なイベントである。indolent ATL の大多数と p15/p16 変異を有する aggressive ATL は p53 変異を有さない。そこで p53 を生理的に分解する MDM2 の阻害剤である Nutlin-3a の ATL 由来細胞株および HTLV-1 感染細胞株に対する効果を検討した。

エピジェネティックな遺伝子発現とクロマチンのリモデリングに関与するヒストン脱アセチル化酵素（HDAC）の阻害剤は、種々のがん細胞の増殖停止と分化による抗がん剤として期待されている。これまでの HDAC 阻害剤よりも低濃度での抗腫瘍効果が示されつつある Hydroxamic acids 系薬剤の LBH589 の ATL に対する有用性を検証するため、ATL 細胞株とプライマリーATL 細胞を in vitro とマウスで検討した。

（倫理面への配慮）

ヘルシンキ宣言およびヒトゲノム・遺伝子解析研究、疫学研究、臨床試験に関するわが国の倫理指針に従って研究を実施する。

C. 研究結果

1）JSPFAD バンキング

収集した検体は細胞と血漿に分離され、ATL のがん幹細胞と臨床病態との関連を明らかにする研究に用いられるまで冷凍バンキングされた。

2）indolent ATL の長期予後と臨床・分子病態

indolent ATL90 例（慢性型 65 例、くすぶり型 25 例）の生存期間中央値は 4.1 年であり、12 例が 10 年以上生存していた。推定 5 年、10 年、15 年生存割合はそれぞれ 47%、25%、14%であり、生存曲線にプラトーは認めなかった。死因は、約 75%が ATL であり、その他は重複がん、慢性肺疾患、日和見感染症、自己免疫性疾患などであった。予後不良因子は全身状態、好中球数、LDH 値、節外病変数、総病変数であった。一部の症例ではゲノム異常が解析され、染色体異常またはがん抑制遺伝子 p53、p15/p16 異常を有する場合は早期に急性転化し、予後不良であった。

3）MDM2 阻害剤 Nutlin-3a とヒストン脱アセチル化酵素阻害剤 LBH586 による ATL 細胞のアポトーシス誘導

Nutlin-3a は、p53 変異を有する ATL/非 ATL 細胞株に何ら影響を及ぼさなかったが、p53 変異を有さない細胞株には p53 活性化とアポトーシスまたは細胞周期停止をもたらした。p53 ではなく p15/p16 変異を有する ATL 由来細胞株では、SA-beta-gal 染色陽性で同定されるセネッセンスを認めた。このセネッセンスに伴う p53 関連分子の発現変化を調べたところ、Tp53-induced glycolysis and apoptosis regulator (TIGAR)の亢進を認め、その siRNA はアポトーシス/セネッセンスを抑制した。

LBH586 は、nM のレベルで ATL 細胞株と Primary 細胞にアポトーシスを誘導し、SCID マウスに移植した ATL 腫瘤を縮小した。網羅的発現アレイ解析と特異的 siRNA 解析により、このアポトーシスは、ATL での変異が報告されている FAS や p53 経路を介することなく、RAIDD と caspase-2 が重要な役割を担っていることが明らかとなった。

D. 考察

本研究では、ATL のがん幹細胞の特性に着目しつつ、多様な臨床病態をとる本疾患の分子病態に基づいて、候補となる新規治療法を見出すことを目的とし JSPFAD バンキングを継続した。

HTLV-1 キャリアのうち毎年数千人に 1 人が ATL を発症すると考えられるが、我が国に 100 万人以上現存する HTLV-1 キャリアの高齢化に伴い、新規 ATL 患者は増加している。今回の解析では、急性転化するまでは watchful waiting が標準治療とされてきた indolent ATL の長期予後は不良であった。aggressive ATL に対する標準治療とされる同種造血幹細胞移植療法や多剤抗がん剤併用療法は、毒性が高いことから indolent ATL に対する治療法としては推奨されていない。indolent ATL 患者に対しては、欧米では ATL の標準治療の 1 つとして汎用されていて比較的毒性が低いインターフェロンαとジドブジンの併用療法、さらにはより毒性が低いとされる分子標的療法の臨床開発が期待され

る。今回の indolent ATL の解析で予後因子であった節外病変としては、皮膚病変が最多であった。indolent ATL の中から皮膚型 ATL を独立させることも提唱されており、層別化治療の観点からも分子病態を含めて今後の検討課題である。

ATL のがん幹細胞に予想されるゲノム異常とエピジェネティックな異常を標的とした新規治療法として、それぞれ MDM2 阻害剤 Nutlin-3a と HDAC 阻害剤 LBH586 について検討した。両剤ともが、それぞれ特徴的な分子の活性化を伴って ATL 細胞にセネッセンスまたはアポトーシスを誘導したことから、indolent ATL を含む ATL に対する分子標的療法として期待される。

高齢者に多く多段階発がんによる ATL に対する新規治療法の開発は、今後、Indolent ATL の時期からの検討が望まれる。

E. 結論

JSPFAD バンキングを継続した。

急性転化するまでは watchful waiting が標準治療とされてきた indolent ATL の長期予後は不良であった。

MDM2 阻害剤 Nutlin-3a と HDAC 阻害剤 LBH586 は、それぞれ特徴的な分子の活性化を伴って ATL 細胞にセネッセンスまたはアポトーシスを誘導した。

