表❶　無菌医薬品製造のための空気の清浄度

空気の清浄度レベル			最大許容微粒子数/m³	
USP<1116> P/ft³	ISO クラス	EU-GMP グレード	非作業時 0.5 μm以上	作業時 0.5 μm以上
100	5	A	3,530	3,530
10,000	7	B	3,530	353,000
100,000	8	C	353,000	3,530,000

い構造として，かつ塵埃を発生しないものとする。また，床と壁の境界は曲面として塵や埃を清拭しやすくする。

無菌区域には流しや排水口を設置しない。CPC内で使用する水は滅菌処理したものだけに制限し，使用後は残さず外へ持ち出す。これはカビの発生を抑え，排水口からの外気の逆流を防ぐためである。

2．空調管理

各作業区域の室圧，温度，湿度をコントロールし，無菌状態を維持するためのシステムが必要となる。特に作業区域へ供給される空気の清浄化は交差汚染防止の重要な要素の1つである。作業区域へは，中性能フィルターやHEPA（high efficiency particulate air）フィルターにより処理された清浄な空気を供給する。清浄度は，1立方フィート内に含まれる微粒子の数（P/ft3）で示され，例えば直径0.5 μm以上の粒子数が10万個以下ならクラス100,000の清浄度となる。

各作業区域の清浄度の設定は，原料や製品が空気に暴露される度合いによって規定される。組織や細胞が作業エリア内の空気に直接暴露されるような作業は，クラス100レベルのキャビネット内で作業を行う。キャビネット内をクラス100レベルに保つためにはキャビネットが置かれているエリアをクラス10,000レベルで維持しなければならない。

清浄度は作業を行っていない非作業時だけでなく，実際に作業を行っている時にでも許容限界値を超えないようにレベルを維持しなければならない（表❶）。そのために，適切な換気回数が作業区域ごとに応じて設定されるべきである。クラス100,000レベルの作業区域であれば1時間あたりに最低20回の換気回数が行える気流が一般的な許容レベルとされている。また，空調の吸気系と排気系を完全に独立させることも交差汚染防止につながる。

3．運営管理

(1) バリデーション[用解6)]

各作業工程だけでなくCPC内に設置されている装置についても定期的にバリデーションを実施する。天秤などの測定装置は定期的に校正を行い，遠心機やパーティクルカウンターなども定期的にその特性などを再確認する必要がある。バリデーションは多くの場合，外部の業者やメーカーに依頼することになる。校正結果や試験結果記録は保存管理する。

(2) 教育訓練

CPC内で作業を行う者は各基準書や標準作業手順書（SOP : standards of operating procedure）の内容を十分に理解し，人為的過誤を防止するために教育訓練を受けなければならない。

(3) 入退室管理

クリーンエリアへの入退室はセキュリティシステムなどにより管理し許可を受けた者だけを入室させる。清浄度を維持するためには，各作業エリアへの入室人数も制限する。

(4) 各種作業記録および運転記録

CPC内で実施された各作業記録，設備の使用履歴管理，清掃実施記録や環境モニタリング記録などを保管管理する。また冷蔵庫，冷凍庫，CO_2インキュベーターなどの庫内環境や各作業エリアの空気清浄度などを随時モニターし記録する。

(5) 原料・資材などの保管管理

検収（入荷），入庫，出庫，出荷の手続きが行われる間，変質・汚染の防止などについて管理を行い，ロット管理や有効期限についても厳密に管理

表❷　環境微生物の評価基準

EU-GMP グレード	空中微生物数 (CFU/m³)	最小空中採取量 (m³)	表面付着微生物数 (CFU/24～30cm²)	
			機器・設備	手袋
A	＜1	0.5	＜1	＜1
B	10	0.5	5	5
C	100	0.2	25	−

を行うことにより不適切な原料・資材の使用を避ける。

(6) サニテーションと環境モニタリング

各作業後には，作業区域内の床や使用したキャビネットおよび装置の表面を清掃することは交差汚染防止のための必須事項である。さらに作業の有無に関わらず，清浄区域の定期的な清掃も必要となってくる。清掃には主に70％エタノールによる清拭を行うが，必要な場合には界面活性剤や次亜塩素酸ナトリウムなども用いられる。クリーンエリアでの作業実施後や定期的なサニテーションを行った後には，空中浮遊菌検査や表面付着菌検査などにより環境モニタリングを実施してサニテーション後の評価を行う（表❷）。

(7) 是正処置

作業工程上で生じた不具合や細胞プロセシングを行い出荷した後に生じた問題点やユーザからの苦情などについては，作業工程記録や品質管理記録を見直して原因を究明し，必要な場合には各基準書やSOPの見直しを行う。

おわりに

今後，わが国における細胞治療や再生治療などの開発を行ううえでGMP準拠細胞プロセシングが必須であることを述べてきたが，細胞プロセシングを行う環境の整備を進めるとともに忘れてならないのは，CPCを管理運営するための人材を教育訓練し育てていくことである。基礎研究を行うための研究者は多くの教育・研究機関や企業などから育成されてくるが，研究の成果を臨床へと引き継ぐための掛け橋（トランスレーション）が十分に整備されているとはいえないのが日本の現状であり，GMPに準拠したCPCの設立とともに管理運営を行うためのマンパワーも必要となってくる。CPCの管理運営は研究者が片手間にできるものではなく，またSOPが作成されルーチン化した作業は研究者の手から離れていく。規則の整備とともに管理運営のための人員の育成も必要不可欠な課題の1つである。紙面の都合で，細胞培養に用いるウシ血清使用の問題など，細胞プロセシング技術自体やトランスレーショナルリサーチに特化したGCP（good clinical practice）の指針[4] などについては述べられなかったが，先端的細胞治療・再生治療開発に向けて解決すべき問題は山積している。

謝辞

本研究の一部は，21世紀COEプログラム「融合的移植再生治療を目指す国際拠点形成」の支援を受けた。

用語解説

1．**細胞プロセシング（cell processing）**：細胞療法に用いるヒト細胞の調整，培養，加工などの工程。
2．**GMP（good manufacturing practice）**：医薬品の製造管理および品質管理に関する基準。
3．**改正薬事法**：薬事法は医薬品，医療機器，医薬部外品などの有効性，安全性，品質などの確保を目的として，一定の基準や取り扱いを定め，必要な規制を行うための法律。その規制の対象は製品を製造する企業だけではなく，製品を取り扱う医療機関・医療関係者も対象となる。平成14年7月30日に公布された「薬事法及び採血及び供血あっせん業取締法の一部を改正する法律」（いわゆる改正薬事法）のうち，生物由来製品の安全確保対策に係る部分が，平成15年7月30日から施行されている。
4．**特定生物由来製品**：生物由来製品のうち，販売し，賃借し，または授与した後において当該生物由来製品による保健衛生上の危害の発生または拡大を防止するための措置を講ずることが必要なものであって，厚生労働大臣が薬事・食品衛生審議会の意見を聴いて指定するものをいう。（「改正薬事法第2条第6項」より引用）
5．**生物由来製品**：人その他の生物（植物を除く）に由来するものを原料または材料として製造（小分けを含む）をされ医薬品，医薬部外品，化粧品または医療用具のうち，保健衛生上特別の注意を要するものとして，厚生労働大臣が薬事・食品衛生審議会の意見を聴いて指定するものをいう。（「改正薬事法第2条第5項」より引用）
6．**バリデーション（validation）**：特別な工程，方法，システムが常にあらかじめ決められた判定基準に合致して物を生産できることを高度に保証するための文書化された計画。

参考文献

1）前川　平：医学のあゆみ 205, 361-366, 2003.
2）前川　平：臨床病理レビュー特集第122号, 81-91, 2002.
3）前川　平：臨血 45, 32-38, 2004.
4）2003年8月1日第2回TR懇話会合意に基づく「トランスレーショナルリサーチ実施にあたっての共通倫理審査指針」

参考図書

＊医薬品GMP解説1999, 厚生省医薬安全局監視指導課監修, 薬事日報社
＊医薬品GMP事例集1999, 厚生省医薬安全局監視指導課監修, 薬事日報社
＊医薬品GMP事例集及び関連法令通知2003年版, 薬事日報社

参考ホームページ

・FAD : Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products ; Inspection and Enforcement ; Proposed Rule
http://www.fda.gov/cber/rules/gtp010801pr.pdf
・CBER : Guidance for Industry Sterile Drug Products Produced by Aseptic Processing ? Current Good Manufacturing Practice　DRAFT GUIDANCE
http://www.fda.gov/cder/guidance/1874dft.pdf

