Genetic regulation of the RUNX transcription factor family has antitumor effects

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Runt-related transcription factor 1 (RUNX1) is generally considered to function as a tumor suppressor in the development of leukemia, but a growing body of evidence suggests that it has pro-oncogenic properties in acute myeloid leukemia (AML). Here we have demonstrated that the antileukemic effect mediated by RUNX1 depletion is highly dependent on a functional p53-mediated cell death pathway. Increased expression of other RUNX family members, including RUNX2 and RUNX3, compensated for the antitumor effect elicited by RUNX1 silencing, and simultaneous attenuation of all RUNX family members as a cluster led to a much stronger antitumor effect relative to suppression of individual RUNX members. Switching off the RUNX cluster using alkylating agent–conjugated pyrrole-imidazole (PI) polyamides, which were designed to specifically bind to consensus RUNX-binding sequences, was highly effective against AML cells and against several poor-prognosis solid tumors in a xenograft mouse model of AML without notable adverse events. Taken together, these results identify a crucial role for the RUNX cluster in the maintenance and progression of cancer cells and suggest that modulation of the RUNX cluster using the PI polyamide gene-switch technology is a potential strategy to control malignancies.

Introduction
Runt-related transcription factor 1 (RUNX1), also known as acute myeloid leukemia 1 protein (AML1), is an essential master transcription factor implicated in the differentiation of hematopoietic stem cells (1). RUNX1 is a member of the RUNX family proteins (RUNX1, RUNX2, and RUNX3), and each member forms a heterodimeric complex with core-binding factor β subunit (CBFB) to exert its sequence-specific transactivation function (2). RUNX modulates the transcription of its target genes through recognizing the core consensus binding sequence 5′-TGTGGT-3′, or very rarely, 5′-TGCGGT-3′, within their regulatory regions via its runt domain, while CBFB is a non-DNA-binding regulatory subunit that allosterically enhances the sequence-specific DNA-binding capacity of RUNX (3, 4). According to previous studies, RUNX1 plays a pivotal role in definitive hematopoiesis, as examined by the phenotypes of Runx1-deficient mice (5). On the other hand, the conditional knockout of Runx1 in adult mice showed marginal changes in platelet counts without hematopoietic stem cell exhaustion (6), implying that RUNX1 is dispensable for adult hematopoiesis. With respect to tumorigenicity, RUNX1 has been considered to act as an oncosuppressor during the development of leukemia owing to its relatively higher frequency of mutations and translocations (7, 8). This classical viewpoint has been challenged by our recent observations showing that wild-type RUNX1 is strongly required for the development of AML with inv(16) or with mixed lineage leukemia (MLL) fusions (9–11). These findings first shed light on the oncogenic property of RUNX1 in the initiation of leukemia. Unfortunately, despite these discoveries, the precise molecular basis for how RUNX1 as well as the other RUNX family members could contribute to the maintenance and/or progression of leukemia has been largely unknown.

p53 (TP53), structurally a distant relative of RUNX, is one of the most famous and intensely studied tumor suppressors in human cancers (12). p53 deficiency generally enhances the initiation or progression of cancers, and tumors lacking p53 elicit more malignant phenotypes characterized by poorer cellular differentiation and increased genetic instability and metastatic potential (13). Frequency of p53 mutations varies according to the tumor background, from less than 10% among de novo AML patients to nearly 50% in lung adenocarcinoma patients (14, 15). Although a few reports have discussed the interactions between RUNX and p53 (16, 17), a relationship between RUNX and RUNX1 has been studied in only a few papers (18–20).
Pyrrole-imidazole (PI) polyamides are noncovalent, synthetic oligomers that recognize specific DNA sequences located within the minor groove by virtue of their pyrrole and imidazole pairs interlocked by a hairpin linkage (17). Designing the order of PI pairs enables in vivo delivery of PI polyamides to the targeted site of the genome in a controllable manner. Despite their relatively large molecular weight, PI polyamides are membrane permeant, localize to the cell nucleus, and then affect endogenous gene transcription at nanomolar levels (18). We have recently succeeded in generating potent histone deacetylase (HDAC) inhibitors, suberoylanilide hydroxamic acid–conjugated (SAHA-conjugated) PI polyamides, and demonstrated that they have the ability to specifically stimulate the expression of target genes through enhanced acetylation of their regulatory regions (19, 20). We have also successfully conjugated the nitrogen mustard alkylating agent chlorambucil (Chb) to PI polyamides, and showed that they could have a much stronger sequence-specific DNA-binding capacity and reduce target gene expression (21, 22). Taking advantage of these attractive technologies, we have newly synthesized PI polyamides that target the consensus RUNX-binding sequences and efficiently inhibit the recruitment of RUNX family members to their binding sites, and evaluated their therapeutic efficacy against AML cells as well as several types of solid tumors originating from different organs in vivo.

