Table 3. Effects of clinical factors on OS in Cox analyses

	All patients (n = 90)						Patients had not received chemotherapy (n = 78)					
	Univariate analysis		Multivariate model A		Multivariate model B		Univariate analysis		Multivariate model C		Multivariate model D	
Clinical factor	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
PS												
0	1		1		1		1		1		1	
1	1.5 (0.8-2.7)	.22	1.4 (0.8-2.8)	.27	1.3 (0.7-2.6)	.37	1.4 (0.7-2.7)	.28	1.6 (0.8-3.1)	.21	1.4 (0.7-2.9)	.30
2 or more	2.5 (1.2-5.2)	.01	2.1 (1.0-4.6)	.06	2.1 (1.0-4.6)	.06	1.7 (0.7-4.0)	.26	1.5 (0.6-3.8)	.39	1.6 (0.6-4.2)	.30
Neutrophil counts												
Less than 7.5×10^9/L	1		1		1		1		1		1	
7.5×10^9/L or greater	1.6 (0.9-2.9)	.15	1.3 (0.6-2.7)	.45	1.2 (0.6-2.3)	.58	1.3 (0.6-2.7)	.47	1.5 (0.6-3.8)	.43	1.0 (0.5-2.3)	.94
LDH												
Less than or equal to NI	1		1		1		1		1		1	
Greater than NI	1.7 (1.0-2.9)	.04	1.5 (0.8-2.7)	.16	1.5 (0.8-2.6)	.21	1.5 (0.8-2.8)	.19	1.7 (0.9-3.3)	.12	1.6 (0.8-3.1)	.20
No. of extranodal lesions												
0-2	1		1				1		1			
3 or more	1.5 (0.8-2.8)	.16	0.7 (0.3-1.6)	.41			0.9 (0.4-2.2)	.82	0.5 (0.1-1.6)	.22		
No. of total involved lesions												
1	1				1		1				1	
2 or 3	1.2 (0.7-2.2)	.52			0.8 (0.4-1.6)	.52	1.1 (0.6-2.1)	.67			0.9 (0.4-1.7)	.65
4 or more	1.5 (0.7-3.0)	.26			0.9 (0.4-2.1)	.83	1.0 (0.5-2.3)	.96			0.8 (0.3-2.0)	.67
Chemotherapy												
Not received	1		1		1							
Received	2.6 (1.4-5.1)	.003	2.3 (1.1-4.7)	.03	2.0 (1.0-4.2)	.06						

HR indicates hazard ratio; 95% CI, 95% confidence interval; and NI, normal index.

with more than 3 extranodal lesions was significantly poor than the others ($P = .005$; Figure 2E). The survival rate was worse in patients with more than 4 total involvement lesions than in the others (Table 2). Of the extranodal lesions, we additionally examined the effect of skin lesion and BM involvement on survival rates. The survival rate of patients with BM involvement was significantly poor than of patients without ($P = .04$; data not shown), but that of patients with skin involvement was not different from those without ($P = .66$; supplemental Figure 2).

Although most patients in this study had not been treated until their disease progression was similar to B-cell chronic lymphoid leukemia, 12 patients with chronic ATL were treated with chemotherapy immediately after diagnosis because of elevated LDH levels in 8 patients, severe BM involvement in 2 patients, and severe skin involvements in 2 patients. Among them, 2 patients were treated with VCAP (vincristine, cyclophosphamide, doxorubicin, and prednisone)–AMP (doxorubicin, ranimustine, and prednisone)–VECP (vindesine, etoposide, carboplatin, and prednisone),[3] 2 with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), 4 with CHOP-like, 3 with VEPA (vincristine, etoposide, prednisone, and doxorubicin),[15] and 1 with low-dose etoposide. All of these patients died (MST, 1.4 years; 95% CI, 1.1-2.3 years), and their prognosis was very poor compared with patients not treated ($P = .01$; Figure 2F).

On the basis of results from Kaplan-Meier curves and univariate analysis for each factor, we decided to include PS category, dichotomized neutrophil counts, dichotomized LDH category, dichotomized number of extranodal lesions, the number of total involved lesions, and chemotherapy states into multivariate Cox analysis. Model A included PS category, dichotomized neutrophil counts, dichotomized LDH category, dichotomized number of extranodal lesions, and chemotherapy states. Model B included the same factors as model A except for the number of total involved lesions instead of the number of extranodal lesions. This was because, by definition, a factor of the number of total involved lesions included a factor of the number of extranodal lesions. Results were summarized in Table 3. In model A, advanced PS (≥ 2; HR, 2.1; 95% CI, 1.0-4.6; $P = .06$, borderline significance) and chemotherapy states (HR, 2.3; 95% CI, 1.1-4.7; $P = .03$, significance) were correlated with OS, but the remaining factors were not independent prognostic factors after adjustment for covariate factors. To evaluate effects of clinical factors beyond the effect of chemotherapy states on OS, we also performed additional multivariate analyses for patients who were not received chemotherapy (n = 78; model C and model D in Table 3). We found that there was no clinical parameter that associated with OS.

Discussion

In the present study, we investigated for the first time the long-term clinical course of patients with indolent ATL with a maximum duration of follow-up of 17.6 years. We found that the prognosis of indolent ATL was poor with the MST of 4.1 years, and the estimated 15-year OS rates were 14.1% with no plateau in the survival curve. The prognosis observed in the present study was poorer than expected. Our results confirmed a recent long-term Brazilian study,[6] that showed a poor OS of less than 20% for indolent ATL. In the present study, we showed that 65.1% of patients died of acute ATL with a median time to transformation of 18.8 months. This finding suggests that most patients with indolent ATL will eventually die of aggressive ATL during their long-term course of illness. These findings suggest that even patients with indolent ATL should be carefully observed by frequent clinical visits.

The cause of death in patients with indolent ATL has not been well reported so far. In the present study, patients with indolent ATL died of various causes such as malignancies other than ATL,

chronic pulmonary diseases, opportunistic infections, and autoimmune diseases, in addition to death from acute ATL after transformation. A previous long-term study, which followed-up 50 HTLV-1 carriers with monoclonal proliferation of T lymphocytes (pre-ATL) for 20 years, also reported that 10 patients died of opportunistic infections such as *Pneumocystis* pneumonia or malignancies other than ATL (skin carcinoma, lung cancer, etc).[16] Patients with indolent ATL were also comorbid with a variety of diseases at diagnosis such as chronic pulmonary disease, opportunistic infections, multiple cancers, and autoimmune diseases in the present study. The pathogens responsible for the opportunistic infections were similar to those observed in patients with AIDS associated with HIV. Opportunistic infection was previously reported as a frequent complication in patients with aggressive or with indolent ATL.[2] These findings suggest that helper T-cell function in indolent ATL might be impaired similar to that in AIDS.[17]

We also presented that chronic pulmonary disease, multiple cancers, and autoimmune diseases were frequent as complications at diagnosis in indolent ATL. The reason why indolent ATL had such immune dysregulation remains unknown. It was recently noted that the origin of the ATL cells in a fraction of the patients was from regulatory T cells expressing FoxP3 and CCR4.[18,19] In the present study, 6 patients also had autoimmune diseases. Among them, 3 patients were treated with immunosuppressive drugs, and of those only one patient with smoldering ATL transformed to acute ATL. Therefore, we were not able to evaluate the effect of comorbid autoimmune diseases and immunosuppressive drug therapy on the risk of transformation or poor prognosis so far. Further studies are warranted to elucidate the mechanisms responsible for the development of hyperimmunity or hypoimmunity in patients with indolent ATL.

Although comparison on OS by subtype is not a primary purpose of this study, it was unexpected that survival rates of smoldering ATL (15-year OS, 12.7%) tended to be lower than chronic ATL (15-year OS, 14.7%), and the MST of smoldering ATL (2.9 years) tended to be shorter than chronic ATL (5.3 years; Table 2; Figure 1B). Transformation rates of smoldering ATL and chronic ATL were 60% (n = 15) and 44% (n = 29), respectively (data not shown), which was also unexpected. Although there was no statistically difference in OS, MST, and transformation rate between the 2 groups, our results were different from a previous short-time follow-up study reported by Shimoyama et al[2] (the 4-year survival rates for smoldering type was 62.8%). It was unknown why the rate of smoldering type was poorer than chronic type in the present study. Some previous studies suggested that skin involvements might be a risk factor for poor prognosis of smoldering ATL.[6,20-22] In the present study, the frequency of patients with skin lesion was a little higher in smoldering ATL (n = 14; 56%) than in chronic ATL (n = 32; 49%). The OS of smoldering ATL with skin lesion was worse than that of chronic ATL without skin lesion (supplemental Figure 2), although there was no statistical difference ($P = .5$). Therefore, a possible explanation might be that smoldering ATL with poor conditions (eg, skin involvement) might be disproportionately included in the present study because data were collected at a university hospital, where more advanced cases were referred from city clinics. Another possible explanation might be that the percentage of patients with smoldering-type ATL has increased recently, as shown in Table 1. In recent decades, more patients have been diagnosed with the smoldering type of ATL on the basis of a health examination, including a blood cell count. Some of these patients may have been in the early phase of acute ATL.

Shimoyama et al[2] reported that involved lymph node lesions, extranodal lesions, and total involvement lesions were significantly poor prognostic factors for ATL all together, and low serum albumin, high LDH, or high BUN levels were PPFs for chronic ATL.[13,14] As we expected, patients with at least 1 of 3 known PPFs for chronic ATL (a high level of LDH and BUN and a low level of albumin)[13,14] showed a poor survival rate than patients without (Table 2; Figure 2D). We also confirmed the difference was seen when analyses were performed for chronic ATL only ($P = .03$) but was not seen for smoldering ATL only ($P = .62$; supplemental Figure 3). This suggests that there may be different prognostic factors for smoldering ATL and chronic ATL, respectively. Further detailed studies regarding prognostic factors are needed for individual subtype.

Other than the known 3 potential prognostic factors, an advanced PS, neutrophilia, more than 3 extranodal lesions, more than 4 total involved lesions, and having received chemotherapy were shown to be possible unfavorable prognostic factors for indolent ATL in our Kaplan-Meier analyses (Table 2; Figures 1B, 2A-F). However, in multivariate Cox analyses, only advanced PS and chemotherapy state were associated with OS after adjustment for other covariates (models A and B in Table 3). The poor prognosis in patients with indolent ATL who were treated by chemotherapy was similar to that of the patients with unfavorable chronic ATL who were treated with intensive combination chemotherapy in several clinical trials in Japan.[3,5,23] Although advanced PS was a borderline significant independent poor factor on survival for indolent ATL in the model that used all patients, the factor was not a prognostic factor anymore when data were limited for only untreated patients (models C and D in Table 3). Among 12 patients who received chemotherapy, 7 (58%) had advanced PS at diagnosis. This suggests that patients with advanced PS at diagnosis might have a condition that required treatments, which introduced the disappearance of the effect of advanced PS on survival, even though advanced PS was an independent poor factor.