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トピックス

VI．細胞療法
3．細胞治療・再生治療とトランスレーショナル・リサーチ —輸血部門の変革を求めて—

前川　平　笠井　泰成

要　旨

細胞治療（cell therapy）とは，輸血，造血幹細胞移植，細胞免疫療法，再生治療，遺伝子治療などのヒト細胞を輸注，移植することにより行う治療法の総称である．これらの治療に用いる細胞は，細胞プロセッシングという一定のルールに基づいた過程を経て作製されなければならない．わが国では，この細胞プロセッシングのルールづくりが遅れており，先端医療の開発を進めるために早急に整備されなければならない．

〔日内会誌　93：1404〜1410，2004〕

Key words：先端治療開発，細胞治療，再生治療，遺伝子治療，細胞プロセッシング

はじめに

17世紀になかば実験的に行われた輸血は，その後紆余曲折を経ながら1900年Landsteinerによる ABO式血液型の発見を契機に，補充療法として20世紀の医療の発展に大きく貢献してきた[1]．そして，20世紀末に爆発的な発展をとげた生命科学研究の成果は，21世紀になり細胞治療の開発や移植医療に応用され，社会に還元されつつある．輸血は細胞治療の原型である．21世紀の輸血医学は，分子生物学や細胞生物学の燦然たる研究成果を取り入れ，細胞移植治療や再生治療をはじめとする革新的な分子細胞治療（cell and molecular therapy）へと変貌し，さらなる飛躍を遂げようとしている．

まえかわ　たいら：京都大学医学部附属病院輸血細胞治療部/分子細胞治療センター
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1．細胞治療に必要なインフラストラクチャー

細胞治療（cell therapy）とは，輸血，造血幹細胞移植，細胞免疫療法などのヒト細胞を輸注，移植することにより行う治療法の総称である．現在脚光を浴びている再生治療や遺伝子治療の多くも，ヒト細胞を治療に用いることから細胞治療に包括される（図1）．これらの先端医療開発により恩恵を受けると考えられる人々は1億人を超えると試算する報告もある（図2）[2]．当然，こういった細胞治療に関する探索的臨床試験研究（トランスレーショナル・リサーチ：基礎研究の成果を臨床応用するための臨床研究）の開発には，科学的，倫理的に高い水準と透明性，そして信頼性が要求される．

現在，一般の治療に用いられる医薬品はGMP（good manufacturing practice：医薬品の製造管理および品質管理に関する基準）を遵守して製造されている．新薬の開発は基礎研究の成果を

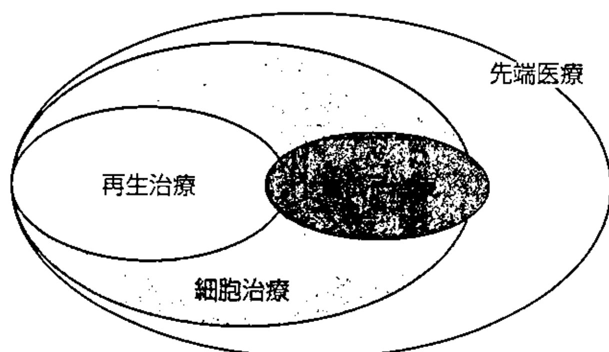

図1．細胞治療，再生治療，遺伝子治療と先端医療

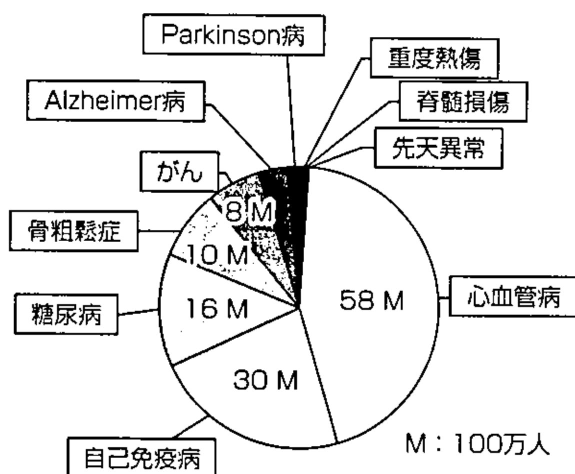

図2．先端医療開発の恩恵にあずかる患者人口の試算（米国）（文献2）を改変）

もとに前臨床試験，フェーズIへと進行する．医薬品はこの段階からGMPグレードで製造されたものが用いられる．治療に用いるヒト細胞を「細胞医薬品」と考えれば，細胞治療に関する探索的臨床試験研究にも，GMP準拠の細胞プロセッシングを受けた細胞を準備して用いる必要があることは容易に理解される．すなわち，治療に用いる細胞は，細胞プロセッシングという一定のルールに基づいた過程を経て作製されなければならない（図3）．したがって，細胞治療や再生治療に関しては，施設やGMP基準など細胞プロセッシングに関するインフラストラクチャーが構築できなければ臨床応用は不可能である．

米国では開発中の新薬はIND（investigational new drug）としてFDA（Food and Drug Administration）から認可されたものが臨床試験に用いられ，わが国でも医薬品に関して同様の体制がとられている．細胞治療に用いるヒト細胞も米国ではINDとして認可され，FDAは細胞を作製する細胞プロセッシングセンターの査察を行う．さらに，治療用ヒト細胞の作製は，2001年1月FDAの提言，cGTP（good tissue practice：current good tissue practice for manufacturers of human cellular and tissue-based products；inspection and enforcement；proposed rule）に準拠して行わねばならないとしている[3]．cGTPはおもに細胞治療による感染症の伝播を危惧したものであり，その防止方策に関係するルールや規制を記載したものである．わが国では，2003年7月から発効した改正薬事法で“生物由来製品については，その感染リスク等を踏まえ，原材料の採取及び製造から市販後に至る各段階において，一般の医薬品，医療機器等における各種基準に加え，以下に掲げる付加的な基準を定めることにより，一層の安全確保を図ることとしたこと．（中略）製造段階においては，構造設備，製造管理及び品質管理の方法について（著者註：いわゆるGMPのこと），生物由来製品の特性に応じた付加的な基準を設けること”とし，これらに関する下位の法令は平成17年4月に公布予定とされている．しかし，これらは血漿分画製剤などを念頭に置いたものであり，血漿分画製剤と治療に用いようとするヒト細胞では，そのプロセッシング方法は大きく異なる．現時点で，わが国では治療用ヒト細胞の作製に関するルールがなく，このことが被験者に対する補償の問題とともに，細胞治療に関する医師主導型の探索的臨床試験研究を大きく遅らせる原因のひとつとなっている．

最近，米国CBER（Center for Biologics Evaluation and Research）からpreliminary concept paperとして，“Sterile drug products produced by aseptic processing draft”が発表され，パブリックコメントを聴取している最中である[4]．序