F. 研究発表

1. 論文発表

1) Takasaki Y, Iwanaga M, Imaizumi Y, Tawara M, Joh T, Kohno T, Yamada Y, Kamihira S, Ikeda S, Miyazaki Y, Tomonaga M, Tsukasaki K. Long-term study of indolent adult T-cell leukemia-lymphoma (ATL). Blood, in press.
2) Kamihira S, Terada C, Sasaki D, Yanagihara K, Tsukasaki K, Hasegawa H, Yamada Y. Aberrant p53 protein expression and function in a panel of hematopoietic cell lines with different p53 mutations. Eur J Haematol, 82(4):301-307, 2009
3) Sakai M, Miyazaki Y, Matsuo E, Moriuchi Y, Hata T, Fukushima T, Imaizumi Y, Imanishi D, Taguchi J, Iwanaga M, Tsushima H, Inoue Y, Takasaki Y, Tsuchiya T, Komoda M, Ando K, Horio K, Moriwaki Y, Tominaga S, Itonaga H, Nagai K, Tsukasaki K, Tsutsumi C, Sawayama Y, Yamasaki R, Ogawa D, Kawaguchi Y, Ikeda S, Yoshida S, Onimaru Y, Tawara M, Atogami S, Koida S, Joh T, Yamamura M, Matsuo Y, Soda H, Nonaka H, Jinnai I, Kuriyama K, Tomonaga M. : Long-term efficacy of imatinib in a practical setting is correlated with imatinib trough concentration that is influenced by body size: a report by the Nagasaki CML Study Group. Int J Hematol, 89(3):319-325, 2009
4) Seki R, Ohshima K, Fujisaki T, Uike N, Kawano F, Gondo H, Makino S, Eto T, Moriuchi Y, Taguchi F, Kamimura T, Tsuda H, Ogawa R, Shimoda K, Yamashita K, Suzuki K, Suzushima H, Tsukasaki K, Higuchi M, Utsunomiya A, Iwahashi M, Imamura Y, Tamura K, Suzumiya J, Yoshida M, Abe Y, Matsumoto T, Okamura T. : Prognostic impact of immunohistochemical biomarkers in diffuse large B-cell lymphoma in the rituximab era. Cancer Sci. 100(10):1842-7, 2009
5) Yamada Y, Okada M, Kamitamari A, Moriuchi H, Yanai M, Hano O, Tsukasaki K, Tsuruda K, Hasegawa H, Yanagihara K, Kamihira S.Multiple Immune Abnormalities in a Patient with Idiopathic CD4+ T-Lymphocytopenia. Intern Med 48(22):1967-71,2009
6) Hasegawa H, Yamada Y, Iha H, Tsukasaki K, Nagai K, Atogami S, Sugahara K, Tsuruda K, Ishizaki A, Kamihira S. : Activation of p53 by Nutlin-3a, an antagonist of MDM2, induces apoptosis and cellular senescence in adult T-cell leukemia cells. Leukamia 23(11): 2090-2101,2009

(総説)

1) 塚崎邦弘：【Current Organ Topics】「Hematologic Malignancies/Pediatric Malignancies 血液,リンパ,腫瘍－T 細胞系腫瘍」IV. 成人 T 細胞白血病／リンパ腫の治療.癌と化学療法 36(5):756-761,2009

2) 塚崎邦弘：【特集　悪性リンパ腫の治療の進歩－限局期症例は治療可能か－】悪性リンパ腫の予後因子．Current Therapy. 27(8): 676-680,2009
3) 福島卓也、塚崎邦弘：Ⅳ．成人Ｔ細胞白血病・リンパ腫．（直江知樹編集,現場で役立つ血液腫瘍治療プロトコール集,医薬ジャーナル社,東京,pp91-97 所収）2009.
4) 塚崎邦弘：【シンポジウム Lymphomas in Asia】Lymphoma in Asia:Smoldering ATL の治療戦略．Skin Cancer 24(2):199-205,2009
5) 石塚賢治、塚崎邦弘：Ⅱ．白血病　G.成人病Ｔ細胞白血病／リンパ腫（ATLL）3.ATL に対するインターフェロン＋抗レトロウィルス剤の位置づけは？(金倉　譲,木崎昌弘,鈴木律朗,神田善伸編集,2010-2011 EBM　血液疾患の治療,中外医学社,東京, pp271-274, 所収）2009.
6) 塚崎邦弘:44. 造血・リンパ組織の腫瘍　5. 慢性リンパ性白血病と類縁疾患．(日本臨床腫瘍学会編集,新臨床腫瘍学－がん薬物療法専門医のために－改訂第 2 版,南江堂,東京, pp703-707, 2009.
7) 塚崎邦弘：Ⅲ　悪性リンパ腫－治療の実際－　1．病型別治療方針－標準的治療、研究的治療　E.成人Ｔ細胞白血病／リンパ腫（ATL）．（飛内賢正、堀田知光、木下朝博編集、悪性リンパ腫治療マニュアル　改訂第 3 版、南江堂、東京、pp188-193,所収）2009.
8) 塚崎邦弘：[特集・難治性悪性リンパ腫の治療戦略]5. 成人Ｔ細胞白血病・リンパ腫．血液フロンティア 20(2),187-195,2010

2. 学会発表

(国際学会)

1) Seto Masao, Umino Akira, Utsunomiya Atae, Oshiro Nakagawa Aya, Takeuchi Ichiro, Ohshima Koichi, Tsukasaki Kunihiro, Nakagawa Masao, Nakamura Shigeo：平成 21 年 7 月 1 日：14th International Conference on Human Retrovirology : HTLV and Related Retroviruses : Salvador-Bahia-Brazil : Oral Presentations 「 Molecular Pathogenesis ATLL」: OP-19 Distinct Genome Profiles of Acute and Lymphoma Type ATLL : An Indication for two Subtypes in Acute Type ATLL.
2) Utsunomiya Atae, Yamamoto Kazuhito, Tsukasaki Kunihiro, Tobinai Kensei, Uike Naokuni, Uozumi Kimiharu, Tomonaga Masao, Matsushima Koji, Shitara Kenya, Akinaga Shiro, Ueda Ryuzo：平成 21 年 7 月 1 日(水)- 7 月 4 日(土)： 14th International Conference on Human Retrovirology : HTLV and Related Retroviruses : Salvador -Bahia-Brazil : : Oral Presentations 「 ATLL Therapy 」: A Novel Anti-Tumor Therapeutic Antibody, Kw-0761, Against Adult T-cell Leukemia-Lymphoma (ATL).