Regarding the effect of the presence of extranodal lesions on poor survival, we previously reported that BM involvement was a prognostic factor for aggressive ATL.[24] Although we did not present the effect of each extranodal lesion on survival in detail, we also confirmed that the survival rate of patients with BM involvement was significantly poor compared with patients those without BM involvement ($P = .04$; data not shown), but the survival rate of patients with skin involvement was not different compared with those without ($P = .66$; supplemental Figure 2). However, some studies reported that the presence of skin lesions was a possible poor prognostic factor in indolent ATL,[6,20-22] as described earlier. Setoyama et al[21] reported that smoldering cases with a deeper infiltration pattern had a more aggressive course than cases with a superficial infiltration pattern. Degree of skin involvement might be associated with prognosis in indolent ATL.

Previously, our study group noted that some patients showed alterations in tumor suppressor genes (p16 INKA[25,26] or p53[27]) or aneuploidy greater than 1 chromosomal locus by comparative genomic hybridization in ATL cells[28] and that such abnormalities were associated with a poor prognosis. Although we could not perform molecular analyses for all patients in the present study, 7 were examined molecularly, and at least one abnormality was found in each patient (data not shown). They had a poor prognosis and died within 2.5 years. Patients with a poor prognosis who died during the first steep slope in the survival curve (Figure 1A) might have had such genetic alterations.

The primary purpose of this study was to analyze prognosis of smoldering and chronic types together as an indolent type of ATL. Therefore, we were not able to present in detail the difference in

prognostic factors between subtypes, which is one of the limitations in this study. The number of cases evaluated in this study was too small to perform detail analyses for prognostic factors in indolent ATL. Further large-scaled studies are warranted.

In conclusion, the long-term prognosis of patients with indolent ATL was not good without a plateau phase in the survival curve. Further studies are warranted to elucidate patients with indolent ATL who require intensive chemotherapy, allogenic hematopoietic stem cell transplantation (in cases of aggressive ATL), or combination therapy with zidovudine and interferon alfa.[29,30] In addition, new molecular targeting treatments, such as histone deacetylase inhibitors,[31] which have shown promise in the treatment of $CD4^+$ cutaneous T-cell lymphoma, should be taken into consideration for treatment of indolent ATL.

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Authorship

Contribution: Y.T. collected and analyzed the data and wrote the manuscript; M.I. analyzed the data and wrote the manuscript; Y.I., M.T., T.J., T.K., Y.Y., S.K., S.I., Y.M., and M.T. made the diagnoses and treated the patients with ATL; and K.T. organized the study.

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Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL

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Objective. **The signaling by thrombopoietin (TPO) via its receptor, c-MPL, plays a crucial role in the maintenance of hematopoietic stem cells (HSCs). Small-molecule c-MPL agonists have recently been shown to be beneficial in the treatment of thrombocytopenia. However, their effects on HSCs have not yet been explored. In this study, we evaluated the effects of NR-101, a novel small-molecule c-MPL agonist, on the ex vivo expansion of human cord blood (hCB) HSCs.**

Materials and Methods. **hCB $CD34^+$ or $CD34^+CD38^-$ hematopoietic stem and progenitor cells were cultured for 7 days in the presence of thrombopoietin (TPO) or NR-101, and then subjected to flow cytometric analyses, colony-forming cell assays, and severe combined immunodeficiency–repopulating cell assays.**

Results. **During a 7-day culture of $CD34^+$ or $CD34^+CD38^-$ hematopoietic stem and progenitor cells, NR-101 efficiently increased their numbers, with a greater than twofold increase compared to TPO, although its effect on megakaryocytopoiesis was comparable to that of TPO. Correspondingly, severe combined immunodeficiency–repopulating cells were increased 2.9-fold during a 7-day culture with NR-101 compared to freshly isolated $CD34^+$ cells, and 2.3-fold compared to that with TPO. Of note, NR-101 persistently activated signal transducer and activator of transcription (STAT) 5 but not signal transducer and activator of transcription 3. Furthermore, NR-101 induced a long-term accumulation of hypoxia-inducible factor-1α protein and enhanced activation of its downstream target genes.**

Conclusion. **This is the first time that a small-molecule c-MPL agonist has been demonstrated to promote net expansion of HSCs. NR-101 is more efficient in ex vivo expansion of HSCs than TPO. NR-101 could be a useful tool for the therapeutic manipulation of human HSCs.** **© 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Thrombopoietin (TPO) is a cytokine initially identified as the primary regulator of megakaryocyte differentiation and platelet production [1–5]. The binding of TPO to its receptor, myeloproliferative leukemia virus protooncogene (c-MPL), triggers the activation of three major pathways: Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT [6–12]. Activation of these signal transduction pathways promotes differentiation toward the megakaryocytic lineage. Recent studies have revealed that c-MPL is expressed not only in the megakaryocytic lineage, but also in hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) and plays a crucial role in the maintenance of HSCs [13–16]. TPO released from the

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bone marrow (BM) niche activated c-MPL on HSCs and regulated the maintenance of quiescent HSCs [17,18]. In addition, mice lacking either *TPO* or *c-MPL* exhibited not only fewer megakaryocytes, but also severe reductions in HSCs and defects in supporting HSC self-renewal [13,16,19]. TPO knockout mice showed a reduced capacity to support the engraftment of wild-type HSCs, which was substantially compensated by the administration of TPO [20]. Also loss of Lnk, which negatively regulates TPO/c-MPL signaling, resulted in the expansion of HSCs [16,21]. These findings have established a critical role for TPO/c-MPL signaling in the development and function of HSCs in vivo.

HSCs are defined by their capacity to self-renew and to differentiate into all blood cell types, and have been applied to HSC transplantation and gene therapy [22–25]. Various attempts have recently been made to expand human cord blood (hCB) HSCs in cultures ex vivo to acquire a sufficient number of transplantable HSCs, particularly to accelerate applications for adult patients [26–29]. Most ex vivo culture systems employ key cytokines, including stem cell factor (SCF), fms-like tyrosine kinase 3 ligand (FL), and TPO. TPO appears to better support the survival and maintenance of HSCs in vitro [14,30,31]. However, attempts to expand HSC numbers ex vivo with these cytokine combinations have not yielded sufficient results. Optimal culture conditions for ex vivo HSC expansion need to be determined.

Although recombinant human TPO (rhTPO) was initially developed to treat thrombocytopenia, clinical trials were unsuccessful due to immunogenicity [32,33]. Alternatively, several nonpeptidyl small-molecule compounds have been developed to activate c-MPL and promote platelet production, e.g., SB-497115 (Eltrombopag), AKR-501, NIP-004, and butyzamide [34–39]. SB-497115 (Eltrombopag), a first-in-class, orally available c-MPL agonist, is being developed as a drug for thrombocytopenia of various etiologies [35,36]. We have also screened such small-molecule compounds and identified several c-MPL agonists [38]. During the course of screening, we noticed that although the overall activity of these small-molecule agonists was similar to TPO, actual signaling downstream c-Mpl could vary. For example, ability to induce megakaryocytic differentiation or ability to induce cell proliferation varied among small-molecule agonists. Based on these observations, we assumed that some compounds may preferentially activate signals that facilitate self-renewal of HSCs. TPO has been characterized as a key factor for human HSCs and applied to ex vivo HSC expansion and gene transduction [26–31]. However, to our knowledge, there has been no report on the effect of nonpeptidyl small-molecule c-MPL agonists on HSCs.

In this study, we evaluated the effects of NR-101, a novel nonpeptidyl small-molecule c-MPL agonist, on the expansion of hCB HSCs, using in vitro proliferation assays and severe combined immunodeficiency (SCID)–repopulation assays. We demonstrated that NR-101 increases numbers of $CD34^+CD38^-$ primitive hematopoietic cells as well as SCID-repopulating cells (SRCs) more efficiently than does TPO. We also found that NR-101 activates unique profiles of signal transduction downstream of c-MPL and induces stabilization of hypoxia-inducible factor-1α (HIF-1α). Our results provide the first evidence that a small-molecule c-MPL agonist can be applied to ex vivo HSC expansion.

Materials and methods

Reagents

NR-101 (5-{[(2E)-2-{1-[5-(3,4-dichlorophenyl)-4-hydroxythiophen-3-yl]ethylidene}hydrazinyl]carbonyl}-N-[(1-methyl-1H-imidazol-4-yl)methyl]thiophene-2-carboxamide; molecular weight: 548.47, Fig. 1), a novel small-molecule agonist of human c-MPL, and SB-497115 (Eltrombopag free acid; 3′-{(2Z)-2-[1-(3,4-dimethylphenyl)-3-methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene]hydrazino}-2′-hydroxy-3-biphenylcarboxylic acid), another c-MPL agonist, were chemically synthesized by Nissan Chemical Industries (Chiba, Japan).

Mice

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from the Sankyo Lab Service (Tokyo, Japan). All experiments using these mice were performed in accordance with our institutional guidelines for the use of laboratory animals.

Cells

Human myeloblastic leukemia cell lines originally obtained from bone marrow, UT-7, UT-7/erythropoietin (EPO), and UT-7/TPO [40–42], were maintained in Iscove's modified Dulbecco's medium (Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum and 10 ng/mL recombinant human (rh) interleukin (IL)-3, 0.5 U/mL rhEPO, and 10 ng/mL rhTPO, respectively. Human cord blood (hCB) $CD34^+$ cells were purchased from Lonza (Basel, Switzerland) and AllCells (Berkeley, CA, USA) or purified from hCB. Fresh hCB cells were obtained from the Tokyo Cord Blood Bank (Tokyo, Japan). Mononuclear cells were separated by density gradient centrifugation. $CD34^+$ cells were immunomagnetically enriched using a magnetic-activated cell sorting CD34 progenitor kit (Miltenyi Biotech, Auburn, CA, USA). The purity of hCB $CD34^+$ cells was >90%. $CD34^+CD38^-$ cells were isolated by fluorescence-activated cell sorting using a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). Purified $CD34^+$ and $CD34^+CD38^-$ cells were cryopreserved or used freshly for experiments. This study was approved by the institutional review committees of the Chiba University.

Human cell culture

hCB $CD34^+$ and $CD34^+CD38^-$ cells were plated at 1×10^4 cells/well in a 24-well plate precoated with 25 μg/mL fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) [43] and cultured in serum-free medium (StemSpan; Stem Cell Technologies, Vancouver, British Columbia, Canada) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Cytokines were added at concentrations of 100 ng/mL for rhSCF, 100 ng/mL for rhFL,

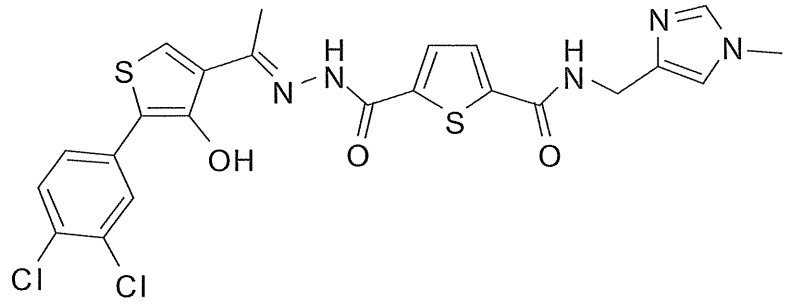

Figure 1. Chemical structure of NR-101, a small-molecule c-MPL agonist.

and 3 to 30 ng/mL for rhTPO, and indicated amounts of NR-101 were added.