Results

RUNX1 depletion-mediated antileukemic effect requires functional p53. We first investigated whether depletion of RUNX1 could have an antileukemic effect on AML cells by taking advantage of the tetracycline-inducible shRNA-mediated RUNXI-knockdown system. As shown in Figure 1, A–C, and Supplemental Figure 1, A–D (supplemental material available online with this article; https://doi.org/10.1172/JCI91788DS1), silencing of RUNXI stimulated cell cycle arrest at the G0/G1 phase and simultaneously induced apoptosis in MV4-11, MOLM-13, and OCI-AML3 cells bearing wild-type P53. These results prompted us to examine whether depletion of RUNXI could affect the proapoptotic p53-mediated cell death pathway. As expected, a remarkable induction of p53 signaling was observed in p53-depleted MV4-11 cells. Nondepleted and RUNXI-depleted MV4-11 cells were treated with 3 μM doxycycline as in B. Total RNA was prepared and then analyzed by real-time RT-PCR for the indicated p53-target genes. Values are normalized to that of control vector–transduced cells (n = 3). Data are the mean ± SEM values. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, by 2-tailed Student’s t test.

Figure 1. p53-dependent antitumor effect of RUNX1 depletion. (A) Growth curves of MOLM-13 and MV4-11 cells transduced with control (sh_Luc) or with RUNX1 shRNAs (sh_RX1 #1 and sh_RX1 #2) in the presence of 3 μM doxycycline (n = 3). (B) RUNX1 depletion–mediated increase in number of cells with G0/G1 DNA content. Nondepleted and RUNX1-depleted MV4-11 cells were cultured in the presence of 3 μM doxycycline. Forty-eight hours after treatment, cells were harvested and analyzed by flow cytometry (n = 3). (C) Early apoptotic cell death induced by RUNX1 silencing. Nondepleted and RUNX1-depleted MV4-11 cells were treated as in B, and the early apoptotic cells (annexin V+ DAPI–) were scored by flow cytometric analysis (n = 3). (D) RUNX1 depletion–mediated stimulation of p53-dependent cell death pathway. Nondepleted and RUNX1-depleted MV4-11 cells were treated as in B. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) Induction of p53-target genes in RUNX1-depleted MV4-11 cells. Nondepleted and RUNX1-depleted MV4-11 cells were treated as in B. Total RNA was prepared and then analyzed by real-time RT-PCR for the indicated p53-target genes. Values are normalized to that of control vector–transduced cells (n = 3). (F) Increase in half-life of p53 in RUNX1-depleted MV4-11 cells. Nondepleted and RUNX1-depleted MV4-11 cells were treated as in B. Total RNA was prepared and then analyzed by real-time RT-PCR for the indicated p53-target genes. Values are normalized to that of control vector–transduced cells (n = 3). Data are the mean ± SEM values. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, by 2-tailed Student’s t test.
causes upregulation of p53-target genes (Figure 1E). Intriguingly, the expression of P53 at the mRNA level remained unchanged regardless of RUNX1 depletion (Figure 1E), raising the possibility that RUNX1 could affect the protein stability of p53. To address this issue, we performed cycloheximide chase experiments. As shown in Figure 1F, the half-life of p53 was prolonged in RUNX1-depleted cells, indicating that RUNX1 negatively regulates p53 protein in AML cells.