(国内学会)

1) 塚崎邦弘：平成 21 年 5 月 23 日：第 25 回日本皮膚悪性腫瘍学会学術集会：岡山コンベンションセンター：シンポジウム３　SY3-2 smoldering ATL の治療戦略.
2) 塚崎邦弘：平成 21 年 7 月 10 日：第 49 回日本リンパ網内系学会総会：淡路夢舞台国際会議場：シンポジウム 2「ATL と HTLV－１研究の最前線」－演題 1 ：ATL の臨床分子病態, 治療戦略.
3) 今泉芳孝、今西大介、田口潤、福島卓也、波多智子、宮﨑泰司、塚崎邦弘、朝長万左男、小川大輔、新野大介、大島孝一：平成 21 年 7 月 11 日：第 49 回日本リンパ網内系学会総会：淡路夢舞台国際会議場：ポスターセッション：リンパ節病変にクリプトコッカス感染症を伴った成人Ｔ細胞白血病リンパ腫.
4) 長谷川寛雄、佐々木大介、塚崎邦弘、山田恭暉、上平憲：平成 21 年 8 月 1 日(土)：第 18 回日本アポトーシス研究会学術集会(日本 Cell Death 学会設立記念学術集会)：長崎大学医学部「良順会館」：白血病細胞株における MDM2 阻害剤 Multin による p53 活性化と細胞死決定のメカニズム.
5) 塚崎邦弘：平成 21 年 8 月 29 日(土)：第 2 回 HTLV-1 研究会・合同班会議：東京大学医科学研究所・大講堂（東京都港区白金台

4-6-1)：ｷｰﾉｰﾄﾚｸﾁｬｰ：ATL 治療の現状.

6) 海野　啓、中川雅夫、片山直之、宇都宮與、塚崎邦弘、瀬戸加大：平成 21 年 8 月 30 日(日)：第 2 回 HTLV-1 研究会・合同班会議：東京大学医科学研究所・大講堂：一般演題口演：急性型 ATLL における多クローンの証明と末梢血腫瘍細胞のクローンの解析.

7) 長谷川寛雄、山田恭暉、塚崎邦弘、森　直樹、朝長万左男、上平　憲：平成 21 年 8 月 30 日：第 2 回 HTLV-1 研究会・合同班会議：東京大学医科学研究所・大講堂：口演：ATL 細胞に対する新規 HDAC 阻害剤 LBH589 の効果. 会・合同班会議ﾌﾟﾛｸﾞﾗﾑ p22,2009

8) 池田柊一、種岡　新、塚崎邦弘、今泉芳孝：平成 21 年 8 月 29 日(土)・30 日(日)：第 2 回 HTLV-1 研究会・合同班会議：東京大学医科学研究所・大講堂：ポスターディスカッション：蔓延地区における HTLV-1 キャリア率の年代変化.

9) Tsukasaki Kunihiro：平成 21 年 10 月 23 日(金)～25 日（日）：第 71 回日本血液学会学術集会：京都国際会議場：発表：23 日（金）・1 日目：ｼﾝﾎﾟｼﾞｳﾑ 3／13:30-16:10：SY3-3 International consensus on the management of ATL.

10) Hasegawa Hiroo, Yamada Yasuaki, Tsukasaki Kunihiro, Mori Naoki, Sasaki Daisuke, Usui Tetsuya, Atogami Sunao, Ishikawa Chie, Machijima Yoshiaki, Sawada Shigeki, Tomonaga Masao, Kamihira Shimeru：平成 21 年 10 月 23 日：第 71 回日本血液学会学術集会：京都国際会議場：一般口演：LBH589, a deacetylase enzymes inhibitor, induces apoptosis in adult T-cell leukemia/lymphoma cells.

11) 糸永英弘、宮﨑泰司、田口　潤、今泉芳孝、福島卓也、波多智子、塚崎邦弘、朝長万左男：平成 21 年 10 月 23 日：第 71 回日本血液学会学術集会：京都国際会議場：一般ポスター：同種造血幹細胞移植後の ATL 及び AML,ALL 症例で生じた CMV 抗原血症の後方視的比較検討.

12) 佐々木大介、今泉芳孝、山田恭暉、尾坂明美、長谷川寛雄、塚崎邦弘、朝長万左男、鶴田一人、柳原克紀、上平　憲：平成 21 年 10 月 24 日：第 71 回日本血液学会学術集会：京都国際会議場：一般ポスター：成人 T 細胞白血病・リンパ腫（ATLL）における EZH2 高発現の病的意義.

G. 知的所有権の出願・取得状況

1. 特許取得　なし

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
石塚賢治、塚崎邦弘	ATLに対するインターフェロン＋抗レトロウィルス剤の位置づけは？	金倉　譲，木崎昌弘，鈴木律朗，神田善伸	2010-2011 EBM　血液疾患の治療	中外医学社	東京	2009	271-274
塚崎邦弘	44．造血・リンパ組織の腫瘍　5．慢性リンパ性白血病と類縁疾患	日本臨床腫瘍学会	新臨床腫瘍学－がん薬物療法専門医のために－改訂第2版	南江堂	東京	2009	703-707
塚崎邦弘	Ⅲ　悪性リンパ腫－治療の実際－　1．病型別治療方針－標準的治療、研究的治療　E.成人T細胞白血病／リンパ腫（ATL）	飛内賢正、堀田知光、木下朝博	悪性リンパ腫治療マニュアル　改訂第 3版	南江堂	東京	2009	188-193