Plasmids

The complementary DNAs (cDNAs) of human TPO receptor (*c-MPL*), human EPO receptor (*EPOR*), human IL-3 receptor α (*IL3RA*), and human granulocyte macrophage colony-stimulating factor receptor β chain (*CSF2RB*) were amplified by reverse transcription-polymerase chain reaction (RT-PCR). Full-length cDNAs were cloned into the vector pcDNA3.1 (Invitrogen).

Proliferation assay

UT-7 and UT-7/TPO cells were starved of cytokines for 17 hours. The cells were resuspended at a density of 6×10^4 cells/mL in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum and incubated with indicated concentrations of NR-101, cytokines, or vehicle (0.1% dimethyl sulfoxide) for 4 days. For chemical screening, CB hCD34$^+$ cells were plated at 1×10^4 cells/well in a 96-well plate with StemSpan medium containing 100 ng/mL rhSCF and library compounds and cultured for 7 days. WST-8 reagent (Kishida Chemical, Osaka, Japan) was used for the measurement of cell proliferation [44].

Cell cycle analysis

The BrdU Flow Kit (BD Biosciences, San Diego, CA, USA) was used for cell cycle analysis of cultured hCB CD34$^+$ cells. After 7 days of culture, the cells were pulsed for 45 minutes with 10 μM bromodeoxyuridine (BrdU), harvested, and stained with antibodies against CD34 or CD38 to identify CD34$^+$CD38$^-$ cells. Cells were fixed and permeabilized to allow staining with the anti-BrdU antibody conjugated to fluorescein isothiocyanate (FITC). Cells were further stained with 7-amino-actinomycin D to assess their position in the cell cycle. To detect Ki-67 expression, cells not treated with BrdU were fixed, permeabilized, and then stained with the anti–Ki-67 antibody conjugated to FITC.

Immunoprecipitation and Western blotting

UT-7/TPO cells were starved of rhTPO for 17 hours and stimulated with 25 μg/mL NR-101, 10 ng/mL rhTPO, and vehicle (0.1% dimethyl sulfoxide) for various periods. Cells were lysed with a lysis buffer (pH 7.0) comprising 20 mM Tris-HCl buffer, 300 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1% NP40, PhosSTOP (Roche Applied Science, Indianapolis, IN, USA), and a protease inhibitor cocktail (Complete Midi, Roche Applied Science), and then sonicated. After removal of insoluble materials by centrifugation, the supernatant was immunoprecipitated with protein G-Sepharose–conjugated anti-c-MPL (IBL, Gunma, Japan), anti-JAK2 (Upstate Technology, Waltham, MA, USA), anti-STAT5a (Upstate Technology), and anti-STAT3 (Upstate Technology). The immunoprecipitate or total cell lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride membranes, and probed with anti-phosphotyrosine antibody (4G10), anti–phospho-AKT (Cell Signaling Technology, Beverly, MA, USA), or anti–phospho-p41/p44 or anti–c-MPL, anti-JAK2, anti-STAT5a, anti-STAT3, anti-AKT, or anti-p42/44 MAPK (Upstate Technology). To detect the nuclear localization of HIF-1α, nuclear extracts were prepared from UT-7/TPO cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA). The nuclear fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed with anti–HIF-1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-histone H3 (Abcam, Cambridge, UK).

Luciferase assay

HEK293 cells were transfected with pcDNA-HuMPL, HuEPOR, or both HuIL-3RA and HuCSF2RB in combination with a STAT5 expression vector, pGCDNsam-STAT5A, and a STAT-responsive luciferase reporter gene, ST5BS-Luc (kindly donated by Dr. T. Kitamura and Dr. H. Nakajima, respectively) [45] by using Lipofectamine Plus reagent (Invitrogen) and starved for 7 hours, after which they were stimulated with indicated cytokines or NR-101 for 17 hours. After the ONE-Glo reagent (Promega, Madison, WI, USA) was added to the cells, luciferase activities were measured.

Colony-forming cell assay

hCB CD34$^+$ cells, which were cultured with NR-101 or rhTPO for 7 days, were plated in Methocult GF H4435 methylcellulose medium containing 50 ng/mL human SCF, 10 ng/mL human granulocyte-macrophage colony-stimulating factor, 10 ng/mL human IL-3, and 3 U/mL human EPO (StemCell Technologies). After 12 to 14 days of culture, the colonies were counted. The number of megakaryocyte colony-forming units (CFU-MK) was assessed using MegaCult-C (StemCell Technologies) according to manufacturer's directions.

RNA extraction and real-time PCR

UT-7/TPO and hCB CD34$^+$ cells were incubated in the presence of rhTPO or NR-101 for predetermined periods, and then total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). The total RNA (1 μg) was reverse-transcribed with

a SuperScript one-step RT-PCR kit (Invitrogen). PCR was carried out for 40 to 45 cycles of 1 minute at 60°C and 15 seconds at 95°C in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). 18S ribosomal RNA (18Sr) or β-2-microglobulin was used as an internal control. Taqman primers and probes were obtained from Applied Biosystems; 18Sr (Hs99999901_s1), β-2-microglobulin (Hs99999907_m1), Oncostatin M (Hs00171165_m1), SOCS3 (Hs00269575_s1), Pim-1 (Hs00171473_m1), EGR (Hs00152928_m1), GATA2 (Hs00 231119_m1), p21 (Hs00355782_m1), p57 (Hs00175938_m1), HBEGF (Hs00181813_m1), TNFRSF12A (Hs00171993_m1), c-Myc (Hs00153408_m1), HOXB4 (Hs00256884_m1), Bmi-1 (Hs00180411_m1), vascular endothelial growth factor (VEGF) (Hs00173626_m1), SLC2A3 (Hs00359840_m1), HIF1A (Hs00936368_m1), CXCL12 (Hs00171022_m1), P4HA1 (Hs00914594_m1), TFRC (Hs00174609_m1), LDHA (Hs0 0855332_g1), PGK1 (Hs99999906_m1), HK1 (Hs00175976_m1), SLC2A1 (Hs00197884_m1), EPOR (Hs00181092_m1), NOS2A (Hs00167257_m1), SERPINE1 (Hs01126606_m1), ALDOA (Hs 00605108_g1), PFKL (Hs00160027_m1), PKM2 (Hs00987255 _m1), ENO1 (Hs00361415_m1).

VEGF enzyme-linked immunosorbent assay

After incubation of the UT-7/TPO cells with indicated concentrations of NR-101, rhTPO, or vehicle (0.1% dimethyl sulfoxide) for 24 hours, the culture medium was collected. The amount of VEGF in the supernatant was measured with a Quantikine kit from R&D Systems (Minneapolis, MN, USA).

Transplantation of hematopoietic cells into NOD/SCID mice

NOD/SCID mice at 8 to 10 weeks age were sublethally irradiated at 2.75 Gy. Limiting doses of fresh hCB $CD34^+$ cells and cultured progenies of hCB $CD34^+$ cells were injected through tail veins. At 8 weeks after transplantation, bone marrow (BM) cells were analyzed by flow cytometry for the presence of human $CD45^+$ cells [46–49]. For the limiting dilution analysis, mice were considered to be positive for the human HSC engraftment when at least 1.0% human $CD45^+$ cells were detected among the BM cells. The data from several experiments were pooled and analyzed by using L-Calc software (StemCell Technologies).

Flow cytometry

Human hCB cells were stained with anti-human CD34-allophycocyanin and anti-human CD38-phycoerythrin or anti-human CD41a-FITC antibodies (BD Pharmingen). Then 1 μg/mL propidium iodide (Sigma, St Louis, MO, USA) was added to exclude nonviable cells. Cells were analyzed on an EPICS-XL flow cytometer (Beckman Coulter, Franklin Lakes, NJ, USA) or a JSAN desktop cell sorter. For analyzing human hematopoietic engraftment in NOD/SCID mice, BM cells were stained with anti-human $CD45^+$-allophycocyanin antibody (BD Pharmingen).

Immunostaining

hCB $CD34^+$ cells in a serum-free medium supplemented with 0.1% bovine serum albumin and either 10 ng/mL rhTPO or 1 μg/mL NR-101 were incubated on fibronectin-coated glass slides for 24 hours at 37°C. After fixation with 2% paraformaldehyde and blocking in 10% goat serum for 1 hour at room temperature, cells were incubated with anti–HIF-1α antibody (Santa Cruz Biotechnology) for 12 hours at 4°C. Cells were washed and incubated with an Alexa 488-conjugated goat anti-mouse antibody (Invitrogen) for 30 minutes at room temperature. 4, 6-Diamidino-2-phenylindole was used for DNA staining. Immunofluorescence was observed with an ECLIPSE SE 80i fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

Data are presented as the mean ± standard error of mean. Statistical significance was analyzed with Student's *t*-test. The level of significance was set at 0.05.

Results

Identification of NR-101 as a novel human c-MPL agonist

To ascertain the effect of small-molecule c-MPL agonists on human HSCs/HPCs, we screened >400 human c-MPL agonists, which were synthesized during the process of identifying NIP-004 [38], for activity to promote the proliferation of hCB $CD34^+$ cells (data not shown). Among several compounds identified, we selected one of the most active, NR-101, and examined its effect on human HSCs/HPCs more closely. NR-101 is a chemically synthesized compound with a structure shown in Figure 1. NR-101 supported the proliferation of UT-7/TPO cells, a human leukemia cell line expressing c-MPL, in a dose-dependent manner, and exhibited a maximum effect at 100 ng/mL (Fig. 2A), which was equivalent to 98% of that of rhTPO at 10 ng/mL. The median effective concentration (EC_{50}) of NR-101, SB-497115 and rhTPO was 0.36 ng/mL (0.65 nM), 49.6 ng/mL (112 nM), and 0.24 ng/mL (0.013 nM), respectively. The activity of NR-101 was dependent on c-MPL as NR-101 did not support the proliferation of other cell lines, UT-7 and UT-7/EPO, which do not express c-MPL but do express IL-3 receptor (IL-3R) and EPOR, respectively (Fig. 2B and C). We also confirmed the specificity of NR-101 activity by using a STAT-reporter gene assay in HEK293 cells expressing c-MPL, IL-3R, or EPOR (Fig. 2D). Luciferase production was induced by NR-101 at a comparable level to that by rhTPO in HEK293 cells expressing human c-MPL, but not in cells expressing human IL-3R or EPOR.

We next tested whether NR-101 affects human megakaryocytopoiesis using colony-forming assays followed by immunostaining of glycoprotein CD41a, a specific marker of the megakaryocyte lineage [50]. NR-101 stimulated the formation of megakaryocyte colonies from hCB $CD34^+$ cells in a dose-dependent manner, and its activity at 1000 ng/mL was comparable to that of rhTPO at 10 ng/mL (Fig. 2E). Similarly, the serum-free culture of hCB $CD34^+$ cells with 300 to 1,000 ng/mL of NR-101 for 10 days induced an increase in the number of $CD41^+$ cells, and the maximum effect was comparable to that of rhTPO (Fig. 2F). Together, these results indicate that NR-101 is a specific agonist of c-MPL and stimulates human megakaryocytopoiesis with full efficacy comparable to rhTPO.