Since RUNX1 is a vital transcription factor, we hypothesized that RUNX1 downregulates p53 through the transcriptional activation of genes implicated in its degradation. To identify genes required for RUNX1-dependent degradation of p53, we first employed 4 independent clinical gene expression array datasets of AML patients and divided their samples into 2 groups based on the expression levels of RUNX1 in their bone marrow. Next, we extracted the top 2,000 commonly upregulated genes in AML patients highly expressing RUNX1 (Figure 2A). Among them, we focused on BCL11A and TRIM24, both of which have been reported to promote p53 degradation either directly or indirectly (23–26). Moreover, we found that expression levels of BCL11A and TRIM24 were positively correlated to those of RUNX1 among AML patient cohorts, underpinning the possible transcriptional regulation of these genes by RUNX1 (Supplemental Figure 3, A and B). In accordance with these observations, knockdown of RUNX1 resulted in a significant downregulation of BCL11A and TRIM24 at both the mRNA and protein levels (Figure 2, B and C). During close inspection of the transcriptional regulatory regions of the BCL11A and TRIM24 genes, we found several RUNX1-binding consensus sites. ChIP-qPCR experiments using anti-RUNX1 antibody revealed that RUNX1 binds to 4 major consensus sites (5′-TGTGGT-3′) located within 3 kb upstream of the transcription start site (TSS) of BCL11A, and 1 major consensus site found within 1.5 kb downstream of the TSS of TRIM24 (Supplemental Figure 4, A and B). Notably, forced expression of BCL11A or TRIM24 in RUNX1-knockdown MV4-11 cells increased their proliferation rates together with the reduction of p53 amount, indicating that BCL11A and TRIM24 play important roles in the RUNX1-mediated pro-oncogenic pathway (Figure 2, D and E, and Supplemental Figure 5, A and B).

As mentioned above, knockdown of RUNX1 potentiated the p53-dependent proapoptotic pathway. To further confirm this notion, we knocked down RUNX1 in additional AML cells including P53-deficient KG1a and HL60 cells and P53-mutated MV4-
RUNX1
cell cycle regulatory function of wild-type p53 is diminished. p21 in these AML cells (Supplemental Figure 6A), it suggests that nutlin-3 treatment did not induce expression of the p53 target gene in p53 were derived from their parental MV4-11 cells (27). Since it has been shown that RUNX family members have redundant functions (9, 29, 30), we sought to examine the expression levels of the other RUNX family members, RUNX2 and RUNX3, in RUNX1-knockdown AML cells. Under our tetracycline-inducible shRNA expression system, the expression levels of RUNX1 started to decline at 24 hours after doxycycline treatment, when the expression levels of RUNX2 and RUNX3 were unchanged. Notably, the doxycycline-dependent increase in the expression levels of RUNX2 and RUNX3 was detectable at 48 hours of incubation (Figure 3A and Supplemental Figure 7A), suggesting that RUNX2 and RUNX3 might compensate for the loss of RUNX1 expression. Although the expression levels of well-established RUNX1-target genes (IL3, CSF2, and CSF2RB) (31–33) were decreased at 24 hours after RUNX1 knockdown, their expression levels were reciprocally increased at 48 hours after RUNX1 knockdown and accompanied by RUNX2 and RUNX3 stimulation (Figure 3A). Forced expression of RUNX1, RUNX2, or RUNX3 suppressed the expression of RUNX2 and RUNX3, RUNX1 and RUNX3, or RUNX1 and RUNX2, respectively (Supplemental Figure 7B). Careful observations in the proximal regulatory regions of RUNX1, RUNX2, and RUNX3 revealed the existence of recurrent consensus RUNX-binding sequences. As expected, a ChIP-qPCR assay confirmed that all of the RUNX family members could bind to their respective binding sites (Supplemental Fig-
expression and completely suppressed their proliferation (Figure 3, B and C, and Supplemental Figure 8E). These results collectively indicate that the expression of all 3 RUNX family members is required for the maintenance of the expanding AML cells, and thus the simultaneous targeting of all RUNX family members as a cluster achieves much more stringent control of leukemia cells. In addition, we found that all RUNX family members bind to the regulatory regions of \( BCL11A \) and \( TRIM24 \) (Supplemental Figure 8, A–C). Moreover, individual RUNX family members consistently suppressed the promoter activity of the other RUNX members (Supplemental Figure 8D), indicating that each RUNX family member physiologically suppresses the function of the other members, probably owing to their functionally redundant roles in leukemia cells. In accordance with these findings, additional knockdown of \( RUNX2 \), \( RUNX3 \), or both of them in \( RUNX1 \)-depleted MV4-11 cells effectively repressed RUNX1-target gene expression and completely suppressed their proliferation (Figure 3, B and C, and Supplemental Figure 8E). These results collectively indicate that the expression of all 3 RUNX family members is required for the maintenance of the expanding AML cells, and thus the simultaneous targeting of all RUNX family members as a cluster achieves much more stringent control of leukemia cells. In addition, we found that all RUNX family members bind to the regulatory regions of \( BCL11A \) and \( TRIM24 \) (Supplemental Figure 8, A–C).
A and B) that and additive inhibition of RUNX2 and RUNX3 to RUNX1 depletion proportionally suppressed the expression of BCL11A and TRIM24 and potentiated p53 activity in AML cells (Supplemental Figure 9A), underscoring the importance of functional redundancy among RUNX family members in the maintenance of tumor cells. Furthermore, deceleration of tumor proliferation rate through p53 pathway activation mediated by RUNX family depletion was observed not only in AML cells, but also in solid tumors of different origins as well (Supplemental Figure 9, B and C). Moreover, considering that the functional alterations of RUNX family members exist in a mutually exclusive manner in AML cells as well as in various cancers (Figure 3D and Supplemental Figure 10), their functional redundancy in the maintenance of AML cells might be generally accepted. If this were the case, regulation of RUNX family as a cluster could be an ideal strategy to control not only leukemia, but also other malignant tumors of different origins. With this in mind, we next addressed this possibility of using the synthetic PI polyamides that could target all RUNX family members.