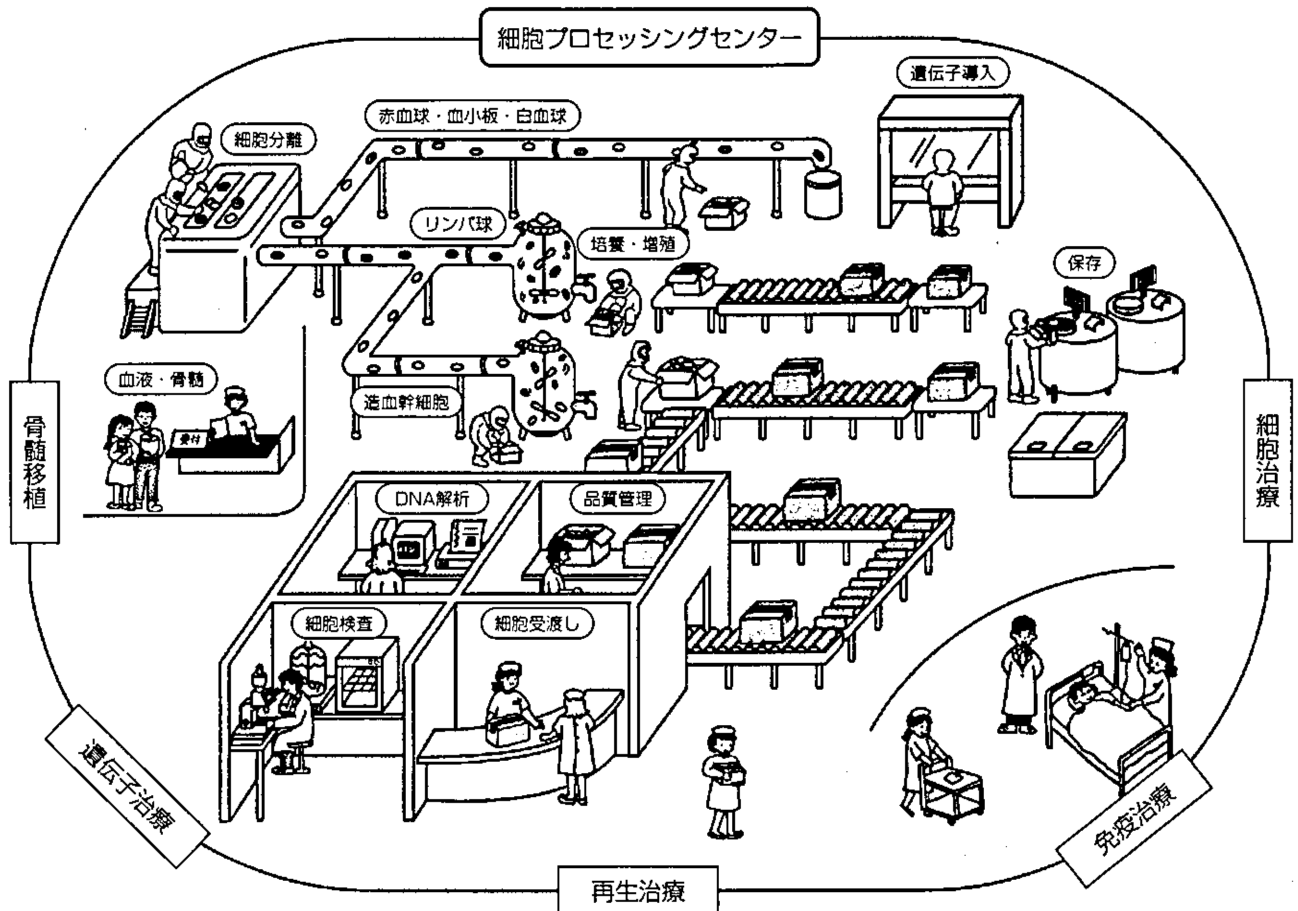

図３．細胞プロセッシングの概念図

文には“Poor cGMP conditions at a manufacturing facility can ultimately pose a life threatening health risk to a patient”と明記されている．この中で，無菌的プロセッシング施設が具備すべき機能やその設計基準，および無菌的プロセッシング技術や管理の必要事項が具体的に示されている．これは主に医薬品の無菌的プロセッシングを念頭に置いてはいるが，細胞のプロセッシングにも適応可能であるように考慮されている．

細胞プロセッシングにGMPは本当に必要なのかとの疑問を聞く．臨床試験研究はICH (International Conference on Harmonisation)-GCP (good clinical practice)を遵守して行う必要があり，それに用いる新薬はGMPグレードで作製したものを用いることを理解しておれば，細胞医薬品であるヒト細胞はどのように作製されたものを用いるべきかは明白であろう．実験室の片隅で，明確な基準や記録もなく，品質管理されずに作製された細胞を，誰も移植されたくはないであろう．

2. Institutional GMPの必要性

「そのようなインフラストラクチャーの構築は大学で行うべきことでなく，企業に任せておけば良い」と言う批判もある．確かに，培養皮膚などすでに臨床応用されているような分野では企業の参入も期待できる．しかし，大学などで開発しようとしているのは，基礎研究の成果をもとにした，まだ実験的医療（トランスレーショナル・リサーチ）の段階のものである．今後治療法として確立されるかどうか分からないのが現状であろう．このような段階の実験的医療開発の細胞プロセッシングにかかわるGMPの構築

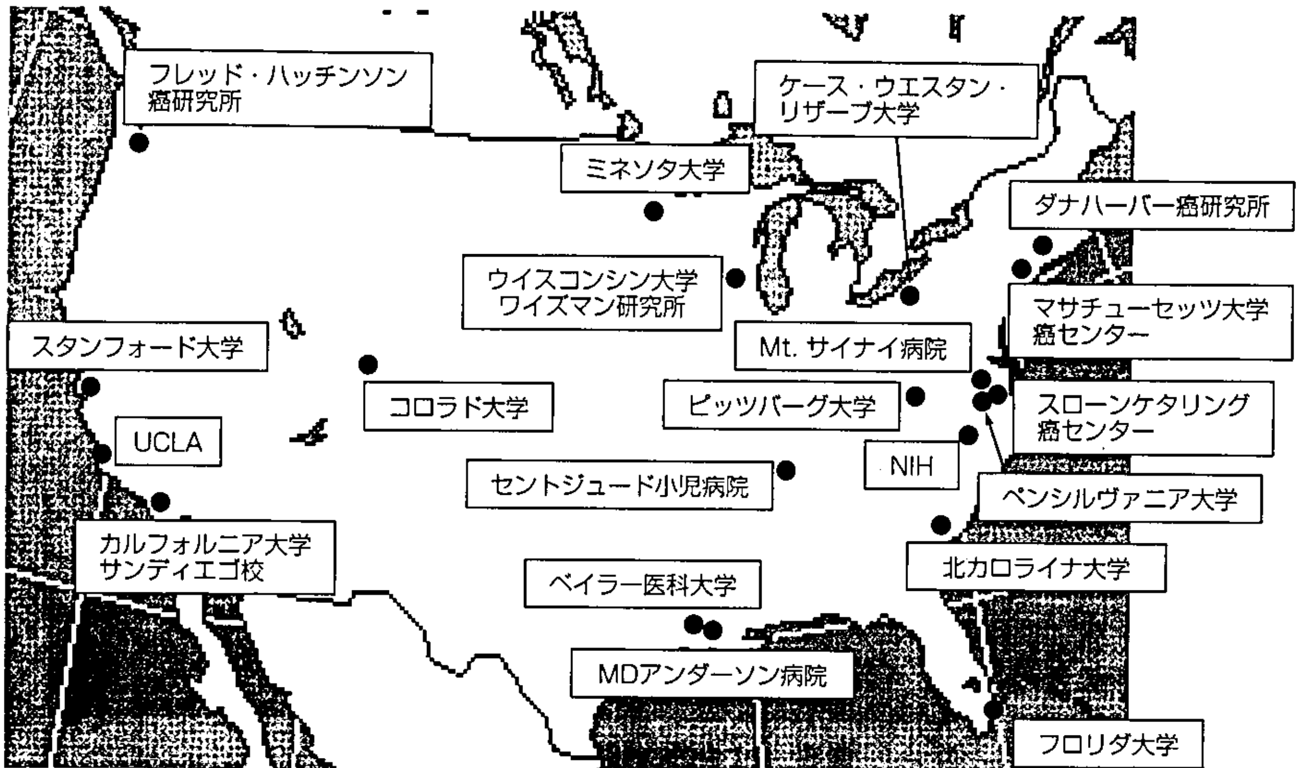

図4．細胞プロセッシングセンターを持つ米国の大学や研究所（2001年3月調査）

に，企業がリスクをとって積極的に参入することは困難である．加えて，企業の中でも，細胞プロセッシングに必要なGMPのノウハウを確立しているところはきわめて少ない．このような現状では，大学や先端医療センターが主導，あるいは施設としての場を提供するかたちで，研究者，臨床研究医，薬剤師，技師，GMPコンサルタント，企業の先端医療開発部門の研究者が一致協力して，世界的ルールにもとづいたものを構築して行く必要がある．

米国では，図4に示すように，先端医療開発を行っている大学や先端医療センターには，細胞プロセッシングセンターを併設しているところが多い．臨床応用可能であると言う目処がつけば，企業も参入が容易になるであろうし，また将来的に治療法として確立され，保険適用もされるようになれば，輸血製剤と同様に，全国にある血液センターが細胞プロセッシングセンターの役割を担い，各病院へ供給するようになると考えている（図5）．

「細胞プロセッシングはサイエンスではない．大学や先端医療研究センターで行う必要はない」と言う意見もある．確かに，細胞プロセッシングはピュアー・サイエンスではない．わが国では，基礎研究の成果を臨床応用しようとしても，そのインフラストラクチャーが不十分であるために欧米の後塵を拝している．基礎研究の成果を社会に還元する「実学」の必要性が認識されるようになり，細胞治療の開発に関しては，そのインフラストラクチャーの一部を細胞プロセッシングが占める．科学知を社会に還元することの必要性を主張し，それを行うのが大学や先端医療研究センターであるべきと考えるならば，先端医療開発のための細胞プロセッシングをどこで行うべきかは明白であろう．

