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造血器悪性腫瘍及び転移性がんで高頻度に異常を来している遺伝子を標的とした新たな治療法の開発に資する研究

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研究代表者　北林　一生

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別紙３

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総合研究報告書

造血器悪性腫瘍及び転移性がんで高頻度に異常を来している遺伝子を標的とした新たな治療法の開発に資する研究

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研究要旨　急性骨髄性白血病(AML)幹細胞に特異的に高発現する表面抗原 Tim-3 および M-CSFR を同定した。Tim-3 および M-CSFR は大多数の AML 幹細胞に高発現しているが、正常造血幹細胞に発現を認めず、理想的な標的候補と考えられた。M-CSF 受容体特異的チロシンキナーゼ阻害剤が発症を抑制することを見出した。Tim-3 陽性細胞株に殺細胞効果を有する抗 Tim-3 抗体を作成し、AML を再構築させたマウスで有効性を検証している。TIM-3 陽性細胞株に対して高い殺細胞効果を有する抗ヒト Tim-3 マウス抗体の作製に成功した。TIM-3 の機能としては AML 細胞自体がリガンドである galectin-9 を autocrine 様式で分泌、作用させていることを見出した。シグナル阻害剤の開発として企業と共同研究中の抗腫瘍薬 OPB-31121 が JAK/Src キナーゼ活性を阻害する事なく STAT3、STAT5 のリン酸化を阻害すること、種々の造血器悪性腫瘍細胞株に対し増殖抑制効果が高いが、正常造血細胞に対する増殖抑制作用は認められないことを細胞株、ヒトプライマリ白血病細胞およびヒト正常臍帯血を移植したマウスモデルを用いて発見した。亜ヒ酸の新たな治療標的の検証のため PAX5-PML 陽性白血病のマウスモデルを作成し、PML 機能の障害の有無、亜ヒ酸の効果について検討した。腫瘍の進展過程でチロシンリン酸化を受ける蛋白質を精力的に解析してきており、実際に転移浸潤にかかわるいくつかの分子の機能を明らかにした。胃がん細胞からは新たに腹膜播種を抑制する ARAP3 を見出して、チロシンリン酸化がその機能に必要であることを明らかにした。また固形腫瘍の足場非依存性と転移性に関わる膜蛋白質 CDCP1 は予後不良群で発現が高いことを見出していたが、その発現制御に Ras-Erk 経路が関わること、足場非依存性の獲得の際にオートファジーの抑制を伴うことなどの重要な知見を得た。CDCP-1 とそのエフェクター分子 PKCδ の結合を阻害するペプチドを設計し、腫瘍細胞の足場非依存性や造腫瘍能を抑制することを示した。

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Ａ．研究目的

近年の診断技術や治療法の進歩により多くのがんで生存率が上昇したが、難治性がんに対しては長期生存率に大きな改善が見られていない。これは、このような難治性がんでは再発や転移が頻繁に生じることが最大の原因である。再発の主な要因は治療耐性を示すがん幹細胞が残存するためであると考えられ、また、転移・浸潤能の獲得にチロシンリン酸化シグナルが関与することが示されている。本研究では、特に再発や転移に深く関与するがん幹細胞やチロシンリン酸化シグナルの制御に関わる分子を標的とした造血器腫瘍や転移性がんに対する新たな治療法の開発を目指す。

重症免疫不全マウスによる異種移植の生着効率改善とマルチカラー・フローサイトメトリー(FACS)を用いた細胞純化技術の進歩により、継代してマウスにヒト急性骨髄性白血病(AML)を再構築させる機能的な AML 幹細胞分画の純化が可能となった。この AML の源である AML 幹細胞のみを治療標的として選択的に死滅させることが可能となれば、骨髄抑制や重症感染症など致死的な副作用を伴わない究極の治療法となり得る。しかしながら、AML 幹細胞は正常の造血幹細胞と細胞特性や表面抗原が極めて酷似しているため、この２つの幹

細胞を鮮明に分離する標的分子が同定されていない時点で、正常造血機構に影響を与えずに、AML 幹細胞を標的とした治療法の開発は困難であった。AML 幹細胞にのみ特異的に発現する表面抗原や機能分子が同定可能となれば、白血病幹細胞を直接標的とした新たな治療法の開発が可能となる。

STAT3 及び STAT5 は細胞内の多くの細胞増殖シグナル経路で共用される主要なシグナル伝達因子であり、多彩な腫瘍で恒常的活性化が認められている。STAT 阻害剤は有用な抗腫瘍薬となりうると予想されるが、臨床治験まで進んだ薬剤はほとんどない。大塚製薬より抗腫瘍薬候補物質としてスクリーニングされてきた新規低分子化合物 OPB-31121 は細胞株で強い STAT3 リン酸化抑制効果と多彩な腫瘍細胞株に対し強い抗腫瘍効果を持つ事、また動物実験で臓器障害など示さず高い安全性が期待できる化合物であったが、STAT リン酸化阻害の機序、本剤による治療対象とする適合癌種、ヒトプライマリ腫瘍細胞に対する効果などが不明の状態であった。本研究では本薬剤の作用機序を明らかにし、proof of concept を確立する事、造血器腫瘍における本薬剤の適合癌種を明らかにし、プライマリ白血病細胞に対する効果を確認し、日本発の抗癌剤開発に寄与する事を目的として研究を行った。亜ヒ酸は急性前骨髄性白血病(APL)の強力な治療薬で APL の原因遺伝子である PML-RARα により破壊された PML nuclear body (PML NB)の再構成を誘導する事で APL 細胞にアポトーシスをもたらすと考えられているが、その作用機序には不明な点が多い。我々は細胞株に PAX5-PML 発現ベクターを導入する事で PAX5-PML が PML NB を破壊する事、亜ヒ酸が破壊された PML NB の再構成をもたらす事、及び PAX5-PML が PAX5 の転写活性化能を阻害する事を示してきた。今回は PAX5-PML を正常造血細胞に導入する事で白血病を発症させられるかを試みる事で PAX5-PML の発癌性を検証すると共に、得られたマウスモデルを用いて、PML NB の破壊が白血病化に関与するのか、破壊された PML NB を亜ヒ酸で再構成させる事が白血病細胞にアポトーシスを誘導する事につながるのかを検証する事で、PML NB の機能を明らかにし、亜ヒ酸による治療の対象として PML-RARα 陽性でなくとも PML 機能が障害されている腫瘍であればその対象となりうるのかを検証した。

腫瘍の増殖異常や転移浸潤能に関わるチロシンリン酸化シグナルを明らかにして、それを媒介するチロシンキナーゼの基質分子の腫瘍における機能の詳細を明らかにするとともに、これらの分子のシグナルをブロックする系を確立する。このシグナルのブロックが in vitro の培養細胞系と in vivo のマウス転移モデルにおいて癌細胞に与える影響を解析することにより、腫瘍特異的な効果をもたらす分子標的治療のモデルを開発する。

B．研究方法

マルチカラー・フローサイトメトリーを用いて，正常ヒト造血幹細胞および前駆細胞の純化方法を確立した。この手法により高純度で単離された正常造血幹細胞および AML 幹細胞($CD34^+CD38^-Lin^-$分画)を用いて、microarray により AML 幹細胞に特異的に高発現する分子を網羅的に探索した。その結果、正常造血幹細胞と比較して AML 幹細胞に約 13 倍高発現する細胞表面抗原 TIM-3 を同定した。FACS による解析では、TIM-3 は正常造血幹細胞には発現していないが、AML 幹細胞に高発現していた。AML 幹細胞の特異的表面抗原と報告されている CD123 や CD33 が正常造血幹細胞においても弱陽性であるのに対し、TIM-3 は AML 幹細胞にのみ発現していることから、理想的な抗体治療の標的分子候補と考えられた。そこで、高い細胞傷害活性を有するヒト TIM-3 に対するマウスモノクローナル抗体を樹立し治療モデルを作成した。さらに TIM-3 の白血病幹細胞における機能解析を行った。このために、TIM-3 リガンドである galectin-9 に着目して研究を進めた。