Cluster regulation of the RUNX family with PI polyamides. PI polyamides have been considered to selectively bind to the designated target sites in the minor groove of the genome sequence at nanomolar levels (17). Taking advantage of the nature of this small molecule, we have designed PI polyamides that can specifically recognize and bind to RUNX-binding consensus sites (5′-TGTGGT-3′ and 5′-TGCGGT-3′) by introducing 4 PI pairs. As shown in Supplemental Figure 11A, we first screened the HDAC inhibitors, SAHA-conjugated PI polyamides targeting RUNX consensus binding sequences (SAHA-M′ targets 5′-TGTGGT-3′ and SAHA-50 targets 5′-TGCGGT-3′), which can enhance the tran-
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Figure 6. In vivo tolerability of Chb-M′. (A) The number of granulocyte (G), macrophage (M), and G/M colonies were scored based on their morphology in a colony-forming cell assay. c-Kit+ bone marrow cells derived from wild-type C57BL/6 mice were cultured in the presence of indicated concentrations of Chb-M′ (n = 3). (B) The number of colonies was scored in a colony-forming cell assay with MLL-ENL-transduced mouse immortalized bone marrow cells in the presence of indicated concentrations of Chb-M′ (n = 3). (C) The number of colonies was scored in a colony-forming cell assay with MV4-11 or MOLM-13 cells in the presence of indicated concentrations of Chb-M′ (n = 3). (D) Schematic representation of treatment and monitoring schedule in wild-type C57BL/6 mice. PB, peripheral blood; BW, body weight. (E and F) Peripheral blood cell counts (E) and body weight (F) in mice receiving Chb-M′ or DMSO treatment for 3 months (n = 3). HGB, hemoglobin; PLT, platelets. (G and H) LSK fraction was determined in bone marrow cells extracted from mice after a 3-month treatment with Chb-M′ or DMSO (n = 6). Representative FACS plots (G) and cumulative data (H) are shown. (I) The number of GM, G, and M colonies were scored based on their morphology in a colony-forming cell assay with c-Kit+ bone marrow cells extracted from mice after a 3-month treatment with Chb-M′ or DMSO (n = 6). Data are the mean ± SEM values. *P < 0.05, **P < 0.01, NS, not significant, by 2-tailed Student’s t test.
simultaneous treatment of Chb-M′ with the p53 inducer PRIMA-1 synergistically reduced the proliferation rates of P53-deficient AML cells (Figure 5C).