では，米国の大学や先端医療開発センターで行われている細胞プロセッシングに，FDAはどのようにかかわっているのであろうか？　基礎研究の成果を臨床応用しようとする際，まず研究者や臨床研究医が臨床試験計画書を作成する

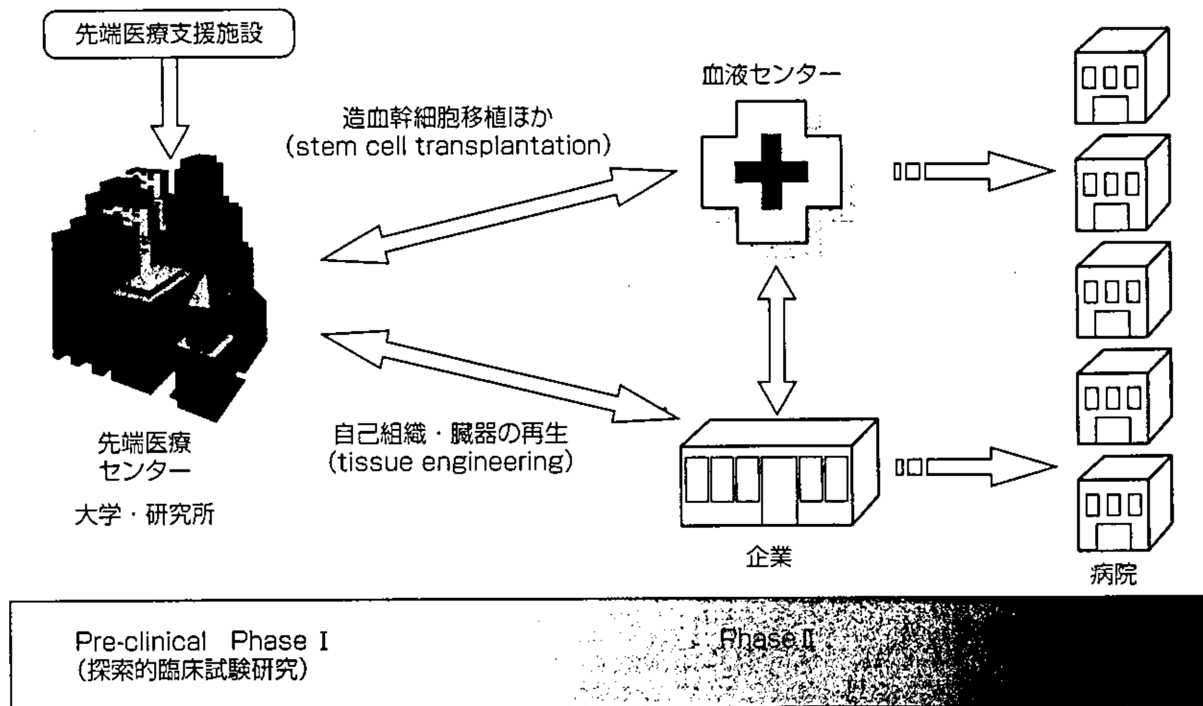

図5．21世紀の細胞治療システム

とともに，それが細胞治療，再生治療，遺伝子治療などにかかわる場合には，細胞プロセッシングに必要な施設の詳細やバリデーション・マスタープラン，品質管理手順書，標準作業手順書（SOP）などGMP準拠細胞プロセッシングに必要な書類をFDAに提出する．FDAはこれらの研究計画に使用される治療用細胞を，INDとして審査を開始するが，同時に施設の査察，およびGMP書類についても指導を行う．この際，FDAの係官は，企業などに要求されるGMP（＝full GMP）の内容をもとにしつつも，大学などの実情を考慮した実験的治療開発に必要なGMP（＝institutional GMP）の作成に協力を惜しまない．たとえば，膵臓Langerhans島細胞移植に必要な細胞プロセッシングに関しては，FDAの係官が施設を査察するとともに，大学の人的余裕などを考慮し，研究者や臨床研究医とともにGMP構築に必要な書類を点検し，最低限必要な事項を示し，不備な点があれば改善するように指導を行っている．full GMPとinstitutional GMPの大きな違いは，前者では違反や不備があれば製造中止命令が出され罰則を伴うが，後者では改善するように勧告を行う，と言うことである．成文化されたinstitutional GMPがあるわけではないが，このように米国では，明確な基準がないために開発研究へのベクトルが弱まることを懸念し，大学などにおけるあたらしい治療法の開発を積極的に支援すると言う国家戦略をとっている．

FDAが行っているようなinstitutional GMPを考慮した教育的指導が現時点では期待できないわが国では，研究者や臨床研究医自らがそのスタンダードを構築する必要がある．わが国においてinstitutional GMPを構築する必要があると主張する所以である（図6）[5]．

3．先端医療開発への輸血部門のかかわり

大学や研究所附属病院で細胞治療や再生治療などの探索的臨床試験研究を行おうとする場合，これらを担当あるいは支援する部門としては既存の大学輸血部を発展させてゆくのが最適であ

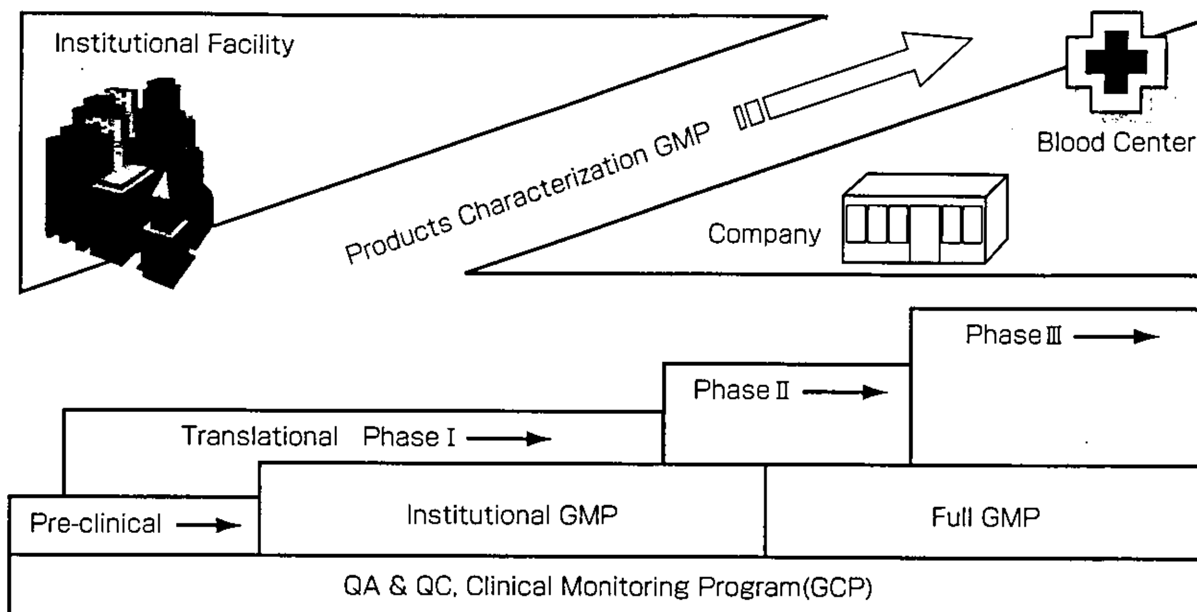

図６．Institutional GMP構築の必要性

ろう．輸血部は院内で治療用細胞を扱う唯一の部門であり，またあたらしい細胞治療や再生治療の開発にはGMP準拠細胞プロセッシングが必須であることを述べてきたが，その根底には安全な輸血療法に通じる考え方が脈々として流れているからである．GMPの原則は，第１に「人為的な誤りを最小限にする」こと，第２に「医薬品（細胞医薬品）に対する汚染および品質変化を防止すること」，第３に「高度な品質を保証するシステムを構築する」ことである．これは，安全で効率的な輸血治療の実践に求められていることに相通じるものである．

輸血部の基本的使命は，院内における安全で効率的な輸血療法の実施とその指導である．輸血にかかわる医師には豊富な臨床経験が要求され，実際に臨床の現場にかかわるべきである．輸血部技官も疾患病態に関する知識を持って，病人の苦しみを知るために臨床の現場に出て行くべきである．そこから，臨床で必要とされるあたらしい検査方法なども開発されよう．従来の輸血部の重要性はあらためて述べることではないが，冒頭に述べたように21世紀の輸血部は従来の輸血サービス管理業務を包括した形で，先端医療開発におけるコア組織のひとつとして大きく発展してゆくと考えている．