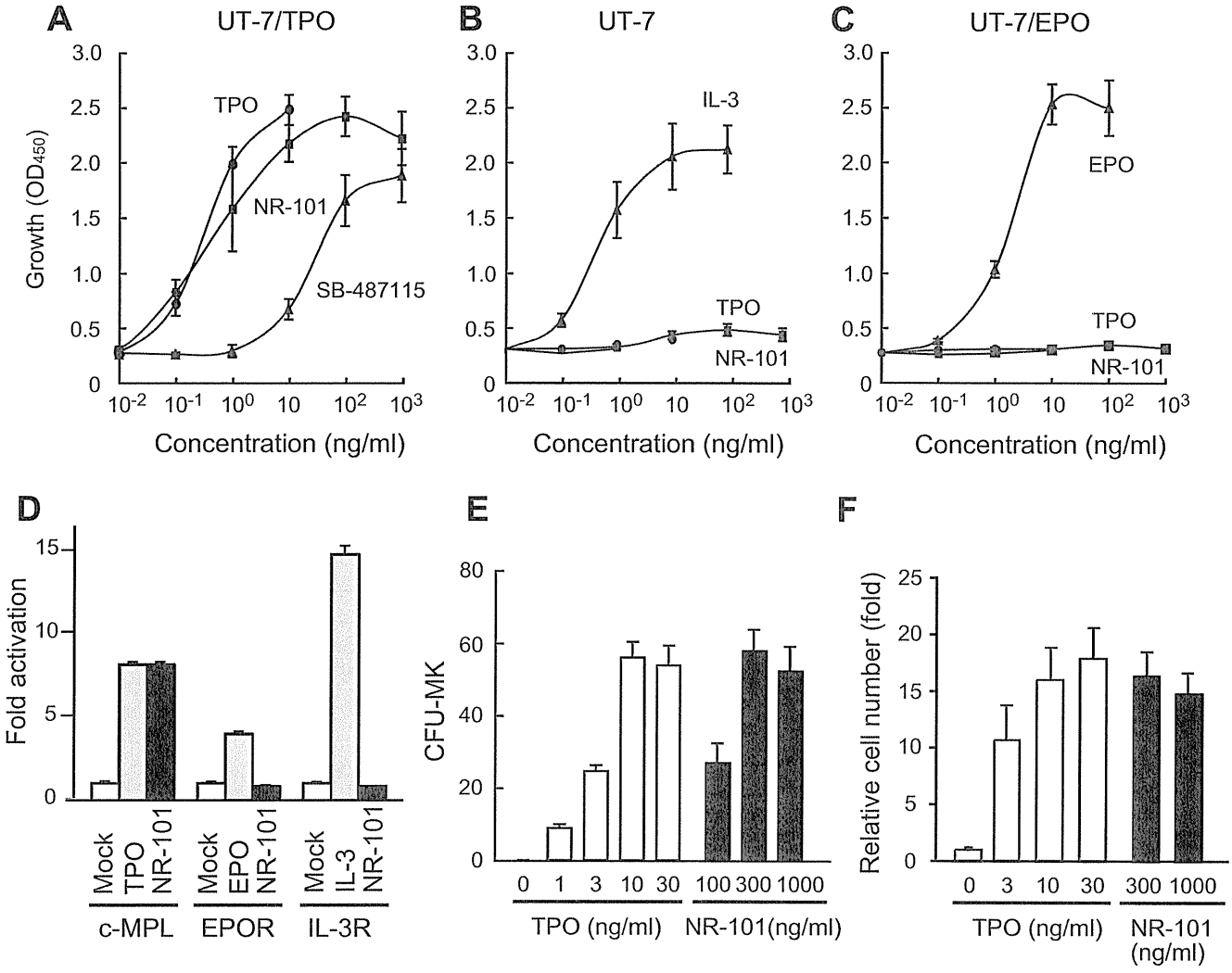

Figure 2. NR-101 specifically activates human c-MPL. Dose-dependent proliferation of UT-7/TPO cells (**A**), UT-7 cells (**B**), and UT-7/EPO cells (**C**) by recombinant human (rh) TPO, rh interleukin (IL)-3, rhEPO, NR-101, and SB-497115. (**D**) signal transducer and activator of transcription (STAT) 5-reporter gene assay with Hek293 cells expressing c-MPL, EPO receptor (EPOR), or IL-3 receptor (IL-3R). Bars represent the fold increase in luciferase activity relative to mock conditions. (**E**) Human cord blood (hCB) $CD34^+$ cells cultured in collagen-gel medium with rhTPO or NR-101 were assessed for megakaryocyte colony-forming units (CFU-MK). Bars represent the number of CFU-MK per 1,500 hCB $CD34^+$ cells. (**F**) hCB $CD34^+$ cells were cultured in serum-free medium supplemented with rhTPO, NR-101, or the same volume of dimethyl sulfoxide (DMSO) (control) for 11 days. Shown is the number of $CD41^+$ megakaryocyte-lineage cells relative to that in the control culture. Data represent the mean ± standard error of mean for three to five independent experiments.

NR-101 expands $CD34^+CD38^-$ cells ex vivo

Although several nonpeptidic c-MPL agonists have been examined with respect to platelet formation [34–39], the effect of c-MPL agonists on the ex vivo expansion of human HSCs/HPCs remains to be tested. We cultured hCB $CD34^+$ cells in serum-free medium supplemented with rhSCF and rhTPO or NR-101 for 7 days and analyzed the phenotypes of the cells. It has been reported that HSCs/HPCs are highly enriched in the $CD34^+CD38^-$ fraction [46–48], thus we first analyzed the population of $CD34^+CD38^-$ cells in the cultured progenies. Although the total number of cells cultured with NR-101 was comparable to that cultured with rhTPO (Fig. 3A), the cultures with 1 μg/mL of NR-101 contained 1.7 and 2.3-fold more $CD34^+$ and $CD34^+CD38^-$ cells, respectively, than those with 10 ng/mL rhTPO (Fig. 3B and C). In contrast, the activity of SB-497115 (Eltrombopag), another c-MPL agonist, was equivalent to that of rhTPO in expanding $CD34^+CD38^-$ cell numbers at the optimum concentration (6 μg/mL) (Fig. 3D). NR-101 still had a 1.7-fold greater effect on $CD34^+CD38^-$ cells than rhTPO even in the presence of rhFL, a cytokine that supports the maintenance of HSCs/HPCs ex vivo (Fig. 3E) [30,51]. Furthermore, we assessed directly the expansion of hCB $CD34^+CD38^-$ cells by using hCB $CD34^+CD38^-$ cells as starting cells for culture. During the 7-day culture, $CD34^+CD38^-$ cells expanded 2.4- ± 0.4-fold and 4.9- ± 0.6-fold with rhSCF plus rhTPO and rhSCF plus NR-101, respectively

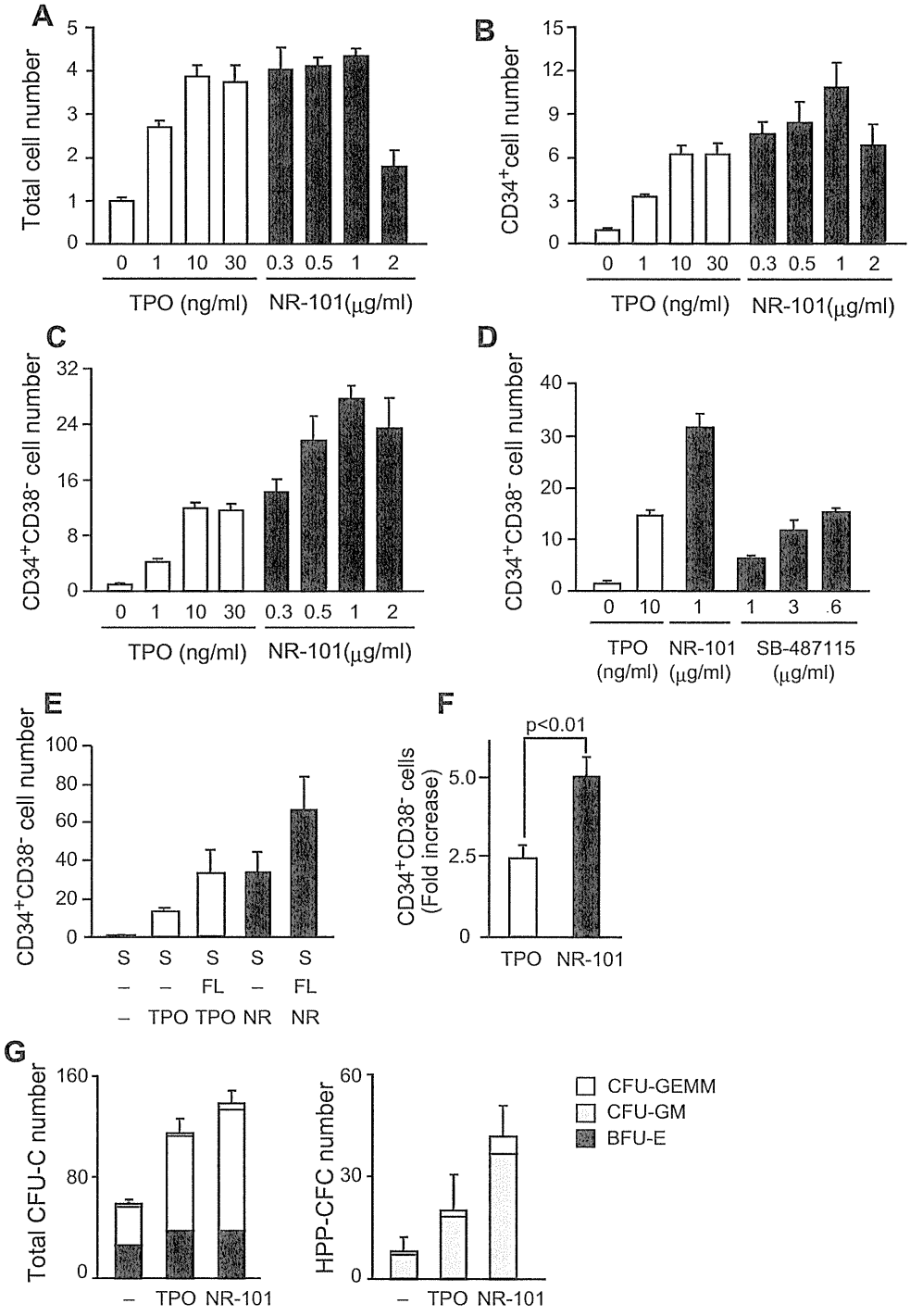

Figure 3. NR-101 expands human $CD34^+CD38^-$ cell numbers more efficiently than thrombopoietin (TPO). Human cord blood (hCB) $CD34^+$ cells were cultured with recombinant human (rh) TPO, NR-101, SB-497115, or the same volume of dimethyl sulfoxide (DMSO) (control) in the presence of rh stem cell factor (SCF) for 7 days, and analyzed by fluorescein-activated cell sorting for CD34 and CD38 expression. Bars represent the relative number of all cells (**A**), $CD34^+$ cells (**B**), and $CD34^+CD38^-$ cells (**C**, **D**) to those in control cultures. (**E**) The effects of the addition of fms-like tyrosine kinase 3 ligand (FL; 100 ng/mL rhFL) on the number of $CD34^+CD38^-$ cells. S, rhSCF; NR, 1 μg/mL of NR-101; TPO, 10 ng/mL rhTPO. (**F**) The number of $CD34^+CD38^-$ cells in 7-day cultures of $CD34^+CD38^-$ cells treated with 10 ng/mL of rhTPO or 1 μg/mL of NR-101. The fold-increase in $CD34^+CD38^-$ cells relative to the input $CD34^+CD38^-$ cells is shown. (**G**) The number of colony-forming units in culture (CFU-C) and high-proliferative potential colony-forming cell (HPP-CFC) in 7-day cultures of hCB $CD34^+$ cells. – indicates the negative control (0.1% DMSO). Data represent the mean ± standard error of mean for three to five independent experiments.