To examine the antitumor efficacy as well as the pharmacological safety of Chb-M′, we next sought to examine the in vivo distribution of Chb-M′. As shown in Supplemental Figure 19, A and B, we prepared FITC-labeled Chb-M′ (FITC-Chb-M′) and visualized its cellular uptake in tumor cells both in vitro and in vivo. FITC-Chb-M′ was readily incorporated in the tumor cells in vitro (Supplemental Figure 19C), and to our surprise, this molecule seemed to accumulate at much higher concentrations in tumor cells than in normal tissue cells when administered intravenously in vivo (Figure 5, D–F), potentially creating a therapeutic window and minimizing the toxic side effects of Chb-M′ in antitumor treatment.

To further justify the use of Chb-M′ in vivo, we examined its acute toxicological effects in mice. As seen in Supplemental Figure 20, A–C, a marginal decrease in the number of platelets was detectable in mice that received up to 10 mg/kg body weight of Chb-M′. This concentration of Chb-M′ was more than 30 times higher than that of the generally accepted dose of PI polyamides (34, 35), indicating that Chb-M′ is highly tolerable even in vivo. We also examined the pharmacokinetic profile of Chb-M′ in vivo in mice and determined the elimination half-life of this molecule as about 6 hours (Supplemental Figure 21). While Chb-M′ treatment significantly reduced the colony-forming capacity in mouse AML cells with MLL-ENL or in human AML cell lines MV4-11 and MOLM-13 at 1 μM, the c-Kit + fraction of bone marrow cells extracted from wild-type mice was insensitive to Chb-M′ at this concentration and could tolerate up to 10 μM Chb-M′ without a significant reduction in colony-forming capacity (Figure 6, A–C). Treatment with Chb-M′ (320 μg/kg body weight injections twice per week) in mice for 3 months did not influence the complete blood counts of peripheral blood, and no significant change in body weight was observed between Chb-M′– or vehicle-treated cohorts (Figure 6, D–F). In addition, no change in the frequency of the LSK (Lin–Sca-1+c-Kit+) fraction was detected in mouse bone marrow cells after a 3-month treatment with Chb-M′ compared with vehicle-treated mice (Figure 6, G and H).

**Figure 7. Antitumor activity of Chb-M′ in vivo.** (A) Schematic representation of treatment schedule in xenotransplanted mice. (B) Overall survival of NOG mice transplanted with MV4-11 cells followed by treatment with DMSO, Ara-C, Chb-S, or with Chb-M′ (n = 7). P < 0.001, by log-rank (Mantel-Cox) test. (C) Overall survival of NOG mice transplanted with SU/SR cells followed by treatment with DMSO, imatinib, or with Chb-M′ (n = 5). P < 0.01, by log-rank (Mantel-Cox) test. (D and E) Human lung cancer xenotransplant model in NOG mice transplanted with A549 cells followed by treatment with DMSO, gefitinib, or with Chb-M′. (D) Overall survival of NOG mice (n = 5). P < 0.01, by log-rank (Mantel-Cox) test. (E) Live animal bioluminescence images at 7, 14, and 21 days after transplantation of A549 cells. Rainbow scale represents relative light units. (F and G) Human stomach cancer xenotransplant model in NOG mice transplanted with MKN45 cells followed by treatment with DMSO or with Chb-M′. (F) Tumor volume of NOG mice (n = 8). (G) Live animal bioluminescence images at 7, 14, and 21 days after transplantation of MKN45 cells. Rainbow scale represents relative light units. Data are the mean ± SEM values.

*P < 0.05, **P < 0.01, ***P < 0.001, by 2-tailed Student’s t test.
Figure 8. Total RUNX expression, equivalent to that of CBFB, is an ideal target for anticancer strategy. (A) Heatmap showing relative expression levels of RUNX1, RUNX2, RUNX3, and CBFB in normal tissues and cancer cell lines of various origins. (B) Expression levels of CBFB in normal tissues (n = 74) and cancer cell lines (n = 38). (C) Correlation between the expression levels of CBFB and RUNX1 + RUNX2 + RUNX3 (Pan_RUNX) (n = 9). P < 0.01, by Spearman’s correlation. (D) Immunoblotting of CBFB and GAPDH in various cancer cells and their corresponding normal tissues. (E) Graphical abstract showing the balanced expression of total RUNX and CBFB (left). Consistent difference in the expression levels of total RUNX or CBFB in between malignant and benign cells provides a pharmacological window to be targeted (right). Data are the mean ± SEM values. ***P < 0.001, by 2-tailed Student’s t test.
We examined its possible effect on gastric cancer cells. For this purpose, human gastric cancer MKN45 cells were subcutaneously injected into immunodeficient mice. We monitored the size of tumors up to 35 days after engraftment and observed that Chb-M′ efficiently controls the growth of gastric tumors (Figure 7, F and G, and Supplemental Figure 24C). Collectively, our results strongly indicate that Chb-M′ is a highly tolerable and potentially novel anticancer drug that targets multiple cancer types through cluster regulation of the RUNX family in vivo.