細胞株を用いて OPB-31121 を添加後の STAT3/5 を含む各種シグナル伝達因子のリン酸化状態の変化を経時的にウエスタンブロットで確認した。種々の造血器悪性腫瘍細胞株に対して本薬剤増殖抑制効果を検討し IC_{50} を算出し比較した。ヒトプライマリ白血病細胞及びヒト正常臍帯血を重度免疫不全マウスである NOG マウスに移植しヒト白血病、あるいはヒト正常造血モデルマウスを作成した。これに対し本薬剤を使用し、プライマリ白血病細胞及び正常造血細胞に対する効果、副作用を検討した。マウス胎児肝臓細胞から pro B 細胞分画を収集し、これに PAX5-PML 発現ベクターをレトロウイルスを用いて導入し、この細胞を放射線照射したマウスに移植し、白血病の発症を観察した。白血病を発症したマウスの白血病細胞を免疫染色して PML NB の破壊を観察し、マウスに亜ヒ酸を投与し、PML NB の変化、腫瘍細胞の減少、マウスの

生存延長の有無を観察した。マウスの白血病細胞より mRNA を抽出し PAX5 転写活性化標的遺伝子の発現を正常 B 細胞と比較し、マイクロアレイで遺伝子発現プロファイルを解析し、これを正常 B 細胞と比較した。

固形腫瘍の転移浸潤に関わるチロシンキナーゼの基質分子について、これまでの解析で見出した Cas、CDCP1、Ossa などの標的分子としての有用性の評価を進めるとともに、解析の進んでいない他の基質分子についても転移能などとの関わりで機能解析を進めた。ヒト腫瘍組織や腫瘍細胞株を用い、これらの基質分子の腫瘍における発現上昇やリン酸化の亢進が、どのような遺伝子変化や活性化シグナルの獲得に伴って誘導されるかを明らかにし、分子標的治療の有効なサブグループを絞り込む。またこのような転移性がんでリン酸化の亢進する分子群の細胞内シグナルをブロックする系を樹立した。特にリン酸化した CDCP1 が PKCδと結合することにより足場非依存性やプロテアーゼ分泌など転移・浸潤に関わる性質をもたらすことを in vitro の系で明らかにしたので、CDCP1 と PKCδのリン酸化依存的結合を阻害する膜透過性ペプチドを用いて CDCP1 のシグナルを抑える系を樹立し、マウスの in vivo モデルで固形腫瘍の転移に対する効果を評価した。

C．研究結果

平成２２年度から平成２３年度にかけては、白血病幹細胞特異的表面抗原としての TIM-3 の同定と、強力な細胞傷害活性を有するモノクローナル抗体の樹立および抗体を用いたヒト AML 治療モデルの確立を行った。平成２４年度は、TIM-3 の AML 以外のヒト造血器腫瘍、特に慢性骨髄増殖性疾患における $CD34^{+}CD38^{-}$幹細胞分画における発現を中心に検討した。この結果、慢性骨髄増殖性疾患の造血幹細胞分画内にも AML における白血病幹細胞同様の TIM-3 陽性の異常表面形質を有する幹細胞が存在し、疾患の進展とともにその割合が増加することを見出した。さらに、平成２５年度は TIM-3 の白血病幹細胞における機能解析を中心に行い、AML 細胞自体が TIM-3 リガンドである galectin-9 を autocrine 様式にて分泌し、TIM-3 を介したシグナルを生じ、NF-kB を含むいくつかの転写因子群を動かしていることを見出した。

OPB-31121 添加後の経時的なウエスタンブロットによる検討では、HEL 細胞株（JAK2 に恒常的活性化変異があり、これにより STAT3、STAT5 が恒常的にリン酸化されている）では薬剤添加後 JAK2 のリン酸化に変化のない時点から STAT3、STAT5 のリン酸化が消失し、本薬剤の作用機序はキナーゼ阻害ではないと考えられた。35 種類の造血器腫瘍細胞株で本薬剤の感受性を検討した所、多発性骨髄腫（3 株中 3 株）、バーキットリンパ腫（3 株中 3 株）における IC_{50} は 10 nM 以下で感受性が高かった。白血病の細胞株では感受性にバラツキがあったが、BCR-ABL、FLT3 変異、JAK2 変異を持つ細胞株では 7 株全てで IC_{50} が 100nM 以下と感受性が高く、それ以外の細胞株では IC_{50} が 100nM 以上の非感受性株を５株（12 株中）認める等感受性は一定しなかった。上記の変異はそれが引き起こす細胞増殖シグナルに STAT3/5 の活性化が重要であることがこれまでの多くの報告で確立されている oncokinase であり、我々はこれを STAT adictive oncokinase (SAO) と名付け、SAO 陽性白血病は本薬剤の適合癌種と考えられた。SAO 陽性白血病のヒト白血病細胞マウスモデルを作成した。AML（FLT3/ITD 陽性） 1 例、ALL（BCR-ABL 陽性）3 例、CML 1 例である。これらに対し OPB-31121 を経口投与し、治療群では腫瘍細胞割合がコントロール群に対し 4～58％に減少していた。OPB-31121 投与群では腫瘍細胞が減少するだけでなく、マウスの正常造血細胞が増加して骨髄の造血細胞の総数が治療群とコントロール群でほとんど変化が無くなっており、OPB-31121 の増殖抑制が腫瘍特異的である事を示唆していた。そこで本剤の正常造血への影響を検討するために CD34 陽性ヒト臍帯血細胞を NOG マウスに移植したモデルを作成し、これに本薬剤を投与してヒト臍帯血細胞に対する増殖抑制効果を調べた所、ほとんど増殖抑制効果を認めなかった（T/C:99%）。この結果は本薬剤のヒト正常造血細胞に対する安全性を示していると考えられた。一方 PAX5-PML 白血病マウスの作成では、コントロールとして GFP 遺伝子のみを導入された pro B 細胞は、移植後３週間で mature B 細胞までの分化が確認された後、移植マウス骨髄、脾臓内からほぼ消失したのに対し、PAX5-PML 発現ベクターを導入された pro B 細胞は 56 日を経ても pro B 段階で分化停止し、マウス骨髄内に残存し続けた。更に移植後約 150 日あまり経過した頃から急性リンパ性白血病（ALL）を発症

して死亡するマウスを認めるようになった。この白血病細胞を別のマウスに移植するとより早期に白血病を発症するようになり、継代可能であった。PAX5-PML 陽性白血病細胞においては PML NB は破壊されていなかった。亜ヒ酸をこの白血病マウスに投与したが、腫瘍細胞の PML NB は変化せず、腫瘍細胞の減少も認めず、マウスの延命効果も認められなかった。PML NB の破壊はこのモデルにおける白血病の発症には寄与していないと考えられた。PAX5 は B リンパ球分化の推進のために多彩な遺伝子の発現を促進する。PAX5-PML 陽性白血病細胞におけるそれら遺伝子の発現を調べると、*CD19*、*CD79a* の発現は軽度抑制されているのみだったが、*BLNK*、*CD23*、*CD72* の発現は強く抑制されていた。PAX5-PML は PAX5 の転写活性を抑制し、これら３つの遺伝子の発現を低下させる事で B 細胞分化を抑制し白血病化に関与すると考えられた。また正常 B 細胞と白血病細胞の mRNA 発現プロファイルをマイクロアレイで比較すると *CDKN2B* の発現低下が重要な変化として抽出された。

腫瘍の進展過程でチロシンリン酸化を受ける蛋白質を多面的に解析して質量分析による同定を進めた。多くの新規分子が同定されたが、。胃がんの腹膜播種部位に発現するチロシンリン酸化タンパク質群の解析から新たに腹膜播種を抑制する ARAP3 を見出して、チロシンリン酸化がその機能に必要であることを明らかにした。また肺がん細胞の足場非依存性と転移性に関わるチロシンリン酸化蛋白質として同定した CDCP1 は、多くの固形腫瘍において活性化した Src ファミリーによりリン酸化されて、エフェクター分子 PKCδを膜にリクルートすることで固形腫瘍の運動能や浸潤能をも制御することがわかった。その制御メカニズムについては CDCP1 及び PKCδがコルタクチンと複合体をつくることで細胞運動に関わり、さらに PKCδとの協調作用によって MMP9 などのメタロプロテアーゼ分泌に関わることも示された。ヒト組織を用いた発現解析では、これまでの肺がんに加えて膵がんの系で、CDCP1 高発現群が低発現群と比較して統計学的に有意に予後が不良であることが明らかになった。肺がんの細胞株や腫瘍組織の解析では、CDCP1 は Ras の活性型変異が存在する場合に Ras の変異がない場合に比べて有意に高い発現が認められた。活性型 Ras を強制発現させると CDCP1 の発現が誘導され、活性型 Ras によって引き起こされた運動能や浸潤能の更新は CDCP1 の発現を抑えることによりキャンセルされた。Ras の下流のシグナルの阻害などの実験で、CDCP1 は Ras-Erk 経路の活性化により発現が誘導され、Ras による転移・浸潤能の獲得は CDCP1 の発現誘導に依存することが確認された。一方、Ras による増殖異常は CDCP1 の発現の明らかな影響を受けないことも確認された。CDCP1 は Ras 経路の活性化による発現制御と Src 経路の活性化によるリン酸化制御の両者によって転移能・浸潤能獲得をもたらすがん遺伝子の共通標的分子であることが示され、更にその重要性が浮き彫りになった。また。乳がんの浸潤モデルにおいては、CDCP1 は細胞外マトリックス分解を伴うアクチンの突起である「invadopodia」という構造体の近くに局在し、この形成に関わることとともにが示された。