おわりに

わが国でも細胞治療や再生治療開発のためには，細胞プロセッシングセンターの必要性がようやく認識されてきたが，なかには単なる実験室のなかにクリーンベンチを置いて，それで大丈夫だとして同じインキュベーターで何人もの細胞を同時に培養したり，あるいは施設の倫理委員会で承認されたから良しとして，ウシ胎児血清で培養したヒト細胞や，マウス細胞と共培養したヒト細胞を人に投与する計画を申請しているところがある．FDAは無血清での培養を基本として指導を行い，またマウス細胞と共培養することは異種移植であるとして原則的に認めていない．未知のウイルスの危険性や，通常なら人への感染は生じないはずの動物ウイルスも，遺伝子が変異することにより，人へ感染する可能性が危惧されるからである．食べるのさえ躊躇しているものを，直接ではないにしろ，移植されたり静注されたりしても良いとは誰も思わ

ないであろう．現時点ではこれを規制する法律はわが国にはないが，だからこそ，安全性や有効性が十分確認されていない先端医療，とくに細胞治療や再生治療で，ヒト細胞を培養したり，遺伝子導入したりといった操作を行う場合は，GMP準拠の規格を有するクリーンルームで，GMPの管理手順に従って行う必要があり，先端医療開発にたずさわるものすべてが遵守しなければならない基本的ルールである．安全性や治療効果もまだわからない実験的探索医療であるからこそ，このような厳格な規制は必要であり，最初からルーズなやり方では取り返しがつかないことになる．規制に従って行い，ここまでは大丈夫だということが明らかになってはじめて徐々に規制を緩和して行く方向に持って行くべきである．

謝辞：本研究の一部は，21世紀COEプログラム「融合的移植再生治療を目指す国際拠点形成」の支援を受けた．

文　献

1) 前川　平：輸血療法の基礎と実際．三輪血液病学．浅野茂隆，他．文光堂，東京，2004（印刷中）．
2) Perry D: Patient's voice: The powerful sound in the stem cell debate. Science 287: 1423, 2000.
3) Current good tissue practice for manufactures of human cellular and tissue-based products; inspection and enforcement; proposed rule. 21 CFR Part 1271, January 8, 2001 (http://www.fda.gov/cber/rules/gtp010801pr.pdf).
4) Sterile drug products produced by aseptic processing draft—preliminary concept paper not for implementation. CBER, September 27, 2002 (http://www.fda.gov/cber/gdlns/steraseptic.pdf).
5) Maekawa T: Current good manufacturing practices (cGMP) controlled cell processing for the development of novel advanced cell and gene therapy. Education program book pp. 43–48, 2003. The 65th Annual meeting of Japanese Society of Hematology and The 45th Annual meeting of Japanese Society of Clinical Hematology.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 279, No. 24, Issue of June 11, pp. 24986–24993, 2004
Printed in U.S.A.

Roles for c-Myc in Self-renewal of Hematopoietic Stem Cells*

Received for publication, January 14, 2004, and in revised form, March 18, 2004
Published, JBC Papers in Press, April 2, 2004, DOI 10.1074/jbc.M400407200

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Notch and HOXB4 have been reported to expand hematopoietic stem cells (HSCs) *in vitro*. However, their critical effector molecules remain undetermined. We found that the expression of *c-myc*, cyclin D2, cyclin D3, cyclin E, and E2F1 was induced or enhanced during Notch1- or HOXB4-induced self-renewal of murine HSCs. Since c-Myc can act as a primary regulator of G_1/S transition, we examined whether c-Myc alone can induce self-renewal of HSCs. In culture with stem cell factor, FLT3 ligand, and IL-6, a 4-hydroxytamoxifen-inducible form of c-Myc (Myc/ERT) enabled murine $Lin^-Sca\text{-}1^+$ HSCs to proliferate with the surface phenotype compatible with HSCs for more than 28 days. c-Myc activated by 4-hydroxytamoxifen augmented telomerase activities and increased the number of CFU-Mix about 2-fold in colony assays. Also, in reconstitution assays, HSCs expanded by c-Myc could reconstitute hematopoiesis for more than 6 months. As for the mechanism of *c-myc* induction by Notch1, we found that activated forms of Notch1 (Notch1C) and its downstream effector recombination signal-binding protein-J κ (RBP-VP16) can activate the *c-myc* promoter through the element between −195 bp and −161 bp by inducing the DNA-binding complex. Together, these results suggest that c-Myc can support self-renewal of HSCs as a downstream mediator of Notch and HOXB4.

Hematopoietic stem cells (HSCs)[1] are characterized by two distinct abilities: self-renewal ability and pluripotentiality. With these activities, HSCs supply all lineages of hematopoietic cells throughout their life. In a murine experimental model, only a single HSC with the $CD34^{low/-}c\text{-}Kit^+Sca\text{-}1^+$ phenotype can reconstitute whole hematopoiesis *in vivo* (1, 2). To maintain homeostasis of hematopoiesis and protect exhaustion of HSC population, most of the HSCs are kept quiescent, and only a limited number of cells enter the cell cycle to supply mature blood cells (3). As external regulatory factors, various cytokines such as stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin, IL-3, and IL-6 promote the growth of HSCs (4–6). The combination of SCF, FL, thrombopoietin, and IL-6, in particular, was reported to efficiently induce the *in vitro* cell division with characteristics of self-renewal in HSCs (7). In contrast, transforming growth factor-β is known to inhibit the growth of HSCs (8, 9). Besides these cytokines, the direct interactions with stromal cells and extracellular matrix in the bone marrow microenvironment also influence the fates of HSCs (10, 11). As one mechanism responsible for this effect, Bernstein and co-workers (12) showed that the activation of Notch transmembrane receptors expressed on HSCs by their ligand (Jagged 1 or Jagged2) on stromal cells promotes the self-renewal of HSCs.

Furthermore, accumulated evidence indicated that the cell cycle state of HSCs is regulated by intrinsic transcription factors such as c-Myb and GATA-2. c-Myb promotes the growth of HSCs, and c-Myb-deficient mice die at embryonic day 15.5 due to the defect of definitive hematopoiesis (13). Similarly, GATA-$2^{-/-}$ mice are embryonic lethal around embryonic day 11.5 because of the defect in the development and/or maintenance of HSCs (14), whereas functional roles of GATA-2 in the growth of HSCs are still controversial (15, 16). Also, HOXB4, a member of Hox family of transcription factors, was reported to induce the marked *in vitro* expansion of HSCs (17, 18).

During the last decade, a number of cell cycle regulatory molecules such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors have been identified and characterized in various types of cells. As for the roles of these molecules in primitive hematopoietic cells, $p21^{WAF1}$ and $p27^{Kip1}$ keep the quiescence of HSCs and of progenitor cells, respectively, thereby governing their pool sizes (19, 20). Meanwhile, inactivation and/or deletion of $p16^{INK4A}$ and $p15^{INK4b}$ genes are supposed to contribute to leukemogenesis in a substantial proportion of *all* cases (21, 22). These results imply that appropriate cell cycle control, particularly at the early stage of stem/progenitor cells, is required for maintaining normal hematopoiesis.

c-Myc belongs to a family of transcription factors containing basic, helix-loop-helix, and leucine zipper domains. The expression of c-Myc is absent in quiescent cells and is rapidly induced by growth factors in normal tissues, whereas it is continuously overexpressed in a variety of neoplasms (23–25). In addition, c-Myc is highly expressed in various tissues during embryogenesis, and *c-myc*$^{-/-}$ mice die at embryonic day 10.5, indicating that c-Myc is essential for normal embryonic development (26). c-Myc, in conjunction with its heterodimer partner Max, regulates gene transcription through the element called the E box (CACGTG) (27, 28). c-Myc induces a number of target molecules involved in G_1/S transition such as E2Fs, CDC25A, CDK2, CDK4, Cul1, Id2, and Rb (29–35). Also, c-Myc activates cyclin E/CDK2 complexes alone or in combination with Ras-

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[1] The abbreviations used are: HSC, hematopoietic stem cell; 4-HT, 4-hydroxytamoxifen; SCF, stem cell factor; FL, Flt3 ligand; RBP, recombination signal-binding protein; CDK, cyclin-dependent kinase; Ab, antibody; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; BM, bone marrow; h, human; m, murine.

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mediated signals (36–38). With these activities, c-Myc works at several points during the cell cycle. In fact, fibroblasts isolated from *c-myc*-deficient rats revealed a markedly prolonged cell doubling time accompanied by the drastic reduction of CDK4/6 and CDK2 activities (32, 33). Furthermore, ectopic expression of c-Myc in quiescent cells under some conditions is sufficient for inducing S phase entry (39). These lines of evidence indicates that c-Myc plays a central role in G_1/S transition as an upstream regulator of cell cycle regulatory molecules (40).

