Figure 4a) and reduced
proliferation (Figure 3e, Supplementary Figure 4b). Although our ability to express NR4As in other AML lines is limited as compared with Kasumi-1 cells, we did find that expression of NR4As also reduced the viability of HL60 and THP1 AML cells (Supplementary Figures 5a and b) to varying degrees. Together, these results extend the NR4A tumor-suppressive function to human AML cells and describe NR4A mechanism of action as non-synergistic and DBD-dependent.

Transcriptional redundancy of NR4A1 and NR4A3 function is revealed by genome-wide expression profiling

Next, we investigated the downstream molecular targets of NR4A tumor suppression. The rapid expression of NR4A1 and NR4A3 permits evaluation of early transcriptional events in AML cells before any overt cellular effects. We performed transcriptome-wide microarray analysis in Kasumi-1 cells at 14 h post-electroporation, as this time point precedes NR4A-induced Kasumi-1 growth changes (Figure 3a). We employed the biologically motivated Rank Products (RP) algorithm to identify differentially expressed genes. This method is microarray platform independent and easily lends itself to integration with other RP analyzed data sets.28 Strikingly, this analysis revealed nearly complete redundancy in NR4A transcriptional regulatory function with over 97% of genes commonly regulated by NR4A1 and NR4A3, consistent with the NR4A1/3 redundancy observed in conditional-DKO mice (Figure 4a and Supplementary Table 1). We interrogated our microarray results for enrichment of annotated gene sets via Gene Set Enrichment Analysis. Most striking was the strong inverse correlation between NR4A expression and multiple MYC-regulated gene sets, including the multi-cancer derived MYC oncogenic signature (Figure 4h), both of which are critical regulators of HSC homeostasis.31,32 In all cases, we confirmed regulation of genes via quantitative PCR analysis and found this regulation to be DBD-dependent and non-synergistic (Figures 4d and f–h). Collectively, these results identify a large and redundant number of molecular targets of NR4A tumor suppression in AML and highlight a central role for suppression of MYC in NR4A transcriptional regulatory function.

NR4As acutely reprogram a subset of LIC gene signatures including MYC

Next, we sought to determine how NR4A-regulated genes related to genes commonly dysregulated in LICs. To this end, we...
intersected our gene signature with the dysregulated gene expression networks recently shown to commonly distinguish highly purified human LICs from normal HSCs. Importantly, analysis of this LIC data set under the same statistical criteria used in the Kasumi-1 microarray confirmed the general downregulation of NR4As in LICs from distinct cytogenetic backgrounds (Supplementary Figure 10a). We identified 73 genes (P-value < 0.001), for which dysregulated expression is common to all AML LICs and is reversed by acute expression of NR4As (Figure 5a and Supplementary Figure 10b). Remarkably, this analysis identified upregulation of MYC and downregulation of FOXO1 as components of the LIC signature, for which expression is acutely reprogrammed by NR4A expression. Together, these genes may define an LIC submodule for which dysregulation is attributable to NR4A downregulation in AML. These data support a general model where NR4A antagonism of MYC is a central component of NR4A tumor suppression in AML. From this, it stands to reason that AML silencing of NR4A gene expression may contribute to MYC upregulation during AML development. To test this, we acutely knocked out NR4A expression in progenitor bone marrow cells of healthy adult conditional-DKO mice with a 6 h in vitro tamoxifen treatment. We found that acute knockout of NR4A activity led to rapid upregulation of MYC mRNA in normal hematopoietic progenitors (Figure 5b). Additionally, through retroviral expression of NR4A3 in leukemic progenitor cells of conditional-DKO with established AML disease, we found that restored NR4A3 expression effectively suppressed MYC mRNA levels as early as 48 h (Figure 5c). Lastly, small interfering RNA knockdown of MYC resulted in reduced Kasumi-1 viability similar to that of NR4A expression alone, suggesting that MYC repression contributes to NR4A tumor suppression (Figures 5d and e). Collectively, these data support a model where upregulation of MYC is an immediate preleukemic consequence of NR4A silencing in normal hematopoietic progenitors and suggest that MYC suppression functionally contributes to NR4A tumor suppression in AML cells.

**DISCUSSION**

We previously reported that germ line deletion of both NR4A1 and NR4A3 in mice is sufficient to cause rapid early postnatal AML development. The requirement for deletion of two NR4A subfamily genes for disease development suggested a redundant or cooperative role for NR4A1 and NR4A3 in tumor suppression.
Further, silenced expression of NR4As is a common feature of human AMLs regardless of cytogenetics and suggests that NR4A dysregulation may be an obligate event in human AML development. However, a potential role for NR4As as therapeutic targets in AML and their molecular mechanisms of tumor suppression remained to be established. In the current report, we have addressed the therapeutic potential of NR4A activation in AML by retroviral rescue of NR4A expression in a mouse model of AML. We have shown that temporally restricted deletion of NR4A1 and NR4A3 in adult mice leads to development of AML disease similar to that observed after germ line deletion of both genes. Functional LIC maintenance as measured by long-term engraftment of transplanted AML cells is critically dependent on NR4A depletion as retroviral rescue of NR4A3 depletes AML LICs and eradicates AML disease in a competitive transplantation setting (Figure 1). These findings support an obligate role for NR4A suppression in AML maintenance and further suggest that additional irreversible mutations are unlikely to be required for LIC transformation in the absence of NR4As.