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Iwanaga M, Watanabe T, Utsunomiya A, Okayama A, Uchimaru K, Koh K-R, Ogata M, Kikuchi H, Sagara Y, Uozumi K, Mochizuki M, Tsukasaki K, Saburi Y, Yamamura M, Tanaka J, Moriuchi Y, Hino S, Kamihira S, Yamaguchi K, for the Joint Study on Predisposing Factors of ATL Development (JSPFAD) investigators	Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan	***Blood***	in press		2010
Sasaki D, Doi Y, Hasegawa H, Yanagihara K, Tsukasaki K, Iwanaga M, Yamada Y, Watanabe T, Kamihira S.	High Human T Cell Leukemia Virus Type-1 (HTLV-1) provirus load in patients with HTLV-1 carriers complicated with HTLV-1-unrelated disorders.	***Virol J*** (E-journal)	in press		2010
Masuda M, Ohta T, Maruyama T, Ito A, Hayashi T, Tsukasaki K, Kamihira S, Yamaoka S, Hoshino H, Yoshida T, Watanabe T, Stanbridge E-J, Murakam Y.	Cadm1 interacts with tiam1 and promotes invasive phenotype of Human T-cell Leukemia Virus type I (HTLV-1) transformed cells and Adult T-cell Leukemia (ATL) cells : possible involvement of Cadm1 in pathogenesis of ATL.	***J Biol Chem***	in press		2010
Misawa A, Katayama R, Koike S, Tomida A, Watanabe T, Fujita N	AP-1-dependent miR-21 expression contributes to chemoresistance in cancer stem cell-like SP cells	***Oncology Research***	in press		2010

Takasaki Y, Iwanaga M, Imaizumi Y, Tawara M, Joh T, Kohno T, Yamada Y, Kamihira S, Ikeda S, Miyazaki Y, Tomonaga M, Tsukasaki K	Long-term study of indolent adult T-cell leukemia-lymphoma (ATL)	***Blood***	in press		2010
Nishino T, Miyaji K, Ishiwata N, Arai K, Yui M, Asai Y, Nakauchi H, Iwama A	Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL	***Exp Hemato***	37	1364-1377	2009
Okabe M, Otsu M, Ahn DH, Kobayashi T, Wakiyama Y, Morita Y, Onodera M, Eto K, Ema H, Nakauchi H	Definitive proof for direct reprogramming of hematopoietic cells to pluripotency	***Blood***	114	1764-1767	2009
Matsumoto K, Isagawa T, Nishimura T, Ogaeri T, Eto K, Miyazaki S, Miyazaki J, Aburatani H, Nakauchi H, Ema H	Stepwise development of hematopoietic stem cells from embryonic stem cells	***PLoS ONE***	4(3)	e4820	2009
Sogo T, Kawahara M, Ueda H, Otsu M, Onodera M, Nakauchi H, Nagamune T	T cell growth control using hapten-specific antibody/interleukin-2 receptor chimera	***Cytokine***	46	127-136	2009
Yashiro Y, Bannai H, Minowa T, Yabiku T, Miyano S, Osawa M, Iwama A, Nakauchi H	Transcriptional profiling of hematopoietic stem cells by high-throughput sequencing	***Int J Hematol***	89	24-33	2009

Yamazaki J, Mizukami T, Takizawa K, Kuramitsu M, Momose H, Masumi A, Ami Y, Hasegawa H, Hall W-W, Tsujimoto H, Hamaguchi I, Yamaguchi Y	Identification of cancer stem cells in a Tax- transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma	***Blood***	114 (13)	2709-2720	2009
Kawaguchi A, Orba Y, Kimura T, Iha H, Ogata M, Tsuji T, Ainai A, Sata T, Okamoto T, Hall W.W, Sawa H, Hasegawa H *	Inhibition of the SDF-1α-CXCR4 axis by the CXCR4 antagonist AMD3100 suppresses the migration of cultured cells from ATL patients and murine lymphoblastoid cells from HTLV-I Tax transgenic mice	***Blood***	114 (14)	2961-2968	2009
Kato M, Sanada M, Kato I, Sato Y, Takita J, Takeuchi K, Niwa A, Chen Y, Nakazaki K, Nomoto J, Asakura Y, Muto S, Tamura A, Iio M, Akatsuka Y, Hayashi Y, Mori H, Igarashi T, Kurokawa M, Chiba S, Mori S, Ishikawa Y, Okamoto K, Tobinai K, Nakagama H, Nakahata T, Yoshino T, Kobayashi Y, Ogawa S	Frequent inactivation of A20 in B-cell lymphomas	***Nature***	459	712-716	2009

Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, Tamura A, Honda H, Sakata-Yanagimoto M, Kumano K, Oda H, Yamagata T, Takita J, Gotoh N, Nakazaki K, Kawamata N, Onodera M, Nobuyoshi M, Hayashi Y, Harada H, Kurokawa M, Chiba S, Mori H, Ozawa K, Omine M, Hirai H, Nakauchi H, Koeffler HP, Ogawa S	Gain- of-function of mutated C-CBL tumor suppressor in myeloid neoplasms	***Nature***	460	904-908	2009
Kamihira S, Terada C, Sasaki D, Yanagihara K, Tsukasaki K, Hasegawa H, Yamada Y.	Aberrant p53 protein expression and function in a panel of hematopoietic cell lines with different p53 mutations	***Eur J Haematol,***	82	301-307	2009
Hasegawa H, Yamada Y, Iha H, Tsukasaki K, Nagai K, Atogami S, Sugahara K, Tsuruda K, Ishizaki A, Kamihira S	Activation of p53 by Nutlin-3a, an antagonist of MDM2, induces apoptosis and cellular senescence in adult T-cell leukemia cells	***Leukemia***	23	2090-2101	2009

IV. 研究成果の刊行物・別刷

ELSEVIER

Experimental Hematology

Experimental Hematology 2009;37:1364–1377

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

Taito Nishino[a], Katsuaki Miyaji[b], Norihisa Ishiwata[c], Kazutaka Arai[a], Makiko Yui[d,e], Yasuyuki Asai[d], Hiromitsu Nakauchi[f], and Atsushi Iwama[e]