CDCP1 を siRNA によって抑制すると足場非依存性に増殖するがん細胞にアノイキスが誘導されるが、この浮遊に伴う細胞死は Caspase 非依存性であった。さらなる解析でアノイキスの誘導とともに LC3-I からオートファゴソーム結合型の LC3-II への変換が観察され、LC3 陽性小胞が誘導された。このような変化はもともと足場依存性の H322 細胞を浮遊状態で培養しただけでも誘導された。この段階ではアノイキス誘導という一種のストレスに抵抗するためにがん細胞がオートファジーというという反応を示したとも考えられたが、オートファジーの阻害剤である３MA で処理すると、予想に反して浮遊状態による細胞死が抑制されることが観察された。このことは足場非依存性に増える A549 細胞で CDCP1 の発現を抑制した場合でも、元々 CDCP1 の発現が低く足場依存性の H322 細胞でも観察され、がん細胞に起こる浮遊状態の細胞死誘導にオートファジーが積極的に関わっていることが示唆された。

また、治療モデルとして CDCP1 と PKCδの結合を阻害する「遮断ペプチド」に膜透過ペプチドを付加する形で合成し、その腫瘍に対する効果を解析した。PKCδのリン酸化チロシン結合ドメインである C２ドメインの理想結合配列（C2B）および実際の CDCP1 の PKCδ結合部位の配列（p762)を用いた結果では、実際に CDCP1 と PKCδの結合を阻害し、足場非依存性増殖を抑制し、マウスモデルにおける腹膜播種の抑制（data not shown）および延命効果が認められ、このシグナルの遮断が転移や腹膜播種の治療に有効であることが示唆された。

D．考察

本研究結果により白血病幹細胞特異的な表面抗原 TIM-3 を同定し、その機能として白血病幹細胞を含む AML 細胞が、autocrine 機構により galectin-9 を分泌し自身の TIM-3 分子にシグナルを生じている事が明らかになった。癌幹細胞自身が、積極的に autocrine 機構を用いている知見はこれまでに報告がなく、癌幹細胞の生物学的理解を深める上で新しいモデルになるものと考えられる。さらに、近年では我々の AML における TIM-3 発現報告に引き続いて、種々の非造血器悪性腫瘍においても腫瘍細胞自身が TIM-3 を異所性に発現し、病理学的に TIM-3 の高発現症例は予後が不良であることが次々に報告されており、腫瘍細胞における TIM-3 の機能解析に関して、非常に注目を集めている。本研究により TIM-3 分子の癌幹細胞を含む腫瘍細胞における機能を明らかにすることは、AML のみではなく、複数の悪性腫瘍における新規治療標的分子の同定につながる可能性が高く、発展性の高い研究であると考えられる。

今回の結果から OPB-31121 の作用機序の一部が明らかにされ、適合癌種が判明し、ヒト造血細胞への安全性が支持された。これらのデータをまとめて論文を作成し、Blood Cancer Journal 誌に掲載された。適合癌種に関しては、STAT の恒常的活性化を認める腫瘍の中にも本薬剤に感受性のものと、そうでないものがあり、判別が難しいものが多かった。これは STAT の活性化が腫瘍の増殖・生存に関わる一次的なものとして起きている腫瘍と、他に腫瘍の増殖に関わる一次的なシグナルの活性化があり、それに伴い二次的に STAT が活性化しているだけの腫瘍があるからであろうと予想された。その違いを個々の腫瘍で判定するのは困難で、過去の研究により STAT シグナルの増殖への関与が確立されている癌遺伝子、すなわち SAO を持つ腫瘍を標的に本剤で治療するのが現実的な戦略であると考えられた。本研究の成果などをもとに大塚製薬では本薬剤の第一相臨床試験を開始した(NCT1406574)。また今回の研究で PAX5-PML が白血病の発症を誘導できる癌遺伝子であることが示された。白血病発症までに 150 日と比較的長い時間がかかるのは PAX5-PML による分化障害に加えて、おそらくは細胞増殖を誘導する何らかの遺伝子異常が入ることが白血病発症に必要であるからと考えられた。そのセカンドヒットとしては *CDKN2B* の発現低下が有力な候補であるが、それを検証していくのは今後の課題である。本白血病における PAX5-PML の発現量は極めて低く、それがこの白血病において PAX5-PML が PML NB を破壊できなかった理由と考えられた。本モデルでは PML NB を破壊する以外の方法で腫瘍細胞の生存を維持する機序が働いていると考えられ、PML の機能を評価するには適さない系であった。むしろ B 細胞分化障害が起こる機序を解析するのに適した系で、今後はその研究を進めていく予定である。

CDCP1 は Src の活性化に応じて足場非依存性を含めた複数の悪性形質を固形腫瘍に付与することにより腫瘍の転移・浸潤に関わっており、膜蛋白質であることとも併せて、転移・浸潤をターゲットとした分子標的の良い候補となりうる。一方、Ras の活性化は固形腫瘍において高頻度に見られ、その発生のみならず進展にも深く関わるが、そのメカニズムや制御分子については実はいまだに明らかになっていないことが多い。CDCP1 が活性化した Ras の下流のエフェクターとして転移・浸潤など腫瘍の悪性形質の獲得に関わる機構を示したことで、現在開発中の CDCP1 のシグナル経路を阻害する低分子化合物が、Ras が活性化した多くの固形腫瘍に対して効果を示す可能性が示唆された。また転移に深く関わる足場非依存性という性質とオートファジーの抑制との関わりを CDCP1 の解析から明らかに出来たので、今後転移浸潤過程におけるオートファジーの意味について更に検討していきたい。ARAP3 は Src キナーゼの基質分子としては例外的に腹膜播種に対して抑制的に働く分子であり、その作用にリン酸化が必要なこととも併せて、ARAP3 ないしそのアゴニストが腫瘍特異的な作用を発揮し、腹膜播種などの治療標的分子になる可能性がある。

E．結論

AML 白血病幹細胞特異的表面抗原 M-CSFR 及び TIM-3 を同定した。急性骨髄性白血病において特に予後が不良であることが知られる MLL 融合遺伝子や MOZ 融合遺伝子が関与する白血病 M-CSF 受容体の発現が高い細胞が白血病幹細胞であり、M-CSF 受容体の阻害剤や抗体医薬による治療が期待される。AML 以外の慢性骨髄増殖性疾患においても TIM-3 を異常発現する細胞が CD34+CD38-幹細胞分画に存在し、疾患の進行にともないその発現率が増加することを見出した。さらに、TIM-3 の白血

病幹細胞における機能に関しても検討を行い、AML細胞自体がTIM-3リガンドであるgalectin-9をautocrine様式にて分泌し、TIM-3を介したシグナルを生じることを見出した。白血病幹細胞を含むAML細胞において、TIM-3はNF-kB経路を含むいくつかの主要な転写因子群の発現を誘導することで白血病幹細胞の生存、自己複製等を強化している可能性が示された。

OPB-31121はSTAT3、STAT5のリン酸化を抑制するが、その阻害は上流のキナーゼ阻害にはよらない。この作用によりSAO陽性の白血病など多くの悪性腫瘍細胞に対し抗腫瘍効果があると期待できる。 PAX5-PMLはPAX5の機能を阻害しリンパ球分化障害から白血病を発症させる癌遺伝子であることが分かった。PML機能の阻害については今回の実験モデルでは明らかにされなかった。

転移性がんの多くで高発現やリン酸化の見られるCDCP1は、がんが足場非依存性に生存する遠隔転移や腹膜播種の過程に必要であり、ヒトの多くの固形腫瘍においてその予後を規定する分子の一つであることを示してきた。CDCP1の発現はRas-Erk経路の下流で誘導され、活性型Rasのもたらす悪性形質の少なくとも一部はCDCP1に依存していることも明らかになった。さらにCDCP1における足場非依存性の獲得はオートファジーの抑制を介することが示された。これらの知見に加えCDCP1とPKCδの結合を阻害する「遮断ペプチド」にこが転移や腹膜播種の治療に有効であることがin vivoの系で示され、CDCP1の転移性がんの治療のための標的分子としての有効性をその作用機構の詳細とともに示すことができた。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