Stable rescue of NR4A expression in human AML cells is hampered by the surprising finding that sequences within the coding sequences of both the NR4A1 and NR4A3 cDNAs contribute to AML cell selective silencing of these genes. Using standard plasmid transfection systems (Supplementary Figure 3a), we found that AML selective silencing of NR4As was retained via their coding regions in human AML cell lines, despite the presence of a highly efficient heterologous promoter and 3′-untranslated region. Importantly, direct electroporation of purified NR4A1 or NR4A3 IVT-RNAs was sufficient to overcome NR4A silencing, suggesting that mechanisms underlying NR4A repression occur primarily at the DNA rather than RNA level. Although the molecular mechanisms underlying NR4A silencing in AML cells are currently being elucidated, understanding these mechanisms and the NR4A sequences involved will be key to achieving stable reactivation of NR4A expression in human AML cells and in animal models of human AML. Notably, the expression level and stability of NR4As varied considerably among cell lines, and NR4A protein exhibited altered mobility that varied with time (Figure 2c and Supplementary Figures 3d and e). In particular, NR4A1 displayed multiple protein species that differed in stability (Supplementary Figure 3f). Previous studies have demonstrated that NR4As are subject to posttranslational modifications that increase NR4A mobility in the same manner observed in this study and are associated with enhanced NR4A degradation. Thus, in addition to DNA-based mechanisms, these results suggest that additional mechanisms operating at the post-translational level also regulate NR4A activity in AML cells.

The IVT-RNA electroporation system has generally proved to be a highly effective method of leukemic cell transfection with over 85% transfection efficiency in all AML cell lines examined. Using this system, we found that acute NR4A expression compromises the leukemogenicity of human AML cells derived from distinct cytogenetic backgrounds, albeit to varying degrees. Further, the IVT-RNA approach leads to extremely rapid protein expression and avoids common problems related to overexpression of exogenously introduced genes. The levels of NR4A1 and NR4A3 in AML cells after IVT-RNA electroporation are approximately 20- to 25-fold higher than NR4A levels we previously reported in human CD34+ stem/progenitor cells. However, we have shown...
that NR4As are highly expressed only in quiescent HSCs and are sharply downregulated (approximately 10-fold) upon HSC proliferation.\(^3\,\text{a}\,\text{f}\) As CD34+ cells represent a mixed population of stem/progenitor cells, the majority of which are proliferating, we calculate that NR4A levels are underrepresented in this population by approximately 10-fold, relative to endogenous levels in quiescent HSCs. Thus, we estimate that IVT-RNA levels of NR4As in AML cells are approximately 2- to 3-fold higher than those in HSCs.

In general, this IVT-RNA electroporation approach provides a unique opportunity to capture early transcriptional responses to gene expression in advance of cellular effects. This is particularly advantageous for delineating temporal responses to gene expression and for investigating molecular targets of constitutively active transcription factors, including ligand-independent orphan nuclear receptors.

Consistent with redundant tumor-suppressive activity of NR4As, global gene-expression profiling in AML cells confirmed remarkable redundancy in NR4A1 and NR4A3 transcriptional activity. Integration of NR4A1/3 regulated gene signatures with remarkable redundancy in NR4A1 and NR4A3 transcriptional expression and for investigating molecular targets of constitutively active transcription factors, including ligand-independent orphan nuclear receptors.

In human AMLs will be required to devise effective strategies toward their reactivation and for therapeutic intervention.

**MATERIALS AND METHODS**

**Mice, retroviral transduction and transplantation**

CreER \(^{-}\)NR4A\(^{-}\)/NR4A\(^{+}\)/NR4A\(^{-}\) mice were generated on a C57BL/6 background. NR4A\(^{+}\)/mice were a gift from Pierre Chambon, and Rosa26CreER mice were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME, USA). Non-lymphoid hematopoietic neoplasms were characterized according to guidelines of the Mouse Models of Human Cancers Consortium (http://emice.nci.nih.gov/emice/mouse_models). Mice were monitored for onset of disease by performing complete blood counts (Advia 120; Bayer-Siemens, Deerfield, IL, USA) with automated and manual differentials. Animals were killed when they became moribund (indicated by hunched posture, lethargy and difficulty in breathing). Bone marrow cellularity was determined by manual counts. Blood smears, bone marrow and spleen cytospins were stained with Wright-Giemsa Stain (Sigma, St Louis, MO, USA). All mouse experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

**For transplantation experiments, recipient mice were lethally irradiated with 10\(\)gy and cells were transplanted via retro-orbital injections.**

**Cell culture**

Kasumi-1, HL60 and THP-1 cells were purchased from ATCC (Manassas, VA, USA). Cells were maintained in 1640 RPMI plus 10% fetal calf serum, except for Kasumi-1 that were maintained in 20% fetal calf serum. All cellular assays were performed in 10% fetal calf serum.