Once HSCs are induced to enter cell cycle under certain conditions, HSCs are obliged to select either self-renewal or differentiation during cell division. In this process, Notch signals and HOXB4 promote self-renewal of HSCs rather than differentiation as described above. Therefore, it is speculated that there must be a set of genes induced by Notch signals or HOXB4 contributing to self-renewal of HSCs. However, at present, their critical target molecules remain to be determined. Moreover, it remains unknown how this process is regulated by cell cycle regulatory molecules.

To clarify the roles of cell cycle regulatory molecules in the cell division of HSCs, in this study, we investigated the changes of their expression during Notch- or HOXB4-induced self-renewal of HSCs. We found that c-Myc was transcriptionally induced by Notch and HOXB4. In addition, we found that its ectopic expression induced the growth of HSCs without disrupting their biologic properties in terms of surface phenotypes, colony-forming activities, and reconstituting activities. Together, these results suggest that c-Myc plays a major role in the self-renewal of HSCs as an effector molecule of Notch signals and HOXB4.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human (h) interleukin-6 (hIL-6), murine (m) IL-3, and murine stem cell factor (mSCF) were provided by Kirin Brewery Company (Tokyo, Japan). Human flt3-ligand (hFL) was purchased from PEPROTECH (London, UK). Fluorescein isothiocyanate-conjugated rat IgG1 and biotinylated rat IgG2b were purchased from IMMUNOTECH (Marseilles, France). Biotinylated antilineage antibodies (Abs) against Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (145-2C11), Mac1 (M1/70), and Ter119 (TER119); a fluorescence-labeled anti-Sca-1 Ab (D7), an allophycocyanin-labeled anti-c-Kit Ab (2B8), a fluorescein isothiocyanate-conjugated anti-Ly5.1 Ab (A20), a biotinylated anti-Ly5.2 Ab (104), and streptavidin-phycoerythrin were purchased from PharMingen. A fluorescein isothiocyanate-conjugated anti-CD34 Ab was kindly provided by Dr. T. Hirano (Osaka University, Osaka, Japan).

Plasmid Constructs—An expression vector encoding murine-activated Notch 1(NotchIC) (pSG5mNotchIC) was provided by Dr. G. W. Bornkamm (Institute for Clinical Molecular Biology, Munich, Germany); Notch1/ERT (rNERTneo) was provided by Dr. U. Just (Institute for Clinical Molecular Biology, Munich, Germany) (41); FLAG-tagged RBP-VP16 (pSG5FLAG-RBP-VP16) was provided by Dr. E. Manet (Unite de Virologie Humaine, Lyon, France); HOXB4 (pMSCVneoHOXB4) was provided by Dr. R. K. Humphries (British Columbia Cancer Agency, Vancouver, Canada). A retrovirus expression vector for Myc/ERT was generated by subcloning its cDNA into pMSCVneo (Clontech).

Flow Cytometry—The DNA content of cultured cells was examined by staining with propidium iodide and analyzed on FACSort (BD Biosciences). Cell cycle analysis was performed with the program Modfit LT2.0 (BD Biosciences). Surface phenotypes of cultured cells were examined with FACSCalibur (BD Biosciences).

Semiquantitative Reverse Transcription (RT)-PCR Analyses—Semiquantitative RT-PCR analyses were performed as reported previously (42). Briefly, total cellular RNA was extracted from cultured cells (about 10^5 cells) and reverse-transcribed into cDNA with oligo(dT) primers (Amersham Biosciences) using SuperScript II reverse transcriptase (Invitrogen). PCR was performed in a total volume of 30 μl using 1 μl of the cDNA product as a template. The primer sets to amplify *c-myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, E2F1, and β-actin are as follows: *c-myc*, 5'-TCACCAGCACAACTACGCCG-3' and 5'-CAGGATG-TAGGCGGTGGCTT-3'; cyclin D1, 5'-AGGCGGATGAGAACAAGCAG-A-3' and 5'-CAGGCTTGACTCCAGAAGGG-3'; cyclin D2, 5'-AAGGAG-AAGCTGTCCCTGATC-3' and 5'-GAACTGCTGCAGGCTGTTCAG-3'; cyclin D3, GCGTCCCCACCCGAAAGGCG-3' and CCAGGAAGTCGTG-CGCAATC-3'; cyclin E, 5'-CCCAGCAGTAAGAAGGCAGAG-3' and 5'-CAGCTTCTGGAGCACTCAGTG-3'; E2F1, 5'-GACTGTGACTTTGGG-ACC-3' and 5'-TGTTCACCTTCATTCCC-3'; β-actin, 5'-CATCACTAT-TGGCAACGAGC-3' and 5'-ACGCAGCTCAGTAACAGTCC-3'. The samples were denatured at 94 °C for 10 min followed by 20–35 cycles of amplification (94 °C, 30 s for denaturation; 56 °C, 30 s for annealing; 72 °C, 30 s for extension). At first, we adjusted the amount of cDNA products among several samples based on the amount of β-actin PCR products. Then, an equal amount of cDNA products was subjected to the PCR. The amount of each mRNA was evaluated after 29~35 cycles of PCR, during which PCR products were exponentially amplified in all of the samples. The PCR products were electrophoresed on agarose gels, and their amount was evaluated by staining with SYBR GREEN I (BioWhittaker Molecular Applications, Rockland, ME).

Luciferase Assays—To construct reporter genes containing various fragments of murine *c-myc* promoter, we performed PCR and subcloned PCR products into the luciferase plasmid. Luciferase assays were performed with a Dual-Luciferase reporter system (Promega, Madison, WI) as described previously (42). In short, 293T cells (2×10^5 cells) cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum were seeded in a 60-mm dish and transfected with the appropriate effector gene (2 μg) and reporter gene (2 μg) together with 5 ng of pRL-CMV-Rluc, an expression vector of *renilla* luciferase, by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and then serum-deprived for 24 h. Then, the cells were lysed and subjected to the measurement of the firefly and *renilla* luciferase activities on a luminometer LB96P (Berthold Japan, Tokyo, Japan). The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the *renilla* luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments.

Electrophoretic Mobility Shift Assay (EMSA)—Seven types of double-stranded oligonucleotides were used as probes or competitors, and their sequences are shown in the legend for Fig. 6.

Assays for Telomerase Activities—Telomerase activities were measured by the stretch PCR method with a TeloChaser system (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Isolation of Murine Bone Marrow Hematopoietic Stem/Progenitor Cells—We isolated murine bone marrow hematopoietic stem/progenitor cells as reported previously (42). Briefly, bone marrow cells were harvested from 8–10-week-old Ly5.2 mice pretreated with 150 mg/kg of 5-fluoruracil for 4 days. Mononuclear cells were isolated by density gradient centrifugation. Then, Lin (CD3, B220, Ter119, Mac1, and Gr-1)$^-$ Sca-1$^+$ cells were collected using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). At this step, we confirmed that more than 95% of the separated cells were Lin$^-$Sca-1$^+$ cells by flow cytometric analysis.

Retrovirus Transfection into Murine Bone Marrow Cells—At first, we prepared conditioned medium containing high titer virus particles using Plat-E cells as described previously (42). The isolated Lin$^-$Sca-1$^+$ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of mIL-3 (10 ng/ml), mSCF (50 ng/ml), hIL-6 (50 ng/ml), and hFL (30 ng/ml) for 72 h. Then, the cultured cells were cultured with conditioned medium containing high titer retrovirus in the presence of polybrene (8 μg/ml). After 24 h, the cells were washed and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing the same cytokines and 1 mg/ml G418 for 72 h. After the selection with G418, retrovirus-infected cells were cultured under the conditions as indicated.

Colony Assays—Retrovirus-infected cells (1×10^5 cells/35-mm dish) were cultured in the methylcellulose media containing human erythropoietin (3 units/ml), mIL-3 (10 ng/ml), hIL-6 (10 ng/ml), and mSCF (50 ng/ml) (Stem Cell Technologies, Vancouver, BC, Canada). G418 selection was continued during methylcellulose cultures. The number of colonies was counted after 12 days.

Transplantation Assays—Ly5.2 mice were lethally irradiated (950 radians) 24 h before the transplantation. For transplantation assays, we prepared Myc/ERT-transduced bone marrow cells from congenic C57BL/6 (B6-Ly5.1) mice. Myc/ERT-transduced Ly5.1 cells (4×10^6 cells) were injected intravenously in combination with 1×10^5 normal bone marrow cells with Ly5.2 phenotype.