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H．知的所有権の出願・登録状況

1. 特許出願

【職務発明番号】2013-18

【発明の名称】マウス AML モデルを用いたイソクエン酸デヒドロゲナーゼ変異体活性の評価システム

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2. 実用新案登録

なし

3.その他

なし

別紙4

研究成果の刊行に関する一覧表

雑誌

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nature medicine

PU.1-mediated upregulation of *CSF1R* is crucial for leukemia stem cell potential induced by MOZ-TIF2

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Leukemias and other cancers possess self-renewing stem cells that help to maintain the cancer[1,2]. Cancer stem cell eradication is thought to be crucial for successful anticancer therapy. Using an acute myeloid leukemia (AML) model induced by the leukemia-associated monocytic leukemia zinc finger (MOZ)-TIF2 fusion protein, we show here that AML can be cured by the ablation of leukemia stem cells. The MOZ fusion proteins MOZ-TIF2 and MOZ-CBP interacted with the transcription factor PU.1 to stimulate the expression of macrophage colony–stimulating factor receptor (CSF1R, also known as M-CSFR, c-FMS or CD115). Studies using PU.1-deficient mice showed that PU.1 is essential for the ability of MOZ-TIF2 to establish and maintain AML stem cells. Cells expressing high amounts of CSF1R (CSF1R^{high} cells), but not those expressing low amounts of CSF1R (CSF1R^{low} cells), showed potent leukemia-initiating activity. Using transgenic mice expressing a drug-inducible suicide gene controlled by the *CSF1R* promoter, we cured AML by ablation of CSF1R^{high} cells. Moreover, induction of AML was suppressed in CSF1R-deficient mice and CSF1R inhibitors slowed the progression of MOZ-TIF2–induced leukemia. Thus, in this subtype of AML, leukemia stem cells are contained within the CSF1R^{high} cell population, and we suggest that targeting of PU.1-mediated upregulation of CSF1R expression might be a useful therapeutic approach.

Chromosomal translocations that involve the *MOZ* gene[3] (official gene symbol *Myst3*) are typically associated with acute myelomonocytic leukemia and predict a poor prognosis[4]. Whereas MOZ is essential for the self-renewal of hematopoietic stem cells[5,6], MOZ fusion proteins enable the transformation of non–self-renewing myeloid progenitors into leukemia stem cells[7]. We previously generated a mouse model for AML by introducing c-Kit$^+$ mouse myeloid stem/progenitor cells infected with a retrovirus encoding MOZ-TIF2 and EGFP into lethally irradiated mice[8].

To identify leukemia-initiating cells (LICs), we investigated the bone marrow cells of these mice for various cell surface markers by FACS analysis. CSF1R^{high} and CSF1R^{low} cells were present in the bone marrow (**Fig. 1a**) and expressed equivalent amounts of MOZ-TIF2 protein (**Fig. 1b**). To determine the LIC activity of these cell populations, we isolated CSF1R^{high} and CSF1R^{low} cells by cell sorting and transplanted limited numbers (10 to 1×10^4 cells) into irradiated mice. One hundred CSF1R^{high} cells were sufficient to induce AML in all transplanted mice (**Fig. 1c**). Conversely, no mice developed AML after 1×10^3 CSF1R^{low} cells were transplanted per mouse, and only half of the mice developed AML with delayed onset when 1×10^4 CSF1R^{low} cells were transplanted (**Fig. 1d**). Thus, the CSF1R^{high} cells showed a >100-fold stronger LIC activity than CSF1R^{low} cells.

FACS analysis indicated that the CSF1R^{high} cell population had the phenotype of both granulocyte-macrophage progenitors (GMPs, Kit$^+$Sca-1$^-$CD16/CD32$^+$) and differentiated monocytes (Mac-1^{low}Gr-1$^+$) (**Supplementary Fig. 1a**). Comparison of the CSF1R^{high} and CSF1R^{low} cell populations indicated that Mac-1 expression was lower in CSF1R^{high} than in CSF1R^{low} cells (**Fig. 1e**). However, we did not observe significant differences between the CSF1R^{high} and CSF1R^{low} cell populations with respect to their cell morphology (**Fig. 1f**), colony-forming ability in methylcellulose medium (**Fig. 1g**), cell cycle distribution (**Supplementary Fig. 1b**) or homeobox A9 (HoxA9) expression (**Supplementary Fig. 1c**). To investigate whether downstream pathways of CSF1R signaling were activated, we measured phosphorylation levels of signal transducer and activator of traranscription-5 (STAT5) and extracellular signal–regulated kinase (ERK) in CSF1R^{high} and CSF1R^{low} cells. STAT5 was highly phosphorylated in the CSF1R^{high} cell population but not in the CSF1R^{low} population, whereas ERK was equivalently phosphorylated in the two cell populations (**Fig. 1h**).

Side population cells, which are present in some types of normal and malignant stem cell populations, were present in the bone marrow of MOZ-TIF2–induced AML mice (**Supplementary Fig. 2a**). Whereas most side population cells were CSF1R^{high}, the non–side population fraction contained both CSFR1^{high} and CSF1R^{low} cells (**Supplementary Fig. 2b**). LICs were approximately tenfold more enriched in the side population fraction than in the non–side population fraction (**Supplementary Fig. 2c,d**). Because the side population fraction was very small (~0.12% of total bone marrow cells), the fraction of LICs in the side population fraction was also small (~1% of all LICs), and most LICs were present in the non–side population fraction (~99%).

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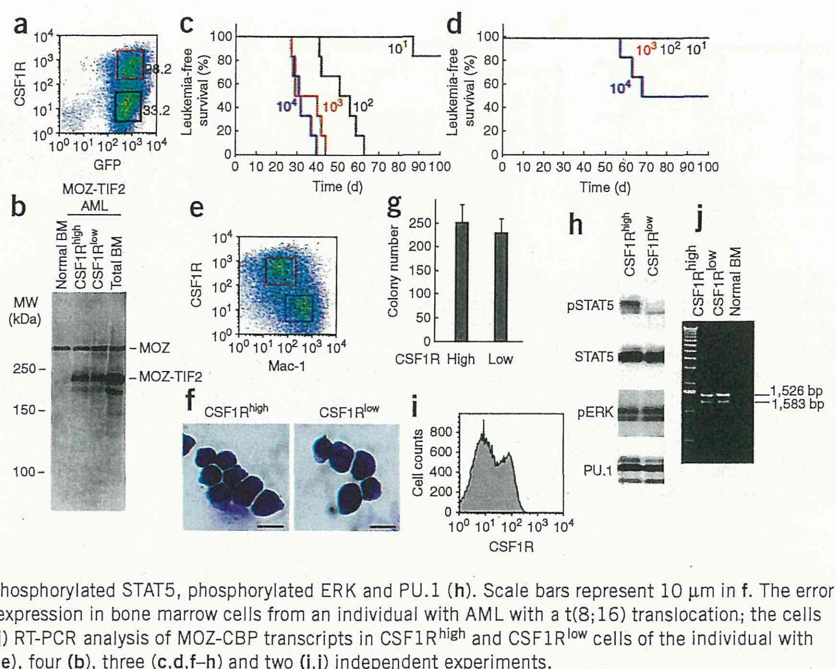

Figure 1 CSF1R^{high} cells show potent leukemia-initiating activity. (a) FACS analysis of bone marrow cells from mice with MOZ-TIF2–induced AML for expression of GFP and CSF1R. The red and black boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (b) Immunoblot analysis of MOZ-TIF2 expression in CSF1R^{high} and CSF1R^{low} cell populations (sorted by flow cytometry) with a MOZ-specific antibody. MW, molecular weight; BM, bone marrow. (c,d) Leukemia-free survival after the indicated numbers of flow-sorted CSF1R^{high} (c) and CSF1R^{low} (d) cells were transplanted into sublethally irradiated mice. $n = 6$, $P = 0.0001$ (1×10^4, 1×10^3 and 1×10^2) and 0.3173 (1×10^1) (CSF1R^{high} versus CSF1R^{low} cells). (e) FACS analysis of Mac-1 and CSF1R expression in bone marrow cells from mice with MOZ-TIF2–induced AML. The red and blue boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (f–h) CSF1R^{high} and CSF1R^{low} cells were sorted and analyzed for morphology by staining with May-Giemsa (f), colony-forming activity in methylcellulose medium (g) and levels of total and phosphorylated STAT5, phosphorylated ERK and PU.1 (h). Scale bars represent 10 μm in f. The error bars represent s.d. in g. (i) FACS analysis of CSF1R expression in bone marrow cells from an individual with AML with a t(8;16) translocation; the cells were cultured for 3 d in 10 ng ml^{-1} human M-CSF. (j) RT-PCR analysis of MOZ-CBP transcripts in CSF1R^{high} and CSF1R^{low} cells of the individual with t(8;16) AML. The results are representative of 25 (a,e), four (b), three (c,d,f–h) and two (i,j) independent experiments.