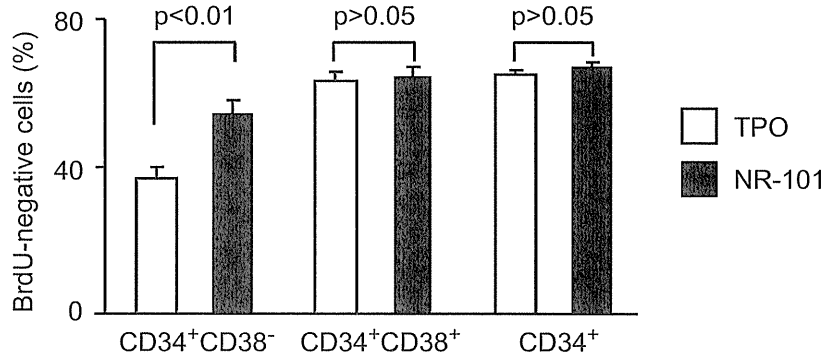

Figure 4. $CD34^+CD38^-$ cells treated with NR-101 show a decelerated cell cycle. Human cord blood (hCB) $CD34^+$ cells were cultured with 10 ng/mL recombinant human thrombopoietin (rhTPO) or 1 μg/mL of NR-101 in the presence of recombinant human stem cell factor (rhSCF) for 7 days. The bromodeoxyuridine (BrdU) incorporated into cultured progenies was determined by a fluorescein-activated cell sorting analysis for $CD34^+CD38^-$ cells, $CD34^+CD38^+$ cells, and $CD34^+$ cells, respectively. Data represent the mean ± standard error of mean (n = 5).

(Fig. 3F), confirming again greater activity of NR-101 in the expansion of HSCs/HPCs than that of rhTPO.

To evaluate the number of functional HSCs/HPCs in cultures with NR-101, we next performed colony assays. The $CD34^+$ cell culture with NR-101 contained all types of myeloid progenitors such as CFU–granulocyte/macrophage/erythrocyte/megakaryocytes, CFU-granulocyte/macrophages, and burst-forming unit-erythrocytes. Of note, high-proliferative potential colony-forming cells, defined by their ability to form large colonies in vitro (diameters >1 mm) [52] and CFU–granulocyte/macrophage/erythrocyte/megakaryocytes that represent the most primitive progenitors detected in vitro, were contained at a higher frequency in cultures with NR-101 than those with rhTPO (Fig. 3G). These results indicate that NR-101 increases the frequency of primitive $CD34^+CD38-$ cells in vitro more efficiently than rhTPO does and maintains the primitive HSC/HPC state during the culture.

NR-101–treated $CD34^+CD38^-$ cells cycle slowly

Cell cycle status is critical to the maintenance of HSCs, i.e., self-renewal vs differentiation fate decision of HSCs at cell division. The advantage of NR-101 in $CD34^+CD38-$ cell expansion prompted us to examine the cell cycle status of NR-101–treated cells. We cultured hCB $CD34^+$ cells with rhTPO or NR-101 for 7 days and measured the cell cycle status by detecting BrdU incorporation. The population of $CD34^+CD38^-$ cells in the G_0/G_1 phases was significantly greater in cultures with NR-101 than in those with rhTPO (Fig. 4). This effect of NR-101 was specific to the $CD34^+CD38^-$ cell fraction, with no effect observed on the $CD34^+CD38^+$ downstream progenitors or total $CD34^+$ cell population. We also examined the effect of rhTPO and NR-101 on the G_0/G_1 ratio using Ki-67 to distinguish cells in the G_0 phase ($Ki\text{-}67^-$) from those in the G_1 phase ($Ki\text{-}67^+$). The G_0/G_1 ratios of $CD34^+CD38^-$ cells cultured with rhTPO and NR-101 for 7 days were 3.73 ± 0.76 and 1.79 ± 0.21, respectively, indicating that more NR-101–treated than TPO-treated $CD34^+CD38^-$ cells were arrested in G_1 (2.1-fold, $p = 0.017$). Collectively, these results show that NR-101–treated $CD34^+CD38^-$ cells cycle more slowly than TPO-treated cells, and NR-101 maintains the $CD34^+CD38^-$ population by suppressing excessive proliferation of the cells.

NR-101 expands SRCs in $CD34^+$ cell cultures

To determine whether NR-101 enhances the self-renewal of HSCs in culture, we performed a NOD/SCID-repopulation assay to estimate the reconstitution capacity of the progeny of hCB $CD34^+$ cells cultured with rhTPO or NR-101 in addition to rhSCF and rhFL. We used limiting numbers of cells for the repopulation assay to estimate the SRC frequencies. Increasing numbers (2.5×10^3, 5×10^3, 1×10^4, 2×10^4, 3×10^4, 4×10^4) of fresh $CD34^+$ cells or the progenies after 7 days of culture corresponding to the same number of input $CD34^+$ cells were transplanted into NOD/SCID recipients (Fig. 5A–C). Figure 5D shows the frequency of engraftment for each cell dose used in each condition. The frequency for SRCs was 1 in 17,387 (95% confidence interval of 1 of 21,731 to 1 of 13,912) in fresh $CD34^+$ cells and 1 in 14,046 (95% confidence interval of 1 of 17,858 to 1 of 11,047) in the culture with rhTPO. In contrast, the frequency for SRCs in the culture with NR-101 was 1 in 6,035 (95% confidence interval of 1 of 8,223 to 1 of 4,430), which was 2.9-fold higher than for fresh $CD34^+$ cells ($p = 0.006$) and 2.3-fold higher than for rhTPO-treated cultures ($p = 0.03$). Correspondingly, the average repopulation levels by human hematopoietic cells were higher in recipient mice infused with NR-101–treated cells than in those infused with fresh $CD34^+$ cells or rhTPO-treated cells (Fig. 5A–C). NR-101–treated cells repopulated both $CD33^+$ myeloid-lineage and $CD19^+$ B-cell lineage cells in immunodeficient mice (Supplementary Figure E1, online only, available at www.exphem.org). These results demonstrate that NR-101 can promote expansion of SRCs in culture while rhTPO only maintains SRCs.

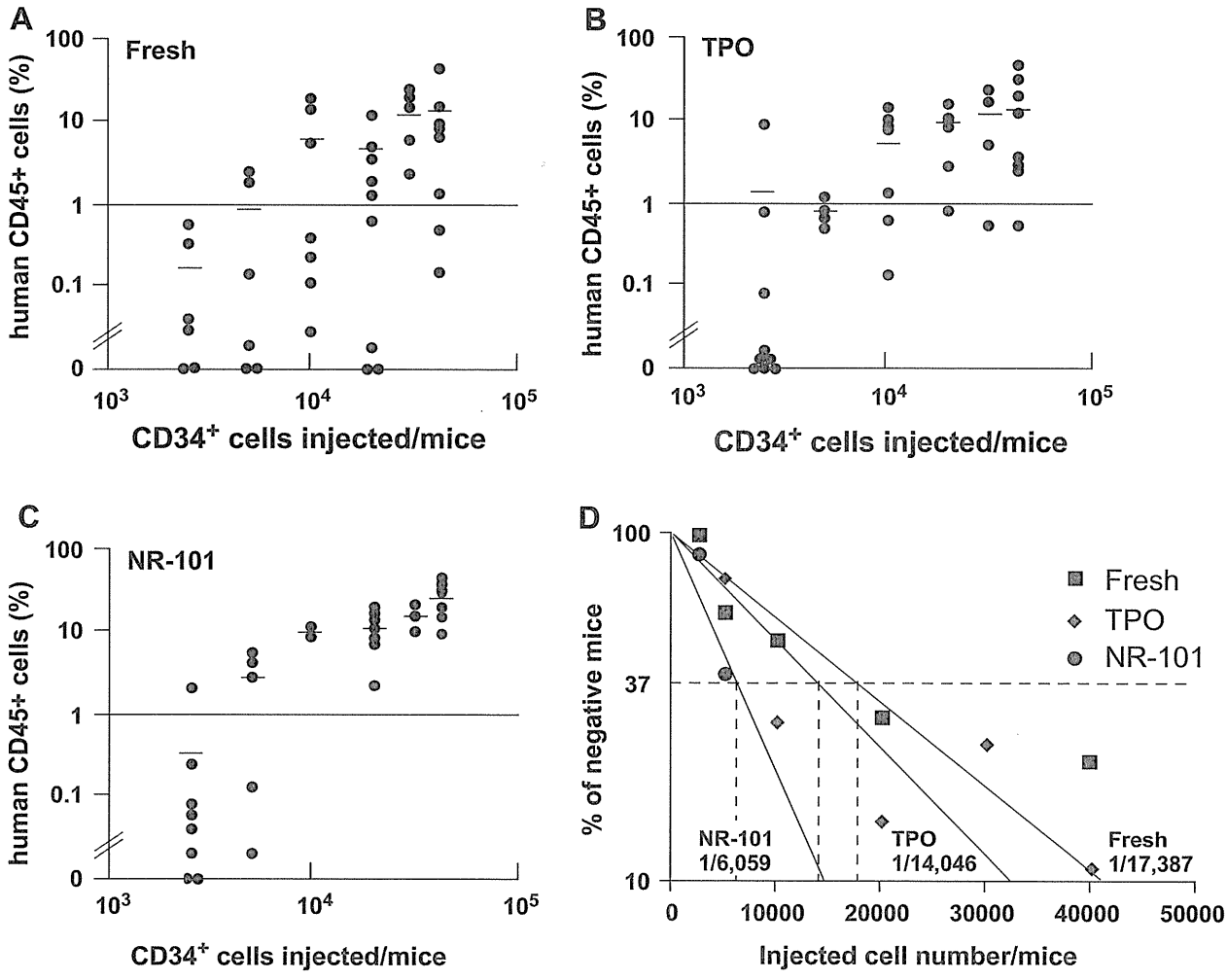

Figure 5. NR-101-treated human cord blood (hCB) $CD34^+$ cells show an increase in the frequency of severe combined immunodeficiency-repopulating cells (SRCs). hCB $CD34^+$ cells were cultured with 10 ng/mL of recombinant human thrombopoietin (rhTPO) or 1 μg/mL NR-101 in the presence of recombinant human stem cell factor (rhSCF) and recombinant human fms-like tyrosine kinase 3 ligand (rhFL) for 7 days. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 114) were injected with increasingly higher doses of freshly isolated $CD34^+$ cells (**A**), cells cultured with rhTPO (**B**), or cells cultured with NR-101 (**C**), and the proportion of human $CD45^+$ cells among recipient bone marrow (BM) cells was analyzed 8 weeks after transplantation. (**D**) Mice with at least 1% human $CD45^+$ cells in BM were considered successfully engrafted, and the frequency of SRCs was determined with a limiting dilution analysis.