[a]*Research Promotion and Coordination Department, Nissan Chemical Industries, Tokyo, Japan;* [b]*Synthesis Research Department, Chemical Research Laboratories, Nissan Chemical Industries, Chiba, Japan;* [c]*Pharmaceutical Research Department, Biological Research Laboratories, Nissan Chemical Industries, Saitama, Japan;* [d]*ReproCELL Inc., Tokyo, Japan;* [e]*Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan;* [f]*Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Japan*

(Received 13 July 2009; revised 31 August 2009; accepted 2 September 2009)

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

Offprint requests to: Atsushi Iwama, M.D., Ph.D., Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8670 Japan; E-mail: aiwama@faculty.chiba-u.jp

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exphem.2009.09.001

0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.09.001

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,

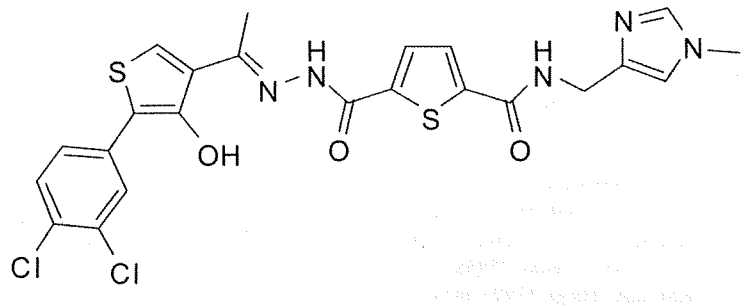

Figure 1. Chemical structure of NR-101, a small-molecule c-MPL agonist.

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 (Hs00231119_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 (Hs00855332_g1), PGK1 (Hs99999906_m1), HK1 (Hs00175976_m1), SLC2A1 (Hs00197884_m1), EPOR (Hs00181092_m1), NOS2A (Hs00167257_m1), SERPINE1 (Hs01126606_m1), ALDOA (Hs00605108_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.

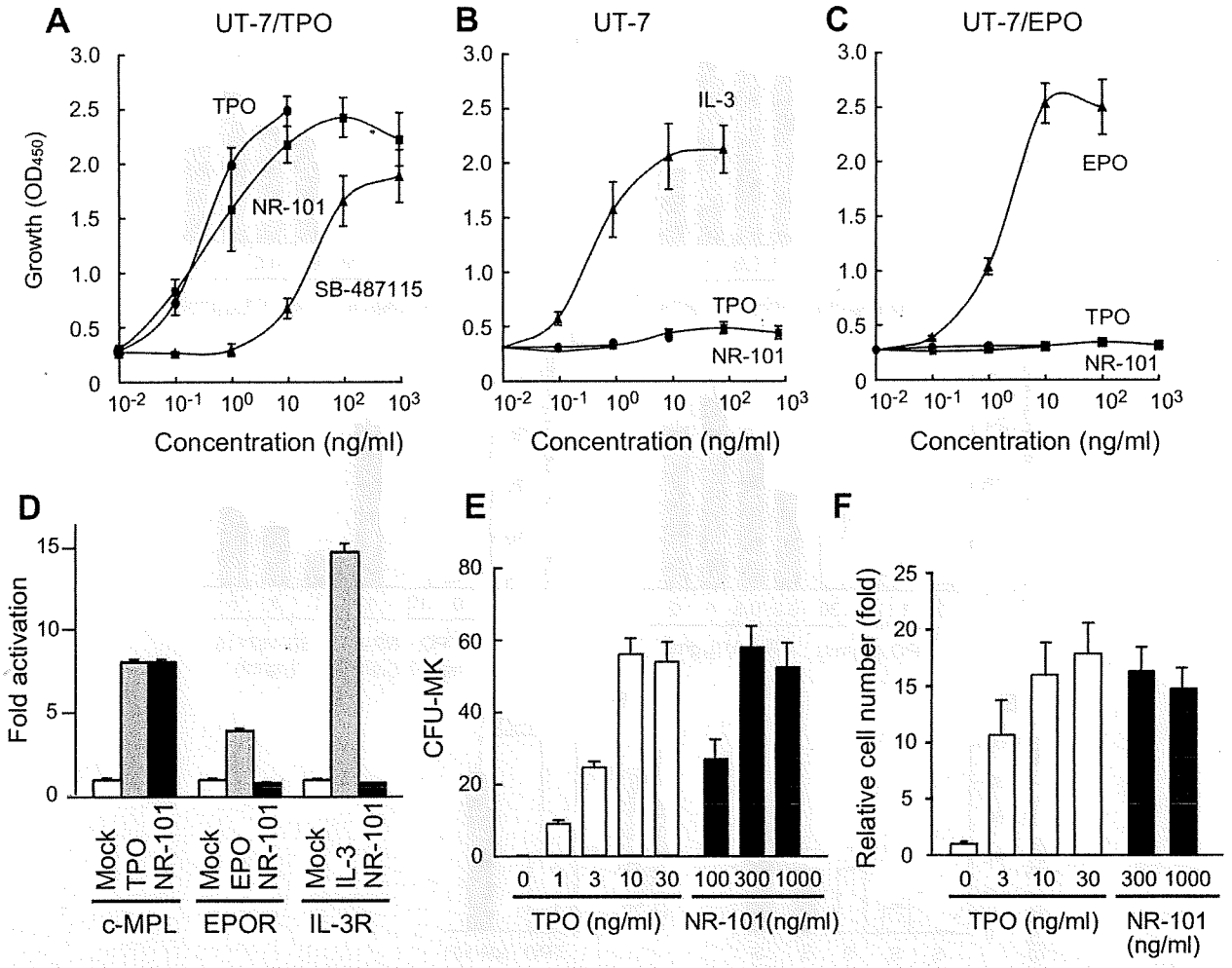

Figure 2. NR-101 specifically activates human c-MPL. Dose-dependent proliferation of UT-7/TPO cells (**A**), UT-7 cells (**B**), and UT-7/EPO cells (**C**) by recombinant human (rh) TPO, rh interleukin (IL)-3, rhEPO, NR-101, and SB-497115. (**D**) signal transducer and activator of transcription (STAT) 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