Statistical Analysis—Statistical analysis was performed with Student's *t* test.

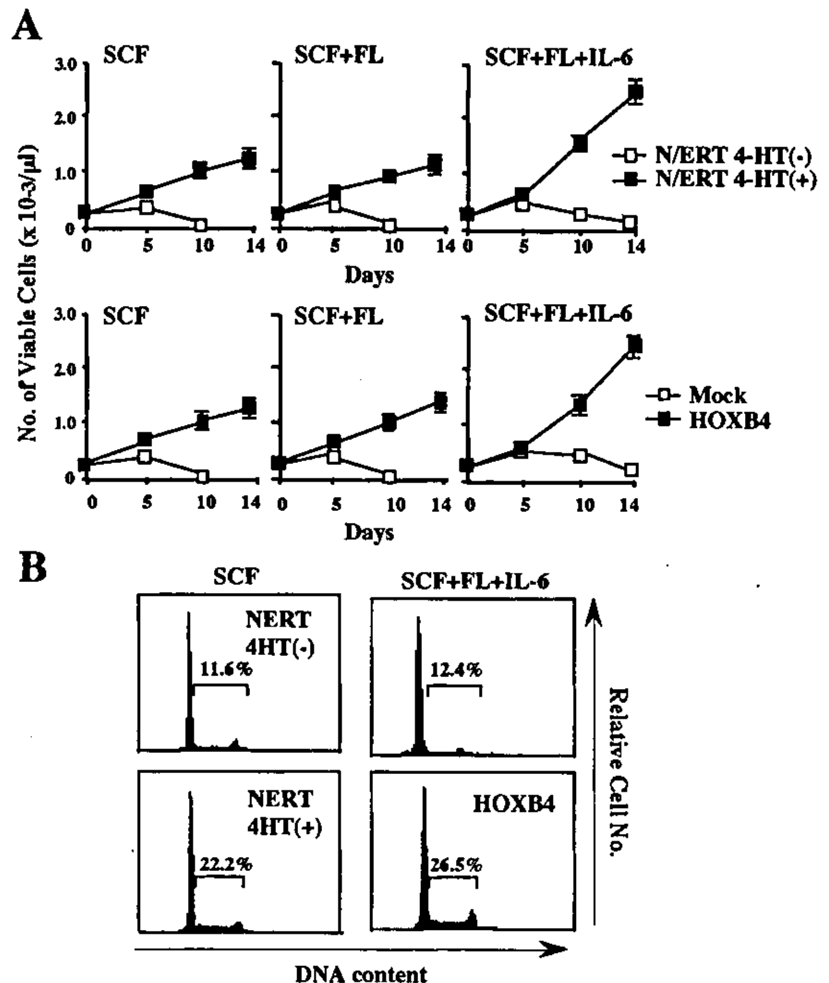


FIG. 1. **Effects of Notch1 and HOXB4 on the growth of murine Lin⁻Sca-1⁺ cells.** *A*, murine Lin⁻Sca-1⁺ cells were infected with retroviruses each containing the indicated gene and cultured for up to 2 weeks. The total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± S.D. of triplicated cultures. *B*, after 7-day cultures under the conditions indicated, the DNA content of the cultured cells was examined by propidium iodide staining. The proportion of the S-G_2/M phase is indicated.

RESULTS

The Effects of Notch1 and HOXB4 on the Expression of Cell Cycle Regulatory Molecules in Murine Lin⁻Sca-1⁺ Cells—First, we analyzed how self-renewal is regulated by cell cycle regulatory molecules in HSCs. For this purpose, we introduced a 4-HT-inducible form of Notch1 (N/ERT, a chimeric molecule consisting of the intracellular domain of Notch1 fused to the estrogen receptor) and HOXB4 into murine Lin⁻Sca-1⁺ cells since both molecules were reported to induce self-renewal in HSCs (17, 43). In the absence of 4-HT, N/ERT-transduced cells could not keep proliferating for 14 days under any cytokine combinations (*i.e.* SCF alone, SCF + FL, or SCF + FL + IL-6) (Fig. 1*A*, *upper panel*). In contrast, 4-HT-treated cells kept growing under the same cytokine combinations during 14 days. Similarly, HOXB4-transduced cells could proliferate for up to 14 days, whereas mock (an empty vector)-transduced cells could not (Fig. 1*A*, *lower panel*). In DNA content analysis, the 4-HT treatment increased the proportion of the cells in S-G_2/M phase from 11.6 to 22.2% in N/ERT-transduced cells under the culture with SCF (Fig. 1*B*, *left panel*). Also, HOXB4 augmented the proportion of the cells in S-G_2/M phase from 12.4 to 26.5% under the culture with SCF + FL + IL-6 (Fig. 1*B*, *right panel*).

Next, we examined the effect of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules by semiquantitative RT-PCR analysis (Fig. 2). With or without the 4-HT treatment, total cellular RNA was extracted from N/ERT-transfected cells after a 7-day culture with SCF. Also, HOXB4-transfected cells were subjected to RT-PCR analysis after a 7-day culture with SCF. At first, we adjusted the amount of

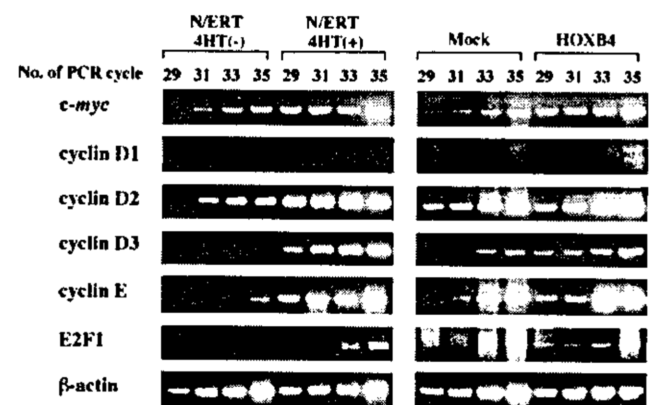


FIG. 2. **Effects of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules.** Murine Lin⁻Sca-1⁺ cells were infected with retroviruses each containing the indicated gene, cultured for 7 days, and subjected to semiquantitative RT-PCR analyses on the expression of c-*myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA. The PCR products were electrophoresed on agarose gels and visualized by staining with SYBR GREEN I.

cDNAs according to the amounts of the β-actin PCR products (Fig. 2, *bottom panel*). Then, we evaluated the expression levels of c-*myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1. After 29–35 cycles of PCR, all of these PCR products were amplified exponentially. As shown in Fig. 2, the 4-HT treatment markedly induced or enhanced the expression of c-*myc*, cyclin D2, cyclin D3, cyclin E, and E2F1, whereas it could not

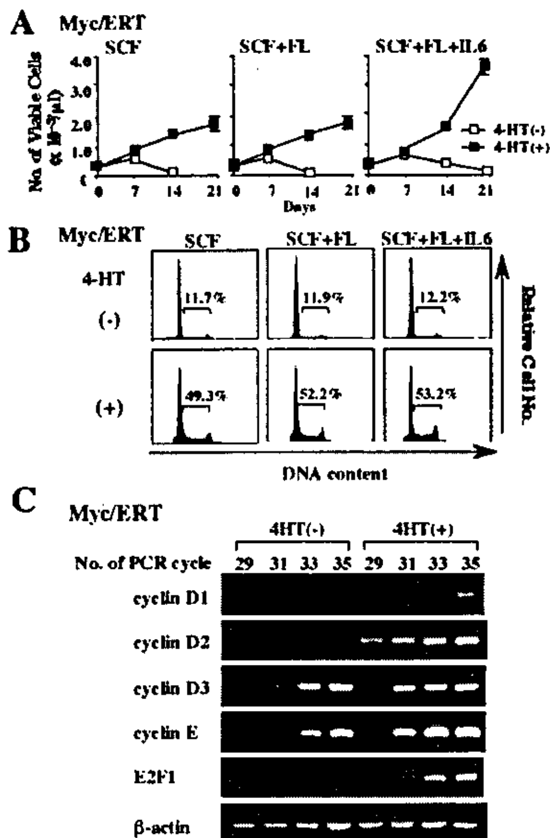


FIG. 3. **Effects of c-Myc on the growth of murine Lin$^-$Sca-1$^+$ cells.** *A*, murine Lin$^-$Sca-1$^+$ cells were infected with retroviruses containing Myc/ERT and cultured with or without 4-HT for up to 3 weeks. The total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± S.D. of triplicated cultures. *B*, after 7-day cultures under the conditions indicated, the DNA content of the Myc/ERT-transduced cells was examined by propidium iodide staining. The proportion of the S-G_2/M phase is indicated. *C*, after 7-day cultures with or without 4-HT, the Myc/ERT-transduced cells were subjected to semiquantitative RT-PCR analyses on the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA.