Unique profiles of c-MPL signaling activated by NR-101

To elucidate the molecular mechanisms by which NR-101 promotes the expansion of HSCs, we first examined whether NR-101 activates the c-MPL signaling pathway in the same manner as TPO in UT-7/TPO cells. NR-101 phosphorylated the major components of TPO-mediated signaling pathways, c-MPL, JAK2, STAT3, STAT5, p42/44 MAPK, and AKT, indicating that NR-101 activates the major pathways of TPO/c-MPL signaling (JAK/STAT, Ras/MAPK, and PI3K/AKT) (Fig. 6). Maximum signal intensity was observed immediately after the treatment with rhTPO (5–60 minutes), whereas, in cells stimulated with NR-101, these signaling molecules became maximally active at later time points (60–240 minutes). Of note, NR-101 treatment sustained high levels of signaling for significantly longer periods. Moreover, of interest, NR-101 scarcely induced STAT3 activation, but selectively and persistently activated STAT5.

Next, we examined the gene expression profiles of UT-7/TPO cells treated with NR-101 by using real-time quantitative PCR. The expression of most early response genes that are responsive to rhTPO, including STAT5-target genes (*OSM*, *SOCS3*, and *PIM1*), MAPK-target genes (*HBEGF*), *TNFRSF12A*, and *EGR*, was gradually upregulated and reached a maximum several hours after the treatment with NR-101 (Supplementary Figures E2A, E3A [online only, available at www.exphem.org], and data not shown). In contrast, levels were markedly increased immediately after treatment with rhTPO and gradually decreased thereafter. rhTPO and NR-101 similarly upregulated expression of a cyclin-dependent kinase inhibitor, *p21*, and a transcription factor, *c-MYC*, which is involved in the regulation of

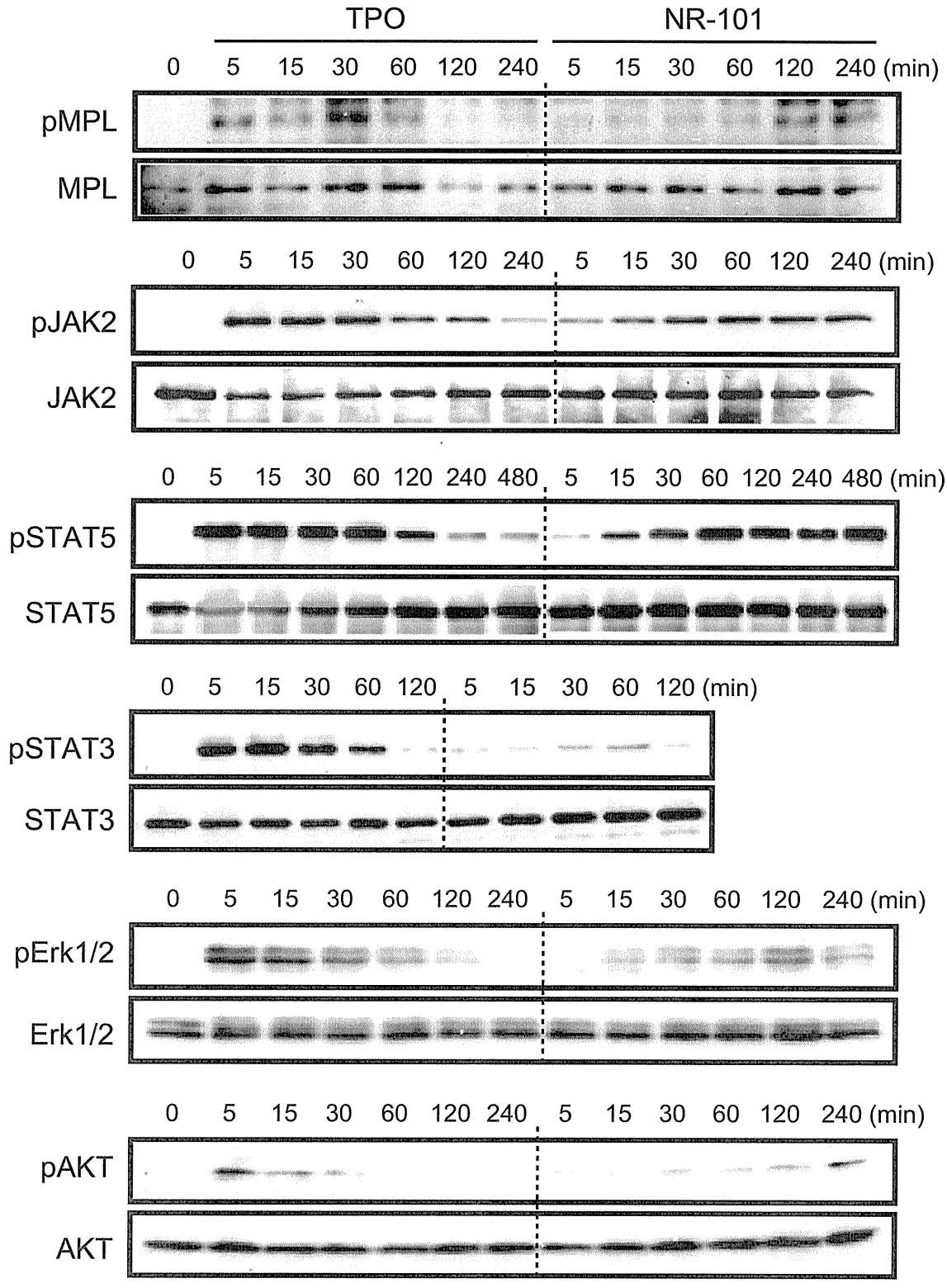

Figure 6. NR-101 shows characteristic signal transduction as compared to thrombopoietin (TPO). UT-7/TPO cells were stimulated with 10 ng/mL recombinant human (rhTPO) (left panels) or 25 μg/mL NR-101 (right panels) for the periods indicated. c-MPL, Janus kinase (JAK) 2, signal transducer and activator of transcription (STAT) 5, and STAT3 were immunoprecipitated and detected with anti-phosphotyrosine and anti–c-MPL, anti-JAK2, anti-STAT5, and anti-STAT3. Whole cell lysate was probed with anti–phospho-Erk1/2, anti-Erk1/2, anti–phospho-AKT, and anti-AKT. Data are representative of at least two independent experiments.

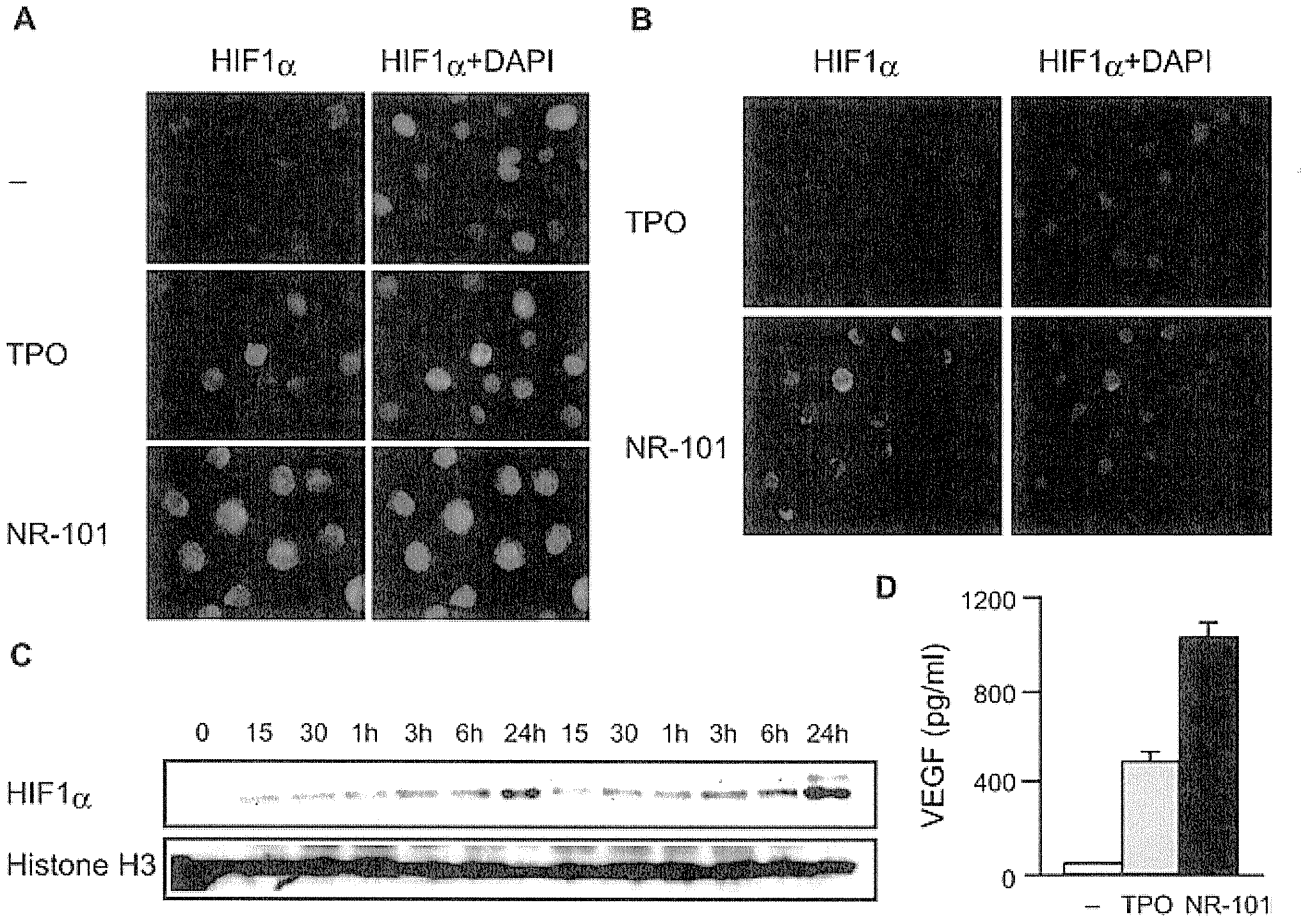

Figure 7. NR-101 stabilizes hypoxia-inducible factor-1α (HIF-1α) protein more efficiently than does thrombopoietin (TPO). UT-7/TPO cells (**A**) and human cord blood (hCB) $CD34^+$ cells (**B**) were stimulated with 10 ng/mL recombinant human TPO (rhTPO) or 1 μg/mL NR-101 for 24 hours. Cells were stained with anti–HIF-1α primary antibody and Alexa488-conjugated secondary antibody (green), and counterstained with 6-diamidino-2-phenylindole (DAPI) to visualize DNA (blue). – indicates the negative control (0.1% dimethyl sulfoxide [DMSO]) (**C**) UT-7/TPO cells were stimulated with 10 ng/mL rhTPO (left) or 1 μg/mL NR-101 (right) for 24 hours. Nuclear extracts were prepared, and then HIF-1α protein levels were analyzed by Western blotting. Histone H3 protein was used as an internal control. (**D**) After 24 hours of stimulation with rhTPO or NR-101, the amount of VEGF released from UT-7/TPO cells into the medium was measured by enzyme-linked immunosorbent assay. – indicates the negative control (0.1% DMSO); TPO, 10 ng/mL rhTPO; NR-101, 1 μg/mL NR-101.

cellular proliferation, and downregulated *p57* expression. rhTPO and NR-101, however, did not significantly alter the expression of HSC regulator genes, *GATA2*, *HOXB4*, and *BMI1* (data not shown). These gene expression profiles may represent the delayed but sustained activation of c-MPL and its downstream pathways, JAK/STAT, Ras/MAPK, and PI3K/AKT, induced by NR-101. The unique gene expression profile activated by NR-101 was also observed in hCB $CD34^+$ cells (Supplementary Figure E2B, online only, available at www.exphem.org).