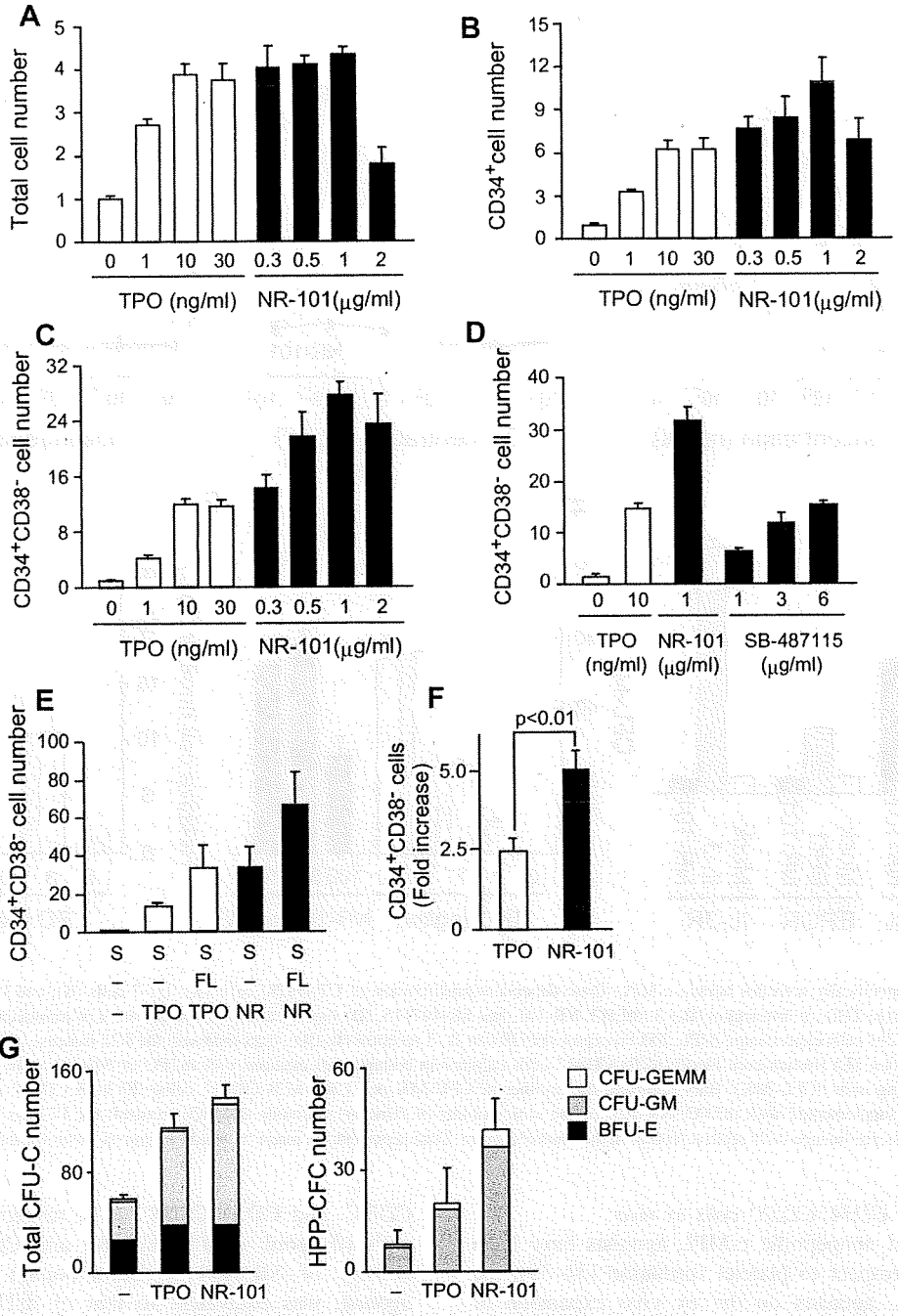

Figure 3. NR-101 expands human $CD34^+CD38^-$ cell numbers more efficiently than thrombopoietin (TPO). Human cord blood (hCB) $CD34^+$ cells were cultured with recombinant human (rh) TPO, NR-101, SB-497115, or the same volume of dimethyl sulfoxide (DMSO) (control) in the presence of rh stem cell factor (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.

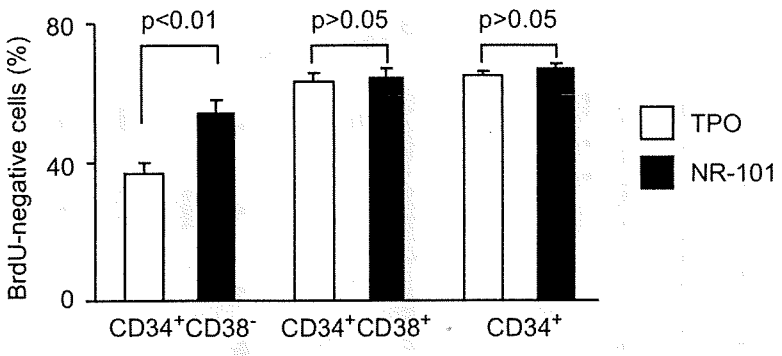

Figure 4. $CD34^+CD38^-$ cells treated with NR-101 show a decelerated cell cycle. Human cord blood (hCB) $CD34^+$ cells were cultured with 10 ng/mL recombinant human thrombopoietin (rhTPO) or 1 μg/mL of NR-101 in the presence of recombinant human stem cell factor (rhSCF) for 7 days. The bromodeoxyuridine (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.