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To determine whether a high level of CSF1R expression also occurs in human AML cells with *MOZ* translocations, we investigated CSF1R expression in bone marrow cells from a subject with AML harboring a t(8;16) translocation, yielding a MOZ-CREB–binding protein (CBP, encoded by the *Crebbp* gene) fusion[9]. FACS analysis indicated that both CSF1R^{high} and CSF1R^{low} cells were present among the bone marrow cells with this translocation (**Fig. 1i**). We detected *MOZ-CBP* fusion transcripts in both the CSF1R^{high} and CSF1R^{low} cell populations (**Fig. 1j**).

These results suggest that leukemia stem cells in this subtype of AML express a high amount of CSF1R, indicating that leukemia might be cured by inducing apoptosis of CSF1R^{high} cells. To test this idea, we used transgenic mice expressing a drug-inducible FK506-binding protein (FKBP)-Fas suicide gene and EGFP under the control of the *CSF1R* promoter[10] (**Fig. 2a**). The suicide gene products are inactive monomers under normal conditions but can be activated by injection of the AP20187 dimerizer, inducing apoptosis of cells expressing high amounts of CSF1R[10]. We infected c-Kit$^+$ bone marrow cells of transgenic mice with the MOZ-TIF2 retrovirus and transplanted them into lethally irradiated wild-type mice. These mice developed AML ~2 months after transplantation. In the bone marrow of these mice, we observed morphologically indistinguishable CSF1R^{high} and CSF1R^{low} cells. As expected, endogenous CSF1R expression was proportional to EGFP and FKBP-Fas expression (**Fig. 2b** and **Supplementary Fig. 3a**).

Next, we transplanted the bone marrow cells of these AML mice (1×10^5 cells per mouse) into secondary sublethally irradiated recipient mice. Seven days after transplantation, we injected the mice with

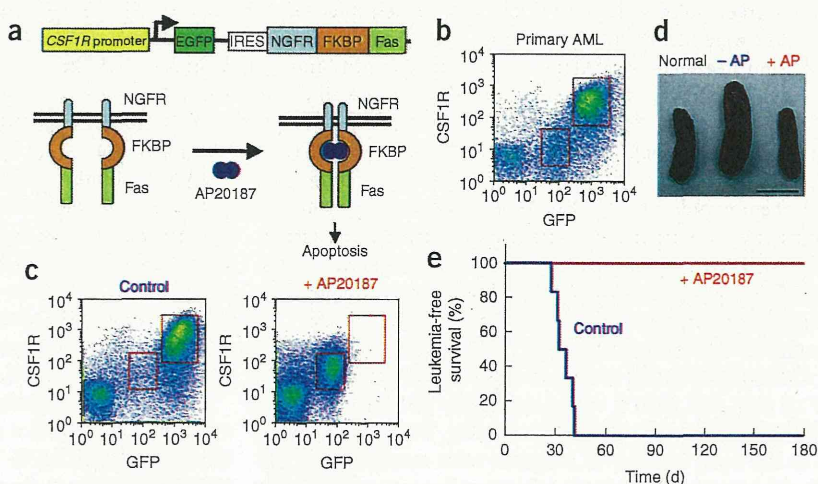

Figure 2 Cure of AML by ablation of CSF1R^{high} cells. (a) Top, structure of the *CSF1R* promoter-EGFP-NGFR-FKBP-Fas suicide construct. Bottom, schematic showing the activation of the NGFR-FKBP-Fas fusion protein: in transgenic mice carrying this suicide construct, ablation of cells expressing high levels of CSF1R can be induced by exposure to the AP20187 dimerizer. (b) FACS analysis of GFP and CSF1R expression in bone marrow cells of mice with AML 2 months after the transplantation of MSCV-MOZ-TIF2-IRES-GFP–transfected bone marrow cells derived from transgenic mice into lethally irradiated C57BL/6 mice. The red boxes signify CSF1R^{high} and CSF1R^{low} cell fractions. (c–e) Bone marrow cells (1×10^5) of primary transplanted mice with AML, generated as in b, were transplanted into sublethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary transplanted mice was started by intravenous injection 3 weeks after transplantation. Expression of GFP and CSF1R in bone marrow cells (c) and spleen sizes (d) were analyzed 4 weeks after transplantation. Scale bars, 1 cm. (e) Leukemia-free survival of the untreated ($n = 6$) and AP20187-treated ($n = 6$) secondary transplanted mice. $P < 0.0001$. The results are representative of five (b), four (c) and three (d,e) independent experiments.

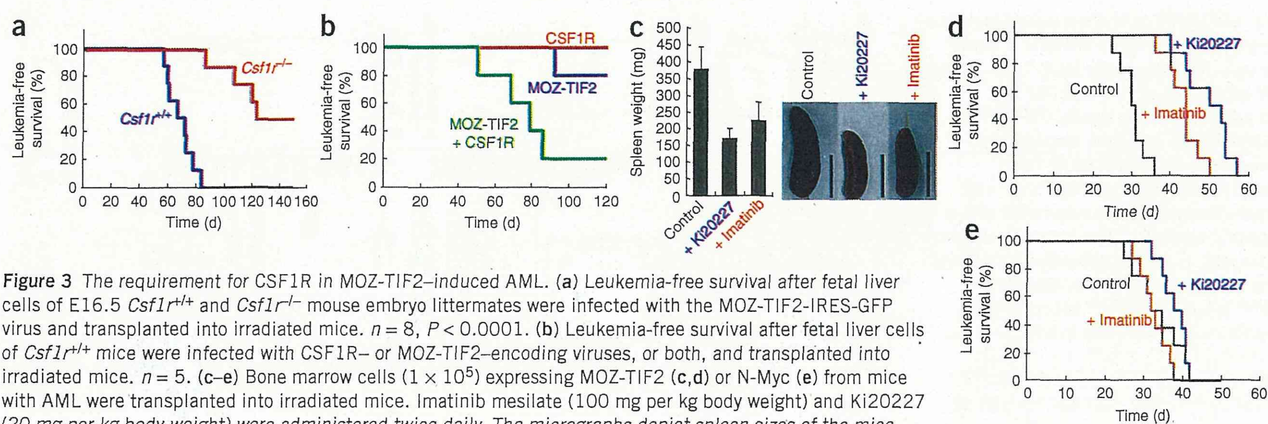

Figure 3 The requirement for CSF1R in MOZ-TIF2–induced AML. (**a**) Leukemia-free survival after fetal liver cells of E16.5 *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ mouse embryo littermates were infected with the MOZ-TIF2-IRES-GFP virus and transplanted into irradiated mice. $n = 8$, $P < 0.0001$. (**b**) Leukemia-free survival after fetal liver cells of *Csf1r*$^{+/+}$ mice were infected with CSF1R– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. $n = 5$. (**c–e**) Bone marrow cells (1×10^5) expressing MOZ-TIF2 (**c**,**d**) or N-Myc (**e**) from mice with AML were transplanted into irradiated mice. Imatinib mesilate (100 mg per kg body weight) and Ki20227 (20 mg per kg body weight) were administered twice daily. The micrographs depict spleen sizes of the mice transplanted with MOZ-TIF2–expressing cells, analyzed three weeks after transplantation (**c**). Scale bars, 1 cm. (**d**,**e**) Leukemia-free survival of the control and drug-treated mice was analyzed. In **d**, $n = 8$, $P < 0.0001$ (control versus + Ki20227 and control versus + imatinib). In **e**, $n = 8$, $P = 0.3825$ (control v.s. + Ki20227) and 0.4051 (control versus + imatinib).