NR-101 efficiently activates and maintains HIF-1 signaling pathways

It has been reported that SRCs expand under hypoxic conditions [53] and TPO activates hypoxia-responsive pathways by increasing the intranuclear level of HIF-1α [54–56], which detects changes in oxygen in the cellular environment and plays an essential role in cellular responses to hypoxia [57,58]. Therefore, we compared HIF-1α protein levels in UT-7/TPO cells stimulated with rhTPO and NR-101 for 24 hours. Immunofluorescence microscopy demonstrated that the levels of intracellular HIF-1α protein in UT-7/TPO cells as well as hCB $CD34^+$ cells were higher in the cells treated with NR-101 than with rhTPO (Fig. 7A, B). A Western blot analysis supported this finding in UT-7/TPO cells (Fig. 7C). However, neither rhTPO nor NR-101 stimulated *HIF1A* gene expression (Supplementary Figure E3A, online only, available at www.exphem.org). Consistent with these findings, we found that a series of HIF-1α–regulated genes, including those related to angiogenesis (*VEGF*), vasomotor control, glycogenesis, glucose transport (*SLC2A3*), and others (*P4HA1*, *PIM1*), were upregulated by NR-101 in UT-7/TPO and hCB $CD34^+$ cells (Supplementary Figures E3A, B, and Supplementary Table E1, online only, available at www.exphem.org). As was the case with other TPO-responsive genes presented in Supplementary Figure E2, HIF-1α–regulated genes were slowly upregulated and reached their maximum levels at later time points after

NR-101 stimulation than after rhTPO stimulation, but their transcriptional levels remained high for long periods. These expression profiles were consistent with the HIF-1α protein levels detected by Western blotting.

Among these HIF-1α–regulated genes, enhanced expression of the *VEGF* gene was confirmed at the protein level by detecting the amount of VEGF secreted into the UT-7/TPO culture supernatant by enzyme-linked immunosorbent assay. NR-101–treated cells produced a higher level of VEGF than did rhTPO-treated cells (Fig. 7D). These results indicated that NR-101 induces a hypoxic response by stabilizing HIF-1α protein more efficiently than TPO does.

Discussion

In the present study, we identified NR-101 as a novel small-molecule c-MPL agonist, which was originally discovered on screening a chemical library for activity to promote the proliferation of $CD34^+$ cells. NR-101 exhibited an EC_{50} of 0.36 ng/mL in the UT-7/TPO cell proliferation assay, which was comparable to that of rhTPO (0.24 ng/mL), and the maximum activity of NR-101 was also equivalent to that of TPO. It is important to note that NR-101 was superior to another small-molecule c-MPL agonist, SB-497115, in inducing the proliferation of UT-7/TPO cells. NR-101 requires a histidine residue (His499) in the transmembrane domain of c-MPL to exert its activity (data not shown). Many other c-MPL agonists, SB-497115, NIP-004, Butyzamide, and AKR-501, also require this histidine residue in the transmembrane domain of c-MPL [34–39], suggesting that NR-101 selectively binds and activates c-MPL. However, further investigations are needed to clarify the mechanism by which NR-101 activates c-MPL because we have not proved direct binding of NR-101 to c-MPL.

We clearly demonstrated that NR-101 prevails over TPO in the ex vivo expansion of HSCs/HPCs. Although there was no significant difference between rhTPO and NR-101 in expanding total cell numbers and inducing megakaryocytic differentiation from human $CD34^+$ cells, NR-101 preferentially expanded the $CD34^+CD38^-$ population and maintained their colony-forming capacity as compared with rhTPO. This positive effect on $CD34^+CD38^-$ cells is specific to NR-101 because the effect of other nonpeptidyl c-MPL agonists, SB-497115, NIP-004, and AKR-501, on $CD34^+CD38^-$ cells was comparable to that of rhTPO (data not shown), suggesting that the specific structure of NR-101 is responsible for the capacity to expand $CD34^+CD38^-$ cells. Moreover, NR-101 induced a 2.9-fold expansion of SRCs in short-term cultures, whereas rhTPO-treated cultures showed no significant change in SRC numbers compared with fresh $CD34^+$ cells (1.2-fold expansion; $p = 0.51$). The observation that TPO did not expand SRC numbers under serum-free conditions is consistent with recent reports [59–61], suggesting that a combination of the cytokines TPO, SCF, and FL is not sufficient to achieve the expansion of human HSCs in the present culture conditions.

To further understand the effect of NR-101 on HSCs/PCs, we analyzed the cell cycle of the $CD34^+CD38^-$ fraction in cultures with rhTPO or NR-101. NR-101 increased the size of the BrdU-negative fraction (G_0 or G_1 phase) in $CD34^+CD38^-$ cells more than did rhTPO, while NR-101 had no effect on the cell cycle in $CD34^+CD38^+$ cells or $CD34^+$ cells overall. Intriguingly, NR-101 appeared to increase the proportion of $CD34^+CD38^+$ cells in the G_1 phase. These observations indicate that NR-101 promotes the cell cycle arrest of primitive HSCs/HPCs in the G_1 phase, but does not induce quiescence (G_0 phase) in vitro. This result is consistent with the finding by Danet et al. that hypoxia promotes the transition from G_0 to G_1 and/or induces cell cycle arrest in G_1 in Lin^-CD34^+ cells [53]. It has been shown that HSCs remain quiescent or undergo division at a slow rate in BM niches to achieve long-term self-renewal and maintain the capacity to differentiate into multiple lineages. By contrast, in ex vivo cultures, HSCs rapidly cycle and undergo terminal differentiation [62,63]. The NR-101–induced slow cycling of HSCs/PCs might help to protect them from excessive differentiation and allow them to self-renew in culture. However, the expression of negative cell cycle regulators, *p21* and *p27*, in $CD34^+CD38^-$ cells was not significantly affected by NR-101 treatment (data not shown), so the underlying molecular mechanisms remain obscure.

In this study, we noted several unique effects of NR-101 that may explain the molecular mechanisms involved in the expansion of HSCs by NR-101. We found that NR-101 activates c-MPL and its downstream signaling molecules very slowly, but maintains high levels of their activated forms for significantly longer periods as compared to rhTPO. It is conceivable that the resistance of chemical compounds to degradation during culture and/or less internalization of c-MPL into the cytoplasm account for the sustained effects of NR-101. Importantly, NR-101 persistently activates STAT5, but hardly activates STAT3. STAT5 plays an essential role in the self-renewal of HSCs [45]. The specific and durable activation of STAT5 by NR-101 might contribute to the augmented self-renewal capacity of HSCs. On the other hand, this finding also suggests that STAT3 is not absolutely essential to the maintenance of self-renewing HSCs in vitro. Taking into account the occasional uncertainty of results obtained by cell line-based evaluation, additional studies using $CD34^+$ and $CD34^+CD38^-$ cells would be necessary to confirm the characteristic cell signaling by NR-101 and actual roles of STAT proteins.

The hypoxic response mediated by HIF-1α is important for the maintenance of HSCs and hypoxic conditions reportedly enhance the reconstitution activity of HSCs in recipient mice [53,64]. TPO is one of the cytokines that activates hypoxia-responsive pathways by increasing the intranuclear level of HIF-1α [54-56]. NR-101 appeared to efficiently stabilize HIF-1α protein and higher levels of HIF-1α protein

were detected in cells treated with NR-101 than those treated with rhTPO. Consistent with these findings, we found that NR-101 induces the gene expression of targets of HIF-1α more intensively than does TPO. Among the targets of HIF-1α, VEGF has been implicated in the survival of HSCs through an internal autocrine loop [65]. Enhanced glycolysis with reduced mitochondrial respiration is a characteristic metabolic shift observed in embryonic stem cells and hypoxic cancer cells with extensive self-renewal capacity [66,67]. Enhanced expression of *VEGF* and genes involved in glycolysis and glucose transport might favor the maintenance and/or expansion of HSCs/HPCs in NR-101–treated cultures. Thus, the induction of hypoxic responses in HSCs/HPCs could be another mechanism by which NR-101 promotes SRC expansion in vitro. However, further study is needed to clarify the mechanism of stabilization of HIF-1α protein by NR-101 and the involvement of sustained activation of STAT5 in this process.

Numerous investigators have attempted to improve the efficiency of ex vivo expansion of human HSCs for clinical purposes, particularly hCB HSC transplantation. Notably, all recent studies have used TPO and SCF in the cell culture. Therefore, it will be beneficial to substitute NR-101 for TPO in the present culture systems to achieve a more efficient ex vivo expansion of HSCs. The approach using NR-101 has an advantage over other strategies in that NR-101 has the capacity to induce HSC self-renewal and is readily available to combine with other technologies. In addition, NR-101 could be also applied to gene therapy using HSCs, which requires the maintenance of HSCs in the process of gene transduction. It will be important to further attempt to expand HSCs by combining NR-101 with other cytokines, ligands, and chemicals capable of inducing HSC self-renewal (e.g., Delta1, angiopoietin-like 5, IGFBP2, 5-aza-2′-deoxycytidine, diethylaminobenzaldehyde, or FP6) [49,59–61,68]. It will also be important to change the chemical structure of NR-101 to achieve optimum activity for the expansion of HSCs.

In conclusion, we have identified NR-101, a novel nonpeptidyl small-molecule compound, which exhibits a selective and sustained activation of c-MPL. The results reported here demonstrate that NR-101 is sufficient to induce the expansion of human HSCs. The approach using NR-101 will provide a wider range of options and be useful for the development of novel and efficient technologies for stem cell and gene therapies.