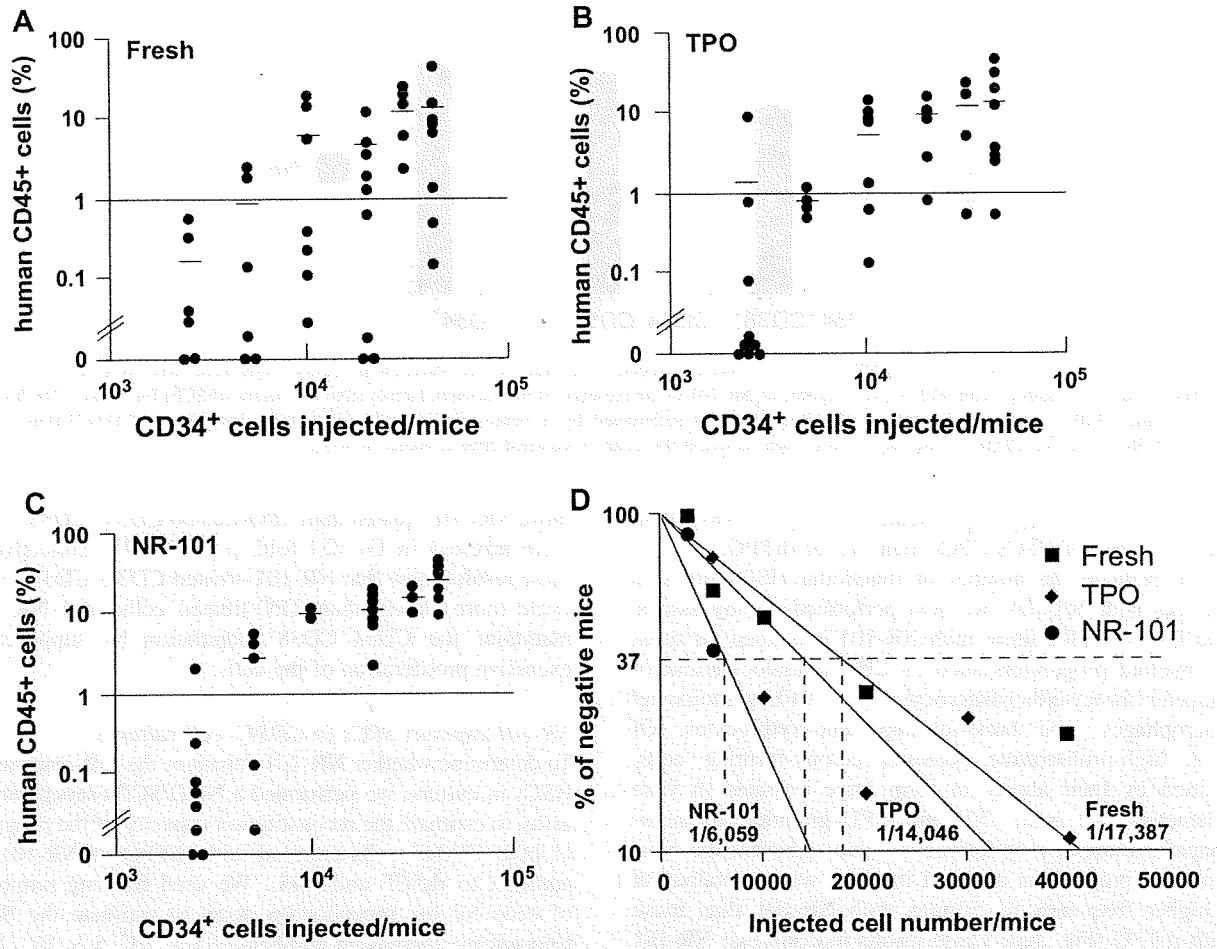

Figure 5. NR-101-treated human cord blood (hCB) $CD34^+$ cells show an increase in the frequency of severe combined immunodeficiency-repopulating cells (SRCs). hCB $CD34^+$ cells were cultured with 10 ng/mL of recombinant human thrombopoietin (rhTPO) or 1 μg/mL NR-101 in the presence of recombinant human 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