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AP20187 or a control solvent, as previously described[10]. We observed an increase in the number of CSF1R^{high} cells (**Fig. 2c**) and splenomegaly (**Fig. 2d**) in the control-treated mice 3 weeks after transplantation. However, we detected neither CSF1R^{high} cells nor splenomegaly in the AP20187-treated mice after a 1-week course of treatment (**Fig. 2c,d**). Although we observed CSF1R^{low} cells in the bone marrow and peripheral blood after the 1-week treatment course, we did not detect these cells after three months of treatment (**Fig. 2c** and **Supplementary Fig. 3b**). All control-treated mice developed AML 4–6 weeks after transplantation, but none of the AP20187-treated mice died of AML within 6 months of transplantation (**Fig. 2e**). These results indicate that ablation of the CSF1R^{high} cells was sufficient to cure MOZ-TIF2–induced AML, and that a high level of CSF1R expression is a key contributor to leukemia stem cell potential.

As it has been reported that N-Myc overexpression rapidly causes AML in mice[11], we next tested the specificity of the requirement for CSF1R^{high} cells in AML progression. We transfected the bone marrow cells of suicide gene–expressing transgenic mice with a retrovirus encoding N-Myc and EGFP, and transplanted the cells into lethally irradiated recipient mice, which developed AML. In these mice, GFP$^+$ leukemia cells were Mac1$^+$Gr1$^+$CSF1R$^-$ blast cells (**Supplementary Fig. 4a,b**), and treatment with AP20187 did not affect AML induction (**Supplementary Fig. 4c**). These results indicate a specific role of CSFR expression in MOZ-TIF2–induced AML.

To investigate the role of CSF1R in the development of MOZ-TIF2–induced AML, we infected wild-type and *Csf1r*$^{-/-}$ (ref. 12) mouse fetal liver cells of embryonic day 16.5 (E16.5) littermate embryos with the MOZ-TIF2 virus and transplanted them into lethally irradiated mice. All mice transplanted with wild-type cells developed AML within 3 months (**Fig. 3a**). In contrast, AML induction was initially suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, but half of the mice developed AML after a longer latency period (**Fig. 3a**). The suppression of AML was rescued by co-infection with the retrovirus encoding CSF1R (**Fig. 3b**). STAT5, which was highly phosphorylated in CSF1R^{high} cells but not in CSF1R^{low} cells (**Fig. 1h**), was phosphorylated in the bone marrow of recipient mice transplanted with *Csf1r*$^{+/+}$ cells but not with *Csf1r*$^{-/-}$ cells (**Supplementary Fig. 5**). To test the specificity of the requirement of CSF1R for AML induction by MOZ-TIF2, we transfected *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ fetal liver cells with the retrovirus encoding N-Myc and transplanted them into irradiated recipient mice. All of the mice transplanted with either *Csf1r*$^{+/+}$ or *Csf1r*$^{-/-}$ cells expressing N-Myc developed AML (**Supplementary Fig. 4d**). These results indicate that CSF1R has a key role in AML induction by MOZ-TIF2, but not by N-Myc.

The above results suggest that signaling through CSF1R might be a therapeutic target for kinase inhibitors in leukemogenesis induced by MOZ fusions. To test this, we used the CSF1R-specific inhibitor Ki20227 (ref. 13) and the tyrosine kinase inhibitor imatinib mesylate (STI571), which inhibits CSF1R1[14–16]. Oral administration of Ki20227 or imatinib inhibited MOZ-TIF2–induced splenomegaly (**Fig. 3c**) and slowed MOZ-TIF2–induced AML onset (**Fig. 3d**). However, the drugs did not affect the progress of N-Myc–induced AML (**Fig. 3e**).

Next, we investigated the molecular mechanism of CSF1R expression in the leukemia cells. Monocyte-specific expression of CSF1R is reportedly regulated by transcription factors such as AML1, PU.1 and CCAAT/enhancer-binding proteins (C/EBPs)[17]. We previously found that MOZ interacts with AML1 and PU.1, but not with C/EBPα or C/EBPε, to stimulate transcription of their target genes[5,18]. Deletion analysis indicated that PU.1 interacted with the N-terminal and central regions of MOZ (**Fig. 4a** and **Supplementary Fig. 6**), and that the acidic amino acid–rich region (DE region) of PU.1 was required for its high-affinity interaction with MOZ (**Fig. 4a** and **Supplementary Fig. 7a–d**). Although binding of PU.1 to N-terminal MOZ (amino acids 1–513) was inhibited by several deletions in the PU.1 protein (**Supplementary Fig. 7c**), binding to full-length MOZ was not completely inhibited by these deletions (**Supplementary Fig. 7b**), suggesting that there may be other PU.1-binding sites in MOZ, its associated proteins or both. A pull-down assay with *Escherichia coli*–produced GST-PU.1 or GST-AML1 and *in vitro*–produced N-terminal MOZ indicated a direct interaction between both PU.1 and MOZ and between AML1 and MOZ (**Supplementary Fig. 8**). However, we cannot rule out a possibility that other factors may facilitate interactions between PU.1 or AML1 and MOZ *in vivo*.

To investigate transcriptional regulation of CSF1R, we performed reporter analysis with a *CSF1R* promoter–luciferase construct and found that MOZ, MOZ-TIF2 and MOZ-CBP could all activate the *CSF1R* promoter in the presence of PU.1 but not in the presence of AML1 (**Fig. 4b**). Moreover, MOZ, MOZ-TIF2 and MOZ-CBP did not activate a *CSF1R* promoter mutant lacking PU.1-binding sites (**Fig. 4c**). These results suggest that MOZ and MOZ fusion