RUNX-related CBFB is a potentially novel molecular marker for pan-cancer. To establish the antitumor strategy targeting all of the RUNX family members, we first checked the expression levels of RUNX1, RUNX2, RUNX3, and CBFB in cancer tissues and in their normal counterparts. Based on previously reported array datasets (reference database for gene expression analysis, RefExA; see ref. 40), we found that the expression levels of CBFB are consistently higher in cancer tissues compared with their normal counterparts, while the expression level of each RUNX family member varied between cancer tissues and their normal counterparts (Figure 8, A and B). Intriguingly, however, the amounts of total RUNX mRNAs (RUNX1 + RUNX2 + RUNX3), as examined by primers selectively amplifying the common region of RUNX family members (Pan_RUNX), were positively correlated to those of CBFB in AML cells, which might be attributable to the heterodimeric complex formation between RUNX family members and CBFB (Figure 8C). Similar results were also obtained by immunoblotting (Supplemental Figure 25, A and B), suggesting that the expression level of CBFB is a molecular marker for predicting the expression of all 3 RUNX family members. Since CBFB is one of the most highly upregulated genes in various cancer tissues, it is likely that CBFB is a reliable molecular marker for a wide variety of cancers (Supplemental Figure 25C).

We also assessed the expression level of CBFB protein in cancer cell lines of various origins and in their normal counterparts. Immunoblotting experiments demonstrated that CBFB is expressed at higher levels in cancer cells than their normal counterparts (Figure 8D). This tendency was further confirmed in the patient-derived samples by immunohistochemistry (Supplemental Figure 26). Finally, we compared the expression of all 3 RUNX family members and CBFB in AML cells and in their normal counterparts (Supplemental Figure 27, A and B). Through this experiment, we have confirmed that the total expression of RUNX family members as well as that of CBFB is consistently higher in malignant cells than in normal ones. Taken together, these results suggest that the entire RUNX family, the total expression of which is equivalent to that of CBFB, is a promising druggable target of antitumor therapy in multiple cancer types (Figure 8E).

**Figure 9.** Chb-M′ as a safe lead compound of a potentially novel anticancer drug category. Chb-M′ targets the consensus RUNX binding sequences on the genome DNA and achieves cluster regulation of RUNX target genes, which offers efficient antitumor activity through potentiating a proapoptotic p53-mediated cell death pathway.
Discussion

Mammalian RUNX transcription factors mediate the homeostasis of both benign and malignant cells through their ability to promote gene activation (2). By further exploring our prior finding that RUNX is a prerequisite for the initiation of leukemia development (10, 11), we now reveal that RUNX plays a pivotal role in the maintenance of leukemia cells as well as tumor cells derived from different origins. According to a previous report, the frequency of RUNX1 point mutations in the Runx domain among de novo adult AML cases is around 10% (41). Some of these mutant RUNX1 (such as D171N and S291fsX300 mutations) and RUNX1 fusion genes (such as RUNX1-ETO and RUNX1-ETO9a) are proven oncogenes in mice with a p53-proficient environment (42). Although these observations remind us of the oncogenic property of RUNX1 alterations, mutations and translocations involving RUNX1 are usually monoclonal and AML cases with homozygous dysfunction of RUNX1 are rarely encountered in humans (43, 44). In addition, we and other groups have previously reported that wild-type RUNX1 is stringently required in leukemogenesis, both in RUNX1-mutated cases (9) and in translocated cases (11, 45). These results suggest that wild-type RUNX1 still exists and could be targeted in RUNX1-mutated leukemia. In addition, we have found and report in this study that functional alterations of RUNX1, RUNX2, and RUNX3 do exist in a mutually exclusive manner in various cancers including AML, indicating that RUNX1-mutated tumors do express wild-type RUNX2 and RUNX3. We believe that these findings collectively rationalize targeting these remaining wild-type RUNX1 or other RUNX family members, even in malignancies harboring RUNX1 mutations.