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Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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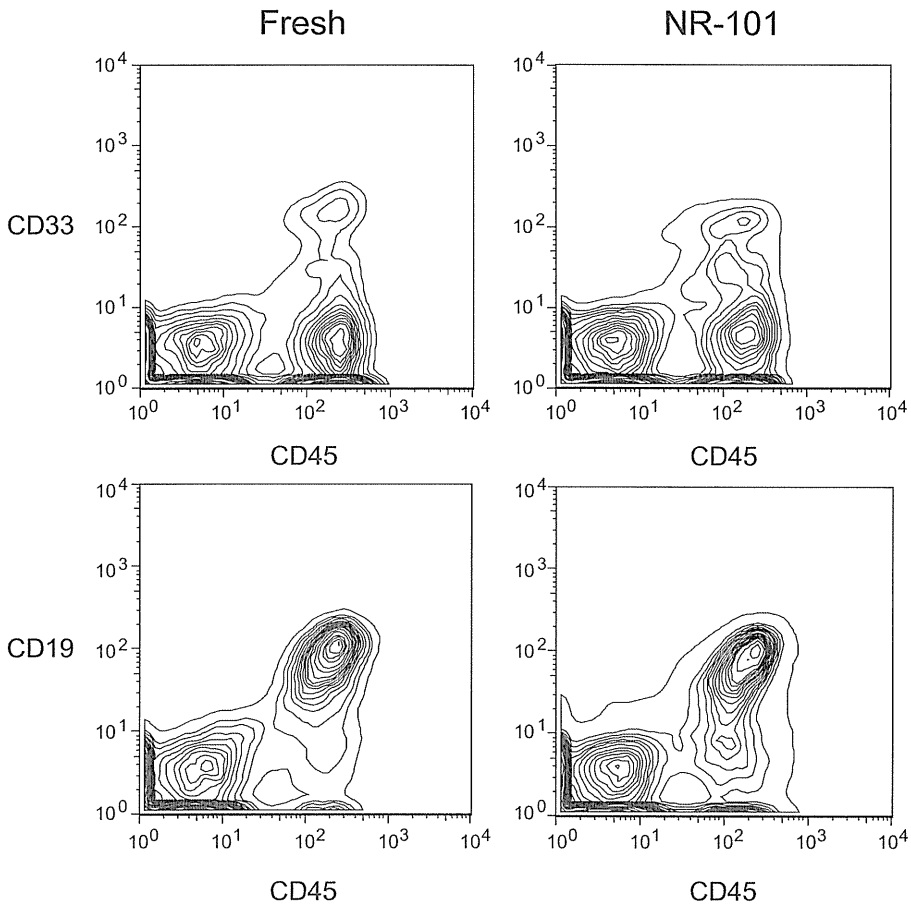
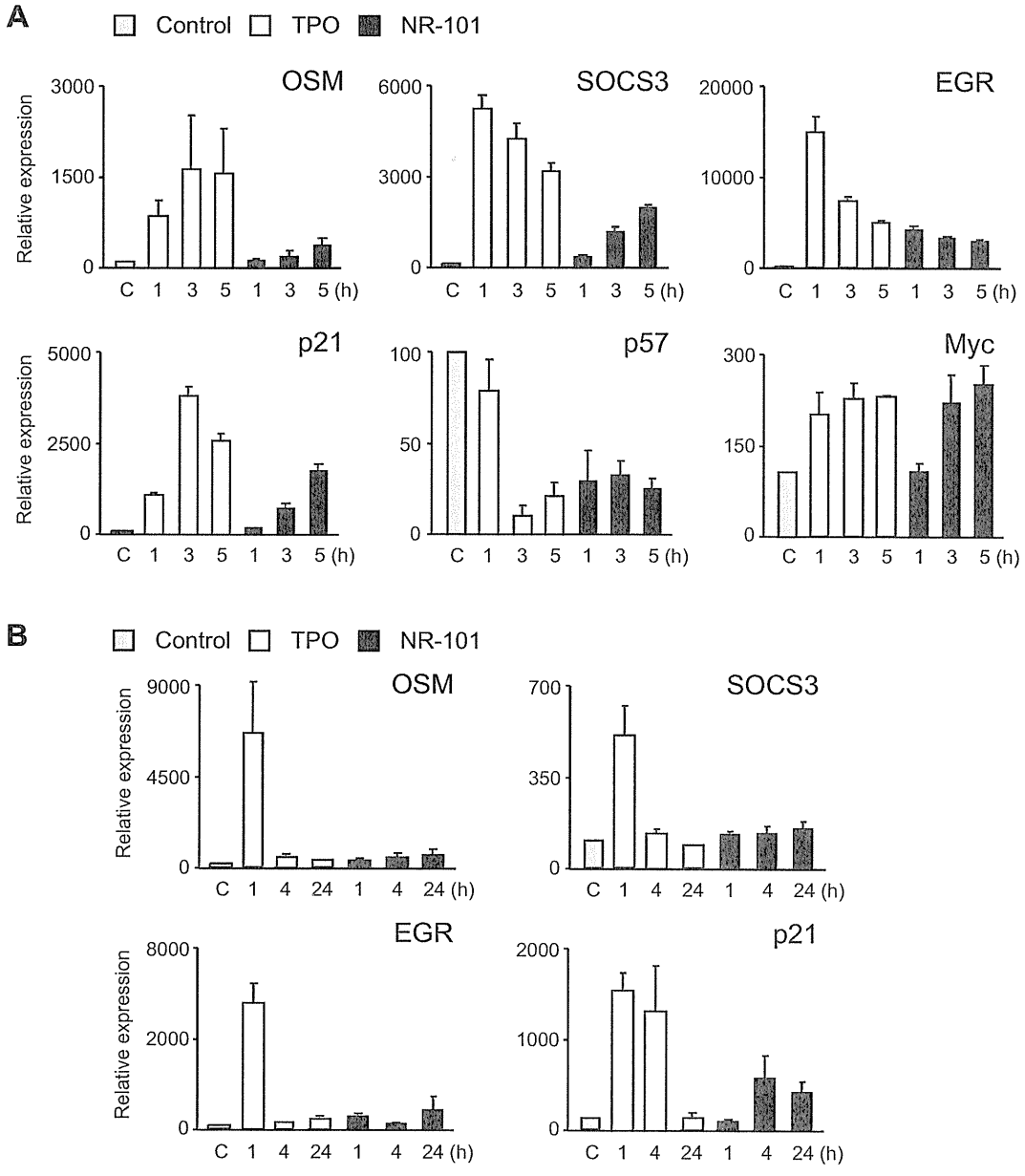
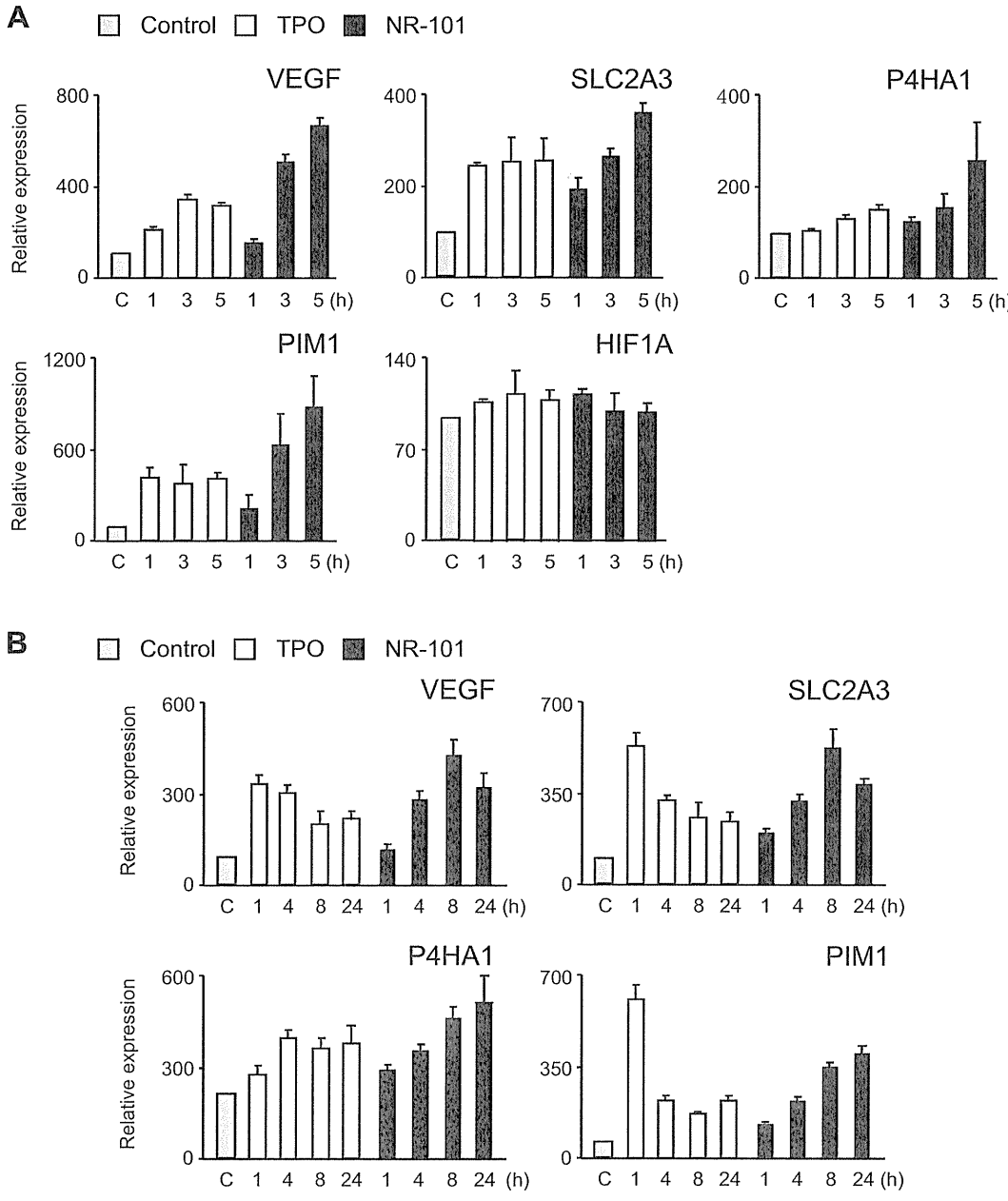


Supplementary Figure E1. NR-101-treated cells repopulate both myeloid-lineage and B cell-lineage cells in immunodeficient mice. Representative flow cytometric dot plots showing the repopulation of human $CD33^+$ myeloid-lineage cells and $CD19^+$ B cell-lineage cells by fresh hCB $CD34^+$ cells and NR-101-treated hCB $CD34^+$ cells in NOD/SCID mouse BM 8 weeks after transplantation.



Supplementary Figure E2. NR-101 enhances the expression of genes downstream of TPO. UT-7/TPO (**A**) or hCB $CD34^+$ (**B**) cells were stimulated with rhTPO or NR-101 for the period of time indicated. Total RNA was then extracted from cultured cells and the relative expression level of genes (*OSM*, *SOCS3*, *EGR*, *p21*, *p57*, and *c-Myc*) was measured by real-time quantitative PCR. 18S ribosomal RNA (18Sr) or beta-2-microglobulin (B2M) was used as an internal control, and a standard curve was used to quantify mRNA. Bars show mRNA levels in rhTPO or NR-101-stimulated cells at the indicated time points relative to those in DMSO (control: C)-treated cells for 1 hour. Data represent the mean ± SEM (n=3-5).



Supplementary Figure E3. NR-101 enhances the expression of genes downstream of HIF-1α. UT-7/TPO (**A**) or hCB CD34$^+$ (**B**) cells were stimulated with rhTPO or NR-101 for the period of time indicated. Total RNA was then extracted from cultured cells and the relative expression level of genes (*VEGF*, *SLC2A3*, *P4HA1*, *PIM1*, and *HIF1A*) was measured by real-time quantitative PCR. 18S ribosomal RNA (18Sr) or beta-2-microglobulin (B2M) was used as an internal control, and a standard curve was used to quantify mRNA. Bars show mRNA levels in rhTPO or NR-101-stimulated cells at the indicated time points relative to those in DMSO (control: C)-treated cells for 1 hour. Data represent the mean ± SEM (n=3–5).