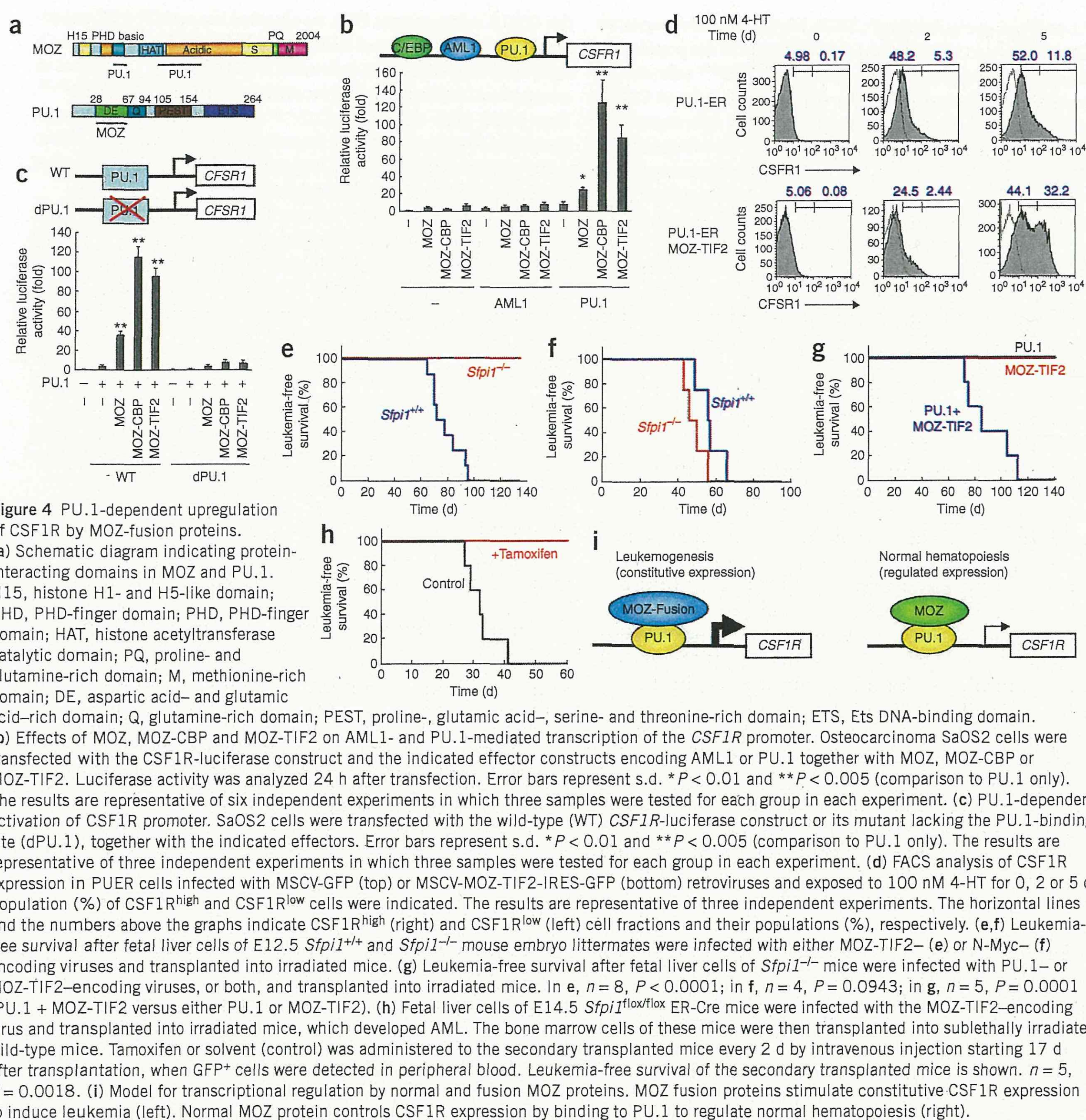

Figure 4 PU.1-dependent upregulation of CSF1R by MOZ-fusion proteins. (**a**) Schematic diagram indicating protein-interacting domains in MOZ and PU.1. H15, histone H1- and H5-like domain; PHD, PHD-finger domain; PHD, PHD-finger domain; HAT, histone acetyltransferase catalytic domain; PQ, proline- and glutamine-rich domain; M, methionine-rich domain; DE, aspartic acid– and glutamic acid–rich domain; Q, glutamine-rich domain; PEST, proline-, glutamic acid–, serine- and threonine-rich domain; ETS, Ets DNA-binding domain. (**b**) Effects of MOZ, MOZ-CBP and MOZ-TIF2 on AML1- and PU.1-mediated transcription of the *CSF1R* promoter. Osteocarcinoma SaOS2 cells were transfected with the CSF1R-luciferase construct and the indicated effector constructs encoding AML1 or PU.1 together with MOZ, MOZ-CBP or MOZ-TIF2. Luciferase activity was analyzed 24 h after transfection. Error bars represent s.d. *$P < 0.01$ and **$P < 0.005$ (comparison to PU.1 only). The results are representative of six independent experiments in which three samples were tested for each group in each experiment. (**c**) PU.1-dependent activation of CSF1R promoter. SaOS2 cells were transfected with the wild-type (WT) *CSF1R*-luciferase construct or its mutant lacking the PU.1-binding site (dPU.1), together with the indicated effectors. Error bars represent s.d. *$P < 0.01$ and **$P < 0.005$ (comparison to PU.1 only). The results are representative of three independent experiments in which three samples were tested for each group in each experiment. (**d**) FACS analysis of CSF1R expression in PUER cells infected with MSCV-GFP (top) or MSCV-MOZ-TIF2-IRES-GFP (bottom) retroviruses and exposed to 100 nM 4-HT for 0, 2 or 5 d. Population (%) of CSF1R^{high} and CSF1R^{low} cells were indicated. The results are representative of three independent experiments. The horizontal lines and the numbers above the graphs indicate CSF1R^{high} (right) and CSF1R^{low} (left) cell fractions and their populations (%), respectively. (**e**,**f**) Leukemia-free survival after fetal liver cells of E12.5 *Sfpi1*$^{+/+}$ and *Sfpi1*$^{-/-}$ mouse embryo littermates were infected with either MOZ-TIF2– (**e**) or N-Myc– (**f**) encoding viruses and transplanted into irradiated mice. (**g**) Leukemia-free survival after fetal liver cells of *Sfpi1*$^{-/-}$ mice were infected with PU.1– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. In **e**, $n = 8$, $P < 0.0001$; in **f**, $n = 4$, $P = 0.0943$; in **g**, $n = 5$, $P = 0.0001$ (PU.1 + MOZ-TIF2 versus either PU.1 or MOZ-TIF2). (**h**) Fetal liver cells of E14.5 *Sfpi1*$^{flox/flox}$ ER-Cre mice were infected with the MOZ-TIF2–encoding virus and transplanted into irradiated mice, which developed AML. The bone marrow cells of these mice were then transplanted into sublethally irradiated wild-type mice. Tamoxifen or solvent (control) was administered to the secondary transplanted mice every 2 d by intravenous injection starting 17 d after transplantation, when GFP$^+$ cells were detected in peripheral blood. Leukemia-free survival of the secondary transplanted mice is shown. $n = 5$, $P = 0.0018$. (**i**) Model for transcriptional regulation by normal and fusion MOZ proteins. MOZ fusion proteins stimulate constitutive CSF1R expression to induce leukemia (left). Normal MOZ protein controls CSF1R expression by binding to PU.1 to regulate normal hematopoiesis (right).

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proteins activate *CSF1R* transcription in a PU.1-dependent manner. It was recently reported that although chromatin reorganization of *Csf1r* requires prior PU.1 expression together with AML1 binding, stable transcription factor complexes and active chromatin can be maintained at the *Csf1r* locus without AML1 once the full hematopoietic program has been established[19]. This might explain why we found that AML1 was not required for MOZ-TIF2–mediated activation of *Csf1r*. Deletion analysis indicated that the DE-rich, Q-rich and ETS DNA-binding domains of PU.1, as well as the histone H1 and H5-like (H15) and the central PU.1-binding domains of MOZ and MOZ fusion proteins, are required for the activation of *CSF1R* transcription (**Supplementary Figs. 7e** and **9**). A truncated version of MOZ (1–1518) lacking the C-terminal region failed to activate transcription, indicating that the transcriptional activity of MOZ-TIF2 and MOZ-CBP, which do not contain that C-terminal region, requires the TIF2 or CBP portion of the fusion protein.

To test the requirement of PU.1 for the expression of endogenous *CSF1R*, we used PU.1-deficient (*Sfpi1*$^{-/-}$) myeloid progenitors expressing the PU.1–estrogen receptor fusion protein (PUER). Upon restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT), PUER cells can differentiate into macrophages[20]. We infected PUER cells with the MOZ-TIF2 retrovirus or control retrovirus, sorted them for GFP expression and cultured the GFP$^+$ cells in the presence of 4-HT. The results of FACS (**Fig. 4d**) and quantitative RT-PCR (**Supplementary Fig. 10**) analyses indicated that CSF1R expression was induced after exposure to 4-HT, and that MOZ-TIF2 enhanced

the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected CSF1R^{high} and CSF1R^{low} cells in the population of PUER cells expressing MOZ-TIF2, but only CSF1R^{low} cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and *Sfpi1*$^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with *Sfpi1*$^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with *Sfpi1*$^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or *Sfpi1*$^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice (*Sfpi1*$^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

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Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in *MOZ*$^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both CSF1R^{high} and CSF1R^{low} cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected *Csf1r*$^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.I. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[10] (Jackson Laboratories), *Csf1r*-deficient mice[12] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[29] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[30] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit^{+} cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

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Cell Stem Cell
Article

TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells

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SUMMARY

Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. Here we identified T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed on LSCs in most types of AML except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). TIM-3$^+$ but not TIM-3$^-$ AML cells reconstituted human AML in immunodeficient mice, suggesting that the TIM-3$^+$ population contains most, if not all, of functional LSCs. We established an anti-human TIM-3 mouse IgG2a antibody having complement-dependent and antibody-dependent cellular cytotoxic activities. This antibody did not harm reconstitution of normal human HSCs, but blocked engraftment of AML after xenotransplantation. Furthermore, when it is administered into mice grafted with human AML, this treatment dramatically diminished their leukemic burden and eliminated LSCs capable of reconstituting human AML in secondary recipients. These data suggest that TIM-3 is one of the promising targets to eradicate AML LSCs.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). LSCs self renew and generate leukemic progenitors that actively divide to produce a large number of immature clonogenic leukemic blasts (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994). This hierarchical stem cell-progenitor-mature cell relationships in AML appears to simulate normal hematopoiesis that originates from hematopoietic stem cells (HSCs) with self-renewal activity. We have shown that like normal HSCs, AML LSCs are quiescent in vivo and appear to reside at the endosteal "osteoblastic" niche in the bone marrow based on our analysis in a xenograft model (Ishikawa et al., 2007). AML LSCs are resistant to chemotherapeutic reagents that usually target cycling malignant cells. In the majority (~90%) of AML patients, the conventional chemotherapies can diminish the leukemic clones to achieve remission. However, ~60% of such remission patients still relapse, and the recurrence of AML in these patients should originate from LSCs that survive the intensive chemotherapies. Therefore, the LSC should be the ultimate cellular target to cure human AML.