**Plasmids and IVT-RNA transfections**

All constructs were PCR-amplified to generate N-terminal FLAG-tagged coding sequence, then cloned into pCR2.1-TOPO TA vectors, and sub-cloned into pcDNA3.1 vectors (Invitrogen, Grand Island, NY, USA). BD mutations were introduced into NR4A1 (C\(^{284E285}\)AA) and NR4A3 (C\(^{309E310}\)AA) with the GeneTailor Site-Directed Mutagenesis system (Invitrogen). In vitro transcription was performed per manufacturer’s instructions with mMESSAGE mMACHINE T7 Kit, polyadenylation was performed with Poly(A) Tailing Kit, and resulting IVT-RNA was purified with MEGA Clear Kit (Applied Biosystems, Carlsbad, CA, USA). For electroporation, cells were suspended to a final concentration of one million cells per 100\(\)\(\mu\)l Dulbecco’s phosphate-buffered saline (DPBS). Cell solution (200\(\)\(\mu\)l) was transferred to 0.4 cm cuvettes (USA Scientific, Ocala, FL, USA), mixed by pipetting with IVT-RNA at a final concentration of 100\(\)nm, and immediately electroporated at 330\(\)V for 5\(\)ms with the GenePulser Xcell system (Bio-Rad, Hercules, CA, USA). Fresh growth media (200\(\)\(\mu\)l) was added to each cuvette, cells rested for 10 min and were then transferred to appropriate culture dishes.

**Cellular assays**

Cell viability was determined by both Trypan Blue exclusion and CellTiter AQueous Non-Radioactive Cell Proliferation Assay (Promega, Fitchburg, WI, USA). Cell death was assayed using fluorescein isothiocyanate (FITC) conjugated Annexin V and propidium iodide. Proliferation was assayed with fluorescein isothiocyanate (FITC) conjugated BrdU. For colony-forming assay 10\(\)000 Kasumi-1 cells were plated in 3\(\)ml of MethoCult H4423 Enriched or MethoCult H4100 (Stem Cell Technologies, Vancouver, BC, Canada) and scored for colony-forming units 8 days later.

Western blot, quantitative reverse-transcriptase PCR (qPCR) and small interfering RNA (siRNA)

Cells were lysed in RIPA buffer plus protease inhibitors, and 10\(\)\(\mu\)g of total protein was used for western blotting. Antibodies used include anti-NR4A sc-990 (Santa Cruz, Santa Cruz, CA, USA) anti-Actin Clone C4 (Millipore, Billerica, MA, USA) and anti-GFP #2555 (Stem Cell Technologies). Secondary antibodies were conjugated to horseradish peroxidase and visualized with chemiluminescence. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed into cDNA using High-Capacity CDNA Reverse Transcription Kits and quantified via TaqMan Gene Expression Assays and Master Mix (Applied Biosystems). MYC and control small interfering RNA (Qiagen) were electroporated into Kasumi-1 cells at a final concentration of 200\(\)nm.
Immunohistochemistry and flow cytometry analysis
Immunohistochemistry and flow cytometry experiments were performed essentially as described in Mullican et al. Flow cytometry data was analyzed with FlowJo (Treestar, Ashland, OR, USA).

Microarray analysis and bioinformatics
Kasumi-1 total RNA was collected with RNeasy Mini Kits (Qiagen). Quality control and processing of human genome U133 Plus 2.0 (Affymetrix; Santa Clara, CA, USA) chips was performed by the Baylor College of Medicine Array Core. Data for LIC gene analysis was collected from the GEO, GSE17054. For all data analysis, probe fluorescence values were normalized and log2-transformed using RMAExpress. Differentially fluorescent probes were identified within the MeV environment using the RP algorithm by criterion of Q-values \( \leq 0.05 \).

For Kasumi-1 microarray analysis, two independent RP tests were performed: EGFP versus NR4A1 and EGFP versus NR4A3. A gene was called significant if it achieved a Q-value \( \leq 0.05 \).

Gene Set Enrichment analysis was performed on signature genes, subject to 100 bootstrapping samples for statistical significance estimation. Gene Set Enrichment analysis was performed with standard parameters on NR4A signature genes, subject to 100 bootstrapping samples for statistical significance estimation.

Chromatin immunoprecipitation assays (ChiPs)
Chromatin immunoprecipitations were performed 4 h after IVT-RNA electroporation using Abcam X-ChIP protocol (Abcam, Cambridge, MA, USA) with 4 μg of anti-NR4A (sc990) or non-specific IgG (sc2345).

Statistical analysis
Data are presented as mean \( \pm \) s.d., except when noted otherwise. The significance of the differences between groups was determined by using Student’s t-test (two-tailed). A P-value \( \leq 0.05 \) was considered significant for all analyses.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We acknowledge the joint participation by Adrienne Helis Malvin Medical Research Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine and the Chemical Genomics of Nuclear Receptors and Coregulators Program. This work was also supported by grants from NIH R01 CA111411 and CPBRT RP110189 to OMC, NIH T32-HL092332 to SPB and, NSF 2000979140 to ARH.

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Supplementary Information accompanies the paper on the Oncogenesis website (http://www.nature.com/oncsis)