During our workup, we have also noticed that RUNX inhibition is solely effective against tumors with functional p53. In this regard, RUNX inhibition therapy should be given according to the pretreatment status of p53, and potentially permits the emergence of resistant cells harboring acquired p53 mutations in future patients. Despite this potential disadvantage, Chb-M′ is highly efficient against p53-proficient tumor cells, and furthermore, we believe this concern could be overcome through adopting the concurrent use of p53 modifiers such as PRIMA-1 with Chb-M′ since this combined therapy was indeed effective against p53-mutated AML cells as we have shown in this report. We are currently preparing for the first clinical trial of Chb-M′ in AML patients, and future tumor cell samples obtained after courses of Chb-M′ treatment might exhibit p53 dysfunctions and further elucidate the close relationships between RUNX family members and p53.

The fact that the total amount of RUNX expression, which is estimated by the expression of CBFB, is considerably higher in leukemia than in their normal counterparts rationalizes our strategy to target the RUNX family by cluster in a variety of cancer patients. In addition, the finding that Chb-M′ is well tolerated in mice even at high doses makes Chb-M′ a safe lead compound for the potentially novel anticancer drug category we refer to as “cluster regulation of RUNX (CROX).” Further studies involving clinical trials with this drug are awaited to validate the CROX strategy against human cancers (Figure 9).

Methods

Cell proliferation. To assess cell proliferation, 1 × 10⁶ cells of the indicated AML-derived cells were seeded in 6-well plates. For the tetra-
clone D6E2), anti-CBFβ (Santa Cruz Biotechnology, clone FL-182), anti-p14ARF (Cell Signaling Technology, catalog 2407), anti-MDM2 (Santa Cruz Biotechnology, clone SMP14), anti-PUMA (Cell Signaling Technology, catalog 4976), anti-cleaved caspase-3 (Cell Signaling Technology, clone SA1E), anti-PARP (Cell Signaling Technology, clone 46D11), anti-p53 (Cell Signaling Technology, clone IC12), and anti-phosphorylated p53 (Ser-15) (Cell Signaling Technology, clone 16G8). For secondary antibodies, HRP-conjugated anti-rabbit IgG and anti–phosphorylated p53 (Ser-15) (Cell Signaling Technology, clone 46D11), anti-p53 (Cell Signaling Technology, clone 1C12), and anti-PUMA (Cell Signaling Technology, catalog 4976), anti–cleaved caspase-3 (Affymetrix) and the ChemiDoc XR+ Imager (Bio-Rad Laboratories) according to the manufacturers’ recommendations. Protein levels were quantified with Image Lab Software (Bio-Rad Laboratories).

Analysis of gene expression microarray. MV4-11 cells were treated with 1 μM Chb-M’, Chb-50, or with DMSO for 6 hours, or transduced with control shRNA (sh_Luc) or with shRNAs targeting RUNX1 (sh_Rx1), RUNX2 (sh_Rx2), and RUNX3 (sh_Rx3), and subsequently incubated with 3 μM doxycycline for 24 hours. After the indicated time of incubation, total RNA was prepared and its quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Cyanine 3-labeled cRNA was generated in the presence of T7 polymerase, purified, and its concentration was measured using a Nanodrop ND1000 v3.5.2 (Thermo Scientific). The resultant cRNA (825 ng) was fragmented and subsequently hybridized with a Human Gene 2.1 ST Array Strip (Affymetrix). The raw data together with the associated sample information were analyzed by GeneSpring GX v12.1.0 (Agilent Technologies), and our microarray data have been deposited in NCBI’s Gene Expression Omnibus (GEO GSE94835). Gene set enrichment analysis (GSEA) was utilized to analyze the microarray data obtained in the present study (46). Gene ontology enrichment analysis was conducted by Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 software according to the provider’s instructions (47, 48).