To eradicate the AML LSC without killing normal HSCs, it is critical to isolate a molecule that is expressed or functions specifically at the AML LSC stage (Krause and Van Etten, 2007). It has been shown that the AML LSCs mainly reside within the CD34$^+$CD38$^-$ fraction of leukemic cells and can reconstitute human AML in immunodeficient mice (Lapidot et al., 1994), although recent studies have suggested that LSCs can exist also in CD34$^+$CD38$^+$ (Taussig et al., 2008) or CD34$^-$ blastic fractions at least in some types of AML (Martelli et al., 2010; Taussig et al., 2010). Normal HSCs with long-term reconstitution activity also have the CD34$^+$ CD38$^-$ phenotype (Bhatia et al., 1997; Ishikawa et al., 2005). However, the expression pattern of other surface molecules in the CD34$^+$CD38$^-$ fraction of AML cells is different from that of normal controls. For example, CD34$^+$CD38$^-$ AML cells possess many phenotypic characteristics analogous to normal granulocyte/macrophage progenitors (GMPs) (Yoshimoto et al., 2009). Previous studies have reported molecules preferentially expressed in AML cells. Such molecules include CLL-1 (van Rhenen et al., 2007), CD25, CD32 (Saito et al., 2010), CD33 (Florian et al., 2006; Hauswirth et al., 2007), CD44 (Florian et al., 2006; Jin et al., 2006), CD47 (Jaiswal et al., 2009; Majeti et al., 2009), CD96 (Hosen et al., 2007), and CD123 (Jin et al., 2009; Yalcintepe et al., 2006). However, in our hands, some of these molecules are expressed in LSCs at a level insufficient for clear distinction, are expressed also in normal HSCs at a considerable level, or are found only in a fraction of AML cases. It is therefore critical to isolate ideal targets for AML LSCs with sufficient specificity and sensitivity.

Here we report a new surface molecule that might be useful to eradicate AML LSCs leaving normal HSC intact. We performed differential transcriptional profiling of AML LSCs and HSCs and extracted the T cell immunoglobulin mucin-3 (TIM-3) as a promising AML LSC-specific target surface molecule. TIM-3 is originally found as a surface molecule expressed in CD4$^+$ Th1 lymphocytes in mouse hematopoiesis and is an important regulator of Th1 cell immunity and tolerance induction (Monney et al., 2002; Sabatos et al., 2003; Sánchez-Fueyo et al., 2003). Murine TIM-3 is also expressed in CD11b$^+$ macrophages and CD11c$^+$ dendritic cells and recognizes apoptotic cells' phosphatidylserine through its IgV domain to mediate phagocytosis (Nakayama et al., 2009).

We found that human TIM-3 was expressed in the vast majority of CD34$^+$CD38$^-$ LSCs and CD34$^+$CD38$^+$ leukemic progenitors in AML of most FAB types, except for acute

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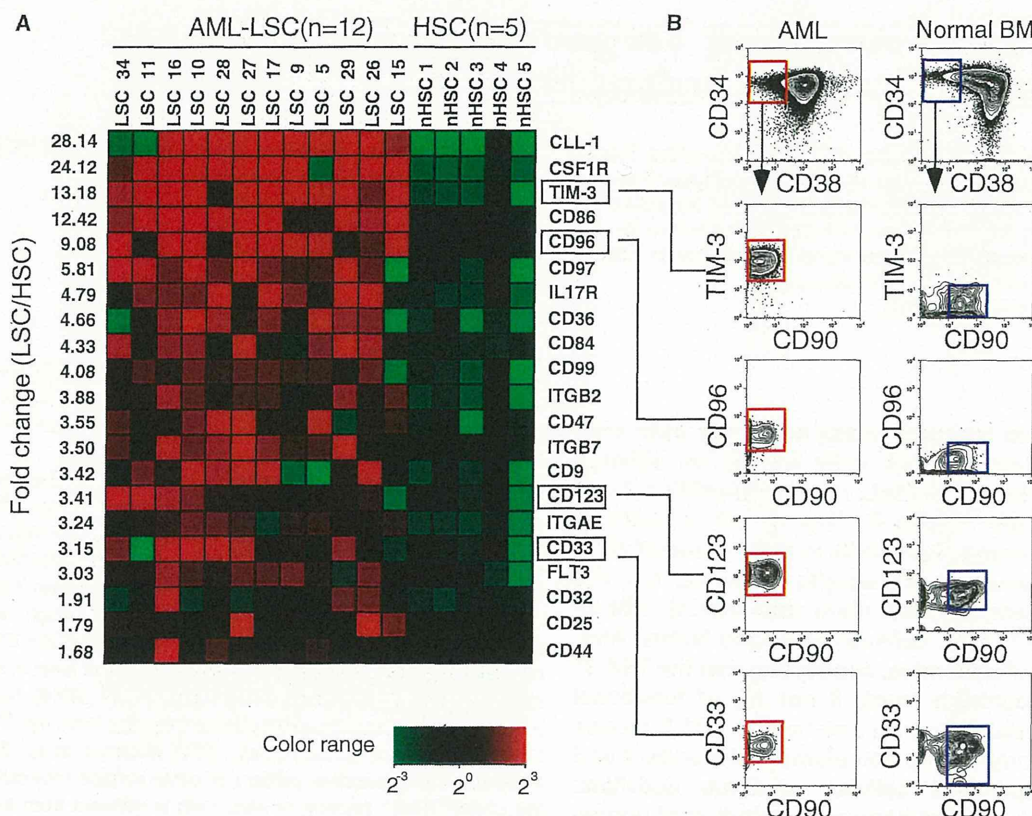

Figure 1. The Expression of LSC-Specific Surface Molecules in AML

The CD34$^+$CD38$^-$ adult bone marrow HSCs and CD34$^+$CD38$^-$ AML LSCs were purified and tested for their surface molecule expression.

(A) Results of cDNA microarray analysis of HSCs and AML LSCs. Representative genes coding surface molecules that are expressed highly in AML LSCs are shown. TIM-3 is expressed specifically in LSCs at high levels in the majority of AML patients. Patient numbers correspond to those in Table S1.

(B) The expression of representative surface proteins in HSCs and AML LSCs on FACS.

promyelocytic leukemia (M3). TIM-3 was not expressed in CD34$^+$CD38$^-$ normal HSCs or the vast majority of CD34$^+$CD38$^+$ normal progenitors. Administration of anti-human TIM-3 mouse antibodies with a complement-dependent cytotoxicity (CDC) and an antibody-dependent cellular cytotoxicity (ADCC) selectively inhibited engraftment and development of human AML in xenograft models. Our data strongly suggest that the use of TIM-3 to target AML LSCs is a promising approach for the improvement of leukemia therapy.

RESULTS

TIM-3 Is Expressed in the CD34$^+$CD38$^-$ Fraction of AML Patients' Bone Marrow Cells

In most types of AML, LSCs are concentrated in the CD34$^+$CD38$^-$ fraction of AML cells (Ishikawa et al., 2007; Lapidot et al., 1994), whose phenotype is common to normal adult HSCs. Patients' characteristics are shown in Table S1 available online. To search for the AML LSC-specific molecules, 10,000 each of purified CD34$^+$CD38$^-$ AML cells and CD34$^+$CD38$^-$ normal HSCs were subjected to cDNA microarray analysis. We extracted 256 genes with >4-fold change between normal HSCs and CD34$^+$CD38$^-$ AML cells and then selected 197 differentially expressed genes with <0.01 of a cut-off p value (Table S2). Genes coding surface molecules that are expressed highly in CD34$^+$CD38$^-$ AML cells were selected for this study. Figure 1A shows the mRNA levels of candidate LSC-specific surface molecules in purified CD34$^+$CD38$^-$ AML cells and normal HSCs. The molecules expressed in CD34$^+$CD38$^-$ AML cells at levels >8-fold higher as compared to normal HSCs included TIM-3 and previously identified LSC-specific molecules such as CLL-1 (van Rhenen et al., 2007), CSF1R (Aikawa et al., 2010), and CD96 (Figure 1A; Hosen et al., 2007). As shown in Figure 1B, TIM-3 protein was highly expressed in CD34$^+$CD38$^-$ AML cells but not in normal HSCs. We focused on TIM-3 not only because it is expressed specifically in CD34$^+$CD38$^-$ AML cells at high levels, but also because it is expressed in the majority of patients with most AML types.

We evaluated the TIM-3 protein expression on cell surface of AML cells by FACS analysis. As shown in Figure 2, the vast majority of the CD34$^+$CD38$^-$ LSCs as well as CD34$^+$CD38$^+$ progenitor fractions in AML M0, M1, M2, and M4 types expressed TIM-3 at a high level in virtually all cases studied. In AML M5, M6, and M7, a considerable fraction of CD34$^+$CD38$^-$ cells