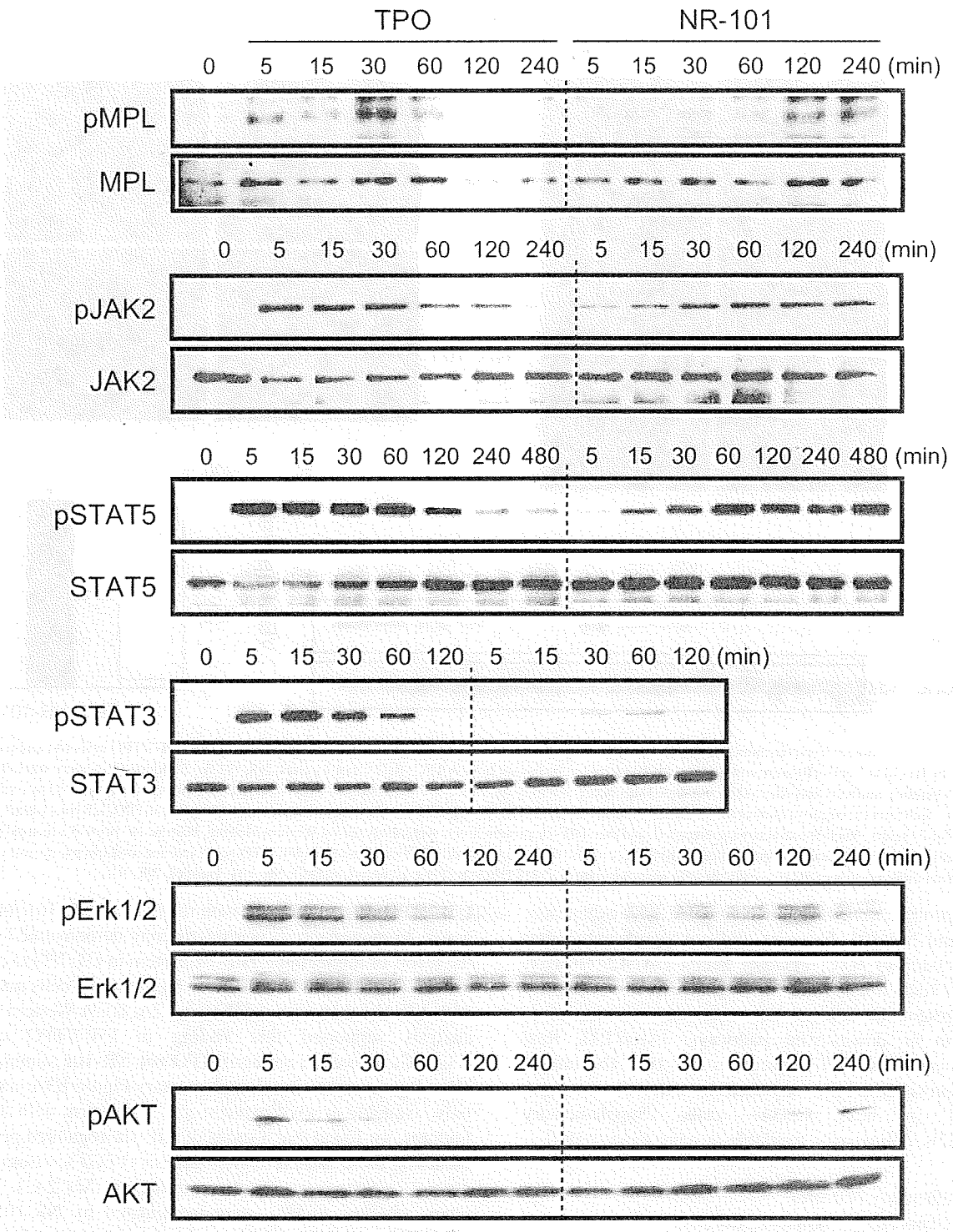

Figure 6. NR-101 shows characteristic signal transduction as compared to thrombopoietin (TPO). UT-7/TPO cells were stimulated with 10 ng/mL recombinant human (rhTPO) (left panels) or 25 μg/mL NR-101 (right panels) for the periods indicated. c-MPL, Janus kinase (JAK) 2, signal transducer and activator 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.

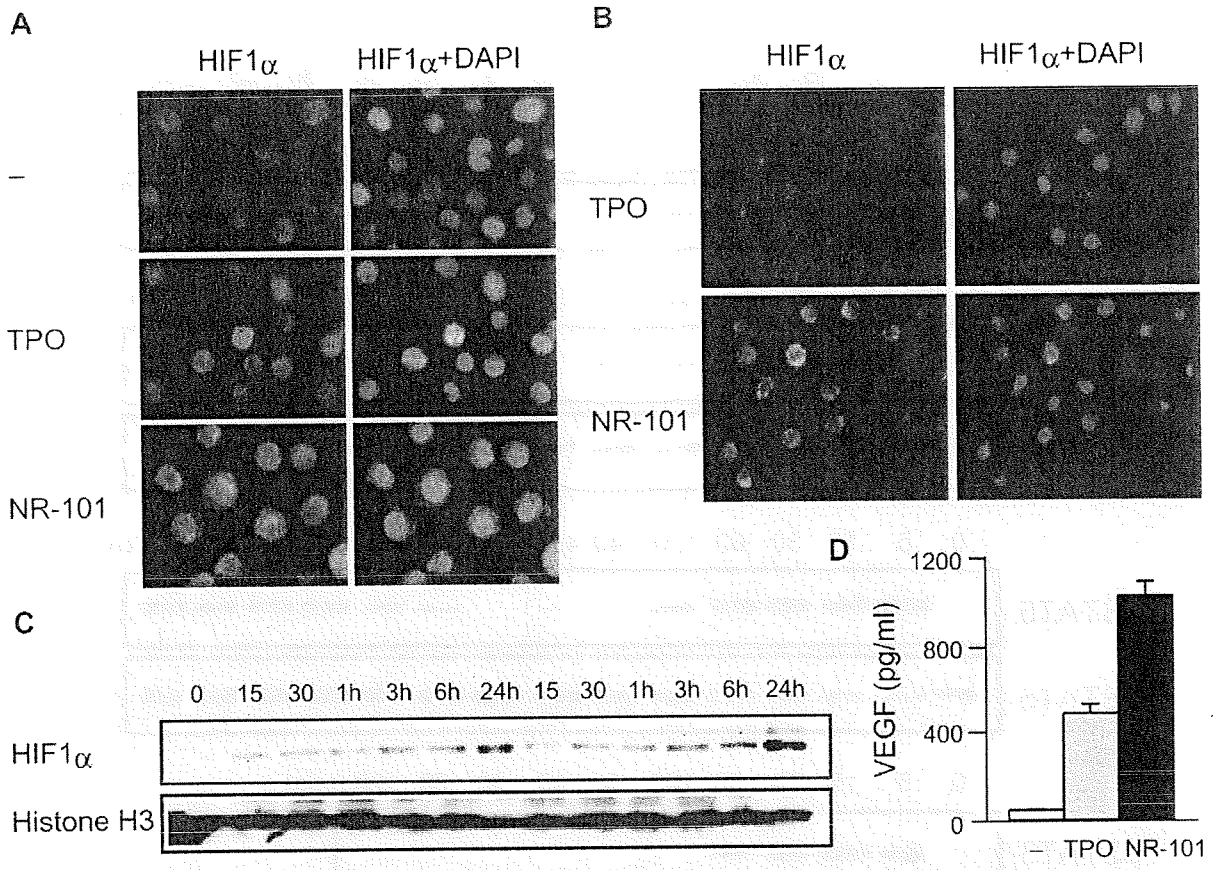

Figure 7. NR-101 stabilizes hypoxia-inducible factor-1α (HIF-1α) protein more efficiently than does thrombopoietin (TPO). UT-7/TPO cells (**A**) and human cord blood (hCB) $CD34^+$ cells (**B**) were stimulated with 10 ng/mL recombinant human TPO (rhTPO) or 1 μg/mL NR-101 for 24 hours. Cells were stained 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