Statistics. Statistical significance of differences between control and experimental groups was assessed by a 2-tailed unpaired Student’s t test, and was declared if the P value was less than 0.05. Equal-ity of variances in 2 populations was calculated with an F test. The results are represented as the average ± SEM values obtained from 3 independent experiments. In transplantation experiments, animals were randomly allocated to each experimental group, and the treatments were given with blinding. The overall survival of mice is shown in a Kaplan-Meier curve. Survival between the indicated groups was compared using the log-rank test. For the measurement of correlation between mRNA or protein expression, Spearman’s rank correlation coefficient was used. To quantify the synergism of Chb-M’ and PRIMA-1, the combination index theorem of Chou-Talalay was calculated using COMPUSYN software. A combination index value less than 1 was considered statistically significant (49).

Mice. C57BL/6j mice were purchased from CLEA Japan, Inc. and NOD/Shi-scid,IL-2RγKO (NOG) mice were from the Central Institute for Experimental Animals, Japan. Crl:CD1(ICR) mice and BALB/c-nu mice were purchased from Charles River Laboratories International, Inc. Littermates were used as controls in all experiments.

Xenograft mouse model. Xenograft mouse models of human cancer cell lines were developed using NOG mice. For leukemia mouse models, 2.5 × 10^6 cells/body of MV4-11 or SU/SR cells were intravenously injected. Peripheral blood (PB) was then collected every week and chimerism was checked by flow cytometer using an anti-human CD45 antibody (BD Biosciences). Seven days after injection, mice were treated with PI polyamides (320 μg/kg body weight, twice per week intravenous injections) or with the equivalent amount of DMSO. For Chb injections, solution was delivered intravenously at 320 μg/kg body weight, twice per week. Administration of imatinib mesylate (Focus Biomolecules) was carried out (100 mg/kg body weight) twice per day (beginning at day 7 until the recipient mice succumb to their disease). For lung cancer mouse models, 1 × 10^6 cells/body of A549 cells were injected intravenously. Cells were then transduced with lentivirus encoding luciferase (Addgene, plenti-luciferase vector). Mice were subsequently injected with D-luciferin (150 mg/kg body weight, Wako Pure Chemical Industries, Ltd.) and their tumor volumes were assessed using an IVIS Spectrum In Vivo Imaging System (PerkinElmer) every week. For gastric cancer xenograft mouse models, 1 × 10^6 cells/body of MKN45 cells were injected subcutaneously at the right dorsal flank. Cells were marked with luciferase, and tumor growth was monitored by IVIS. For biofluorescence assay, BALB/c-nu mice were subcutaneously transplanted with 2 × 10^6 cells/body of MKN45 cells, followed by intravenous injection of FITC-labeled Chb-M’ 320 μg/kg body weight. Biofluorescence was detected by IVIS through the GFP filter.

Synthesis of PI polyamides. Reagents and solvents were purchased from standard suppliers and used without further purification. Flash column purifications were performed by a CombiFlash Rf (Teledyne Isco, Inc.) with a C18 RediSep Rf Flash Column. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) was performed on a Bio-TOF II (Bruker Daltonics) mass spectrometer using positive ionization mode. Machine-assisted polyamide syntheses were performed on a PSSM-8 (Shimadzu) system with computer-assisted operation. Proton nuclear magnetic resonance (1H NMR) spectra were recorded with a JEOL JNM ECA-600 spectrometer operating at 600 MHz and in parts per million (ppm) downfield relative to tetramethylsilane used as an internal standard. The following abbreviations apply to spin multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet). The results of NMR and ESI-TOF-MS are available in Supplemental Figures 28 and 29.

Study approval. All animal studies were properly conducted in accordance with the Regulation on Animal Experimentation at Kyoto University, based on International Guiding Principles for Biomedical Research Involving Animals. All procedures employed in this study were approved by Kyoto University Animal Experimentation Committee (permit number: Med Kyo 14332). See supplemental data for more information.

Author contributions

KM designed research, performed experiments, analyzed data, and wrote the manuscript. KS, SM, AM, GK, JT, YM, AY, YY, HK, CT, HM, RM, MN, MH, and ST helped to collect data. MT, YO, MM, KN, KI, T. Kataoka, T. Kitamura, Y. Kaneda, PL, and SA participated in discussions and interpretation of the data and results, and commented on research direction. TB and HS synthesized and designed the PI polyamides. Y. Kamikubo initiated and designed the study and led the entire project.
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