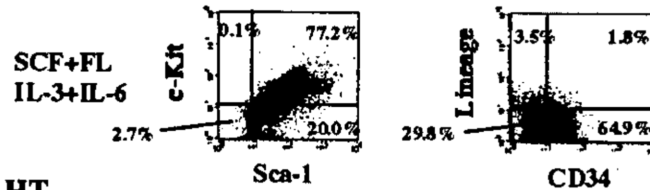
induce the expression of cyclin D1. Similarly, we found that HOXB4 enhanced the expression of *c-myc*, cyclin D2, cyclin D3, cyclin E, and E2F1 but not of cyclin D1.

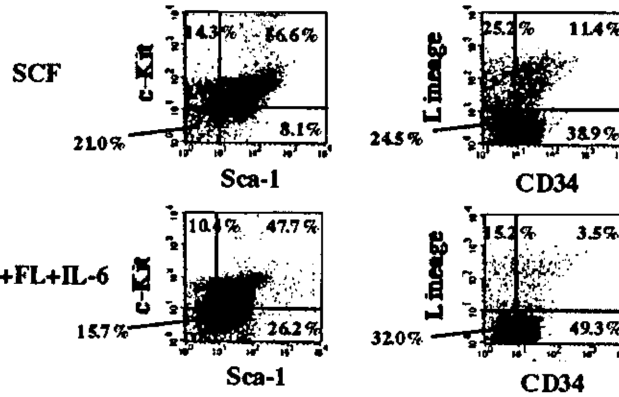
c-Myc Enhances and Supports the Growth of Murine Lin$^-$Sca-1$^+$ Cells—c-Myc is a primary regulator of G_1/S transition, and its ectopic expression can drive quiescent cells to S phase in fibroblasts (as described under the Introduction). Therefore, we examined the effect of c-Myc on the growth and biologic properties of HSCs. We retrovirally transduced a 4-HT-inducible form of Myc (Myc/ERT) to murine Lin$^-$Sca-1$^+$ cells. When treated with 4-HT, Myc/ERT-transduced cells proliferated for more than 21 days under the culture with SCF, SCF + FL, or SCF + FL + IL-6 (Fig. 3*A*). In contrast, 4-HT-untreated cells died within 21 days under the same cytokine combinations. In DNA content analysis, the 4-HT addition prominently increased the cells in S-G_2/M phase in Myc/ERT-transduced cells after 7 days (SCF, from 11.7 to 49.3%; SCF + FL, from 11.9 to 52.2%; SCF + FL + IL-6, from 12.2 to 53.2%) (Fig. 3*B*). Next, we examined the effects of c-Myc on the expression profile of cell cycle regulatory molecules. With or without the 4-HT treatment, we isolated total cellular RNA from Myc/ERT-transfected cells after a 7-day culture with SCF and performed semiquantitative RT-PCR analysis. In Myc/ERT-transduced cells, 4-HT-activated c-Myc induced or enhanced the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA (Fig. 3*C*), suggesting a possibility that c-Myc would be a primary regulator of Notch- or HOXB4-mediated cell growth among various cell cycle regulatory molecules.

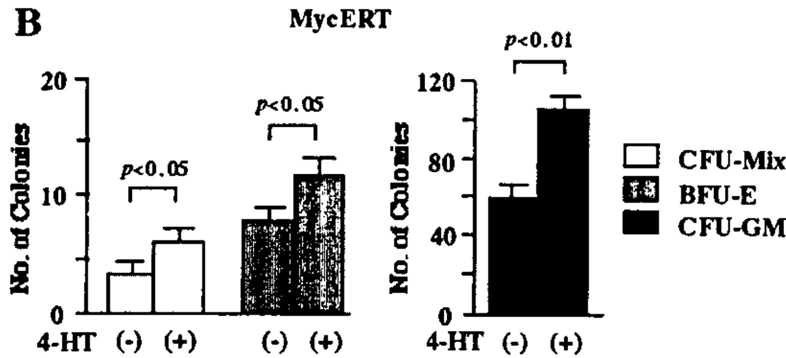
Characterization of the Cells Expanded by Myc/ERT Using in Vitro Assays—The 4-HT treatment was able to keep Myc/ERT-transduced cells viable even after a 28-day culture with SCF or SCF + FL + IL-6 (data not shown). To investigate the biologic properties of these cells, we initially examined their surface phenotypes by fluorescence-activated cell sorter. Before the addition of 4-HT, 77.2% of the Myc/ERT-transduced cells were Sca-1$^+$c-Kit$^+$, and 94.7% (64.9 + 29.8) were Lin$^-$ (including CD34-positive and negative cells). After a 28-day culture with SCF, more than 50% of the cultured cells still revealed immature surface phenotypes: Sca-1$^+$c-Kit$^+$ cells, 56.6%; Lin$^-$ cells, 63.4% (24.5 + 38.9) (Fig. 4*A*). Similar results were also observed after a 28-day culture with SCF+FL+IL-6: Sca-1$^+$c-Kit$^+$ cells, 47.7%; Lin$^-$ cells, 81.3% (32.0 + 49.3). Next, we analyzed the effects of c-Myc on the colony-forming ability of HSCs. After the 3-day selection with G418, Myc/ERT-transduced Sca-1$^+$Lin$^-$ cells were seeded into methylcellulose media (containing IL-3, IL-6, SCF, and erythropoietin) and cultured with or without 4-HT for 12 days. As shown in Fig. 4*B*, 4-HT-activated Myc/ERT increased the number of CFU-Mix with a statistically significant difference $p < 0.05$. Also, Myc/ERT significantly increased burst-forming unit-E ($p < 0.05$) and colony forming units-granulocyte/macrophage ($p < 0.01$). We also analyzed changes of telomerase activity in the Myc/ERT-transduced cells. Quiescent HSCs have been reported to exhibit a low level of telomerase activity, which is up-regulated in response to cytokine stimulation (44, 45), whereas it declines during the maturation process (46). After a 7-day culture in the presence of SCF with or without the 4-HT treatment, we isolated cell extracts from Myc/ERT-transduced cells and evaluated telomerase activity by the stretch PCR method. As observed in a positive control, Hela, the 4-HT treatment induced telomerase activity in Myc/ERT-transduced cells, of which specificity was confirmed by its heat instability. In contrast, Myc/ERT-transduced cells cultured without 4-HT did not have telomerase activity (Fig. 4*C*). Together, these results suggest that c-Myc-induced cell growth does not spoil the immature characteristics of HSCs.

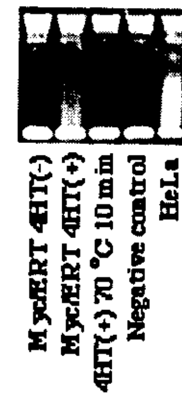
Reconstitution Assays Using HSCs Expanded by Myc/ERT—Next, we evaluated the *in vivo* function of Myc/ERT-expanded HSCs with reconstitution assays. After 12-day cultures, 4-HT-treated or untreated Myc/ERT-transduced cells (4×10^6 cells) with Ly5.1 phenotype were transfused into lethally irradiated Ly5.2 mice in combination with 1×10^5 Ly5.2$^+$ normal bone marrow cells. When 4-HT-treated cells were transplanted, 11.3% of the peripheral blood cells were Ly5.1$^+$ in the recipient mice after 4 weeks (Fig. 4*D*, *upper left panel*). In contrast, Ly5.1$^+$ cells were hardly detected in the mice transplanted with 4-HT-untreated cells after 4 weeks (Fig. 4*D*, *upper right panel*). Furthermore, Ly5.1$^+$ cells were still detected in 7.9% of the peripheral blood cells in the mice transplanted with 4-HT-treated cells even after 12 weeks (Fig. 4*D*, *lower left panel*). In this Ly5.1$^+$ fraction, 15% of the cells were Macl/Gr-1$^+$, 37.1% were CD4/8$^+$, and 50.7% were B220$^+$, indicating that HSCs expanded by Myc/ERT has an ability of multilineage reconstitution. Also, these cells were able to supply hematopoietic cells in the recipient mice for more than 6 months (data not shown). Collectively, these results suggested that ectopically introduced c-Myc can induce self-renewal of HSCs.

The Effects of Notch Signaling and HOXB4 on c-myc Promoter Activities—Since c-Myc was considered to be important for self-renewal of HSCs, we next tried to clarify the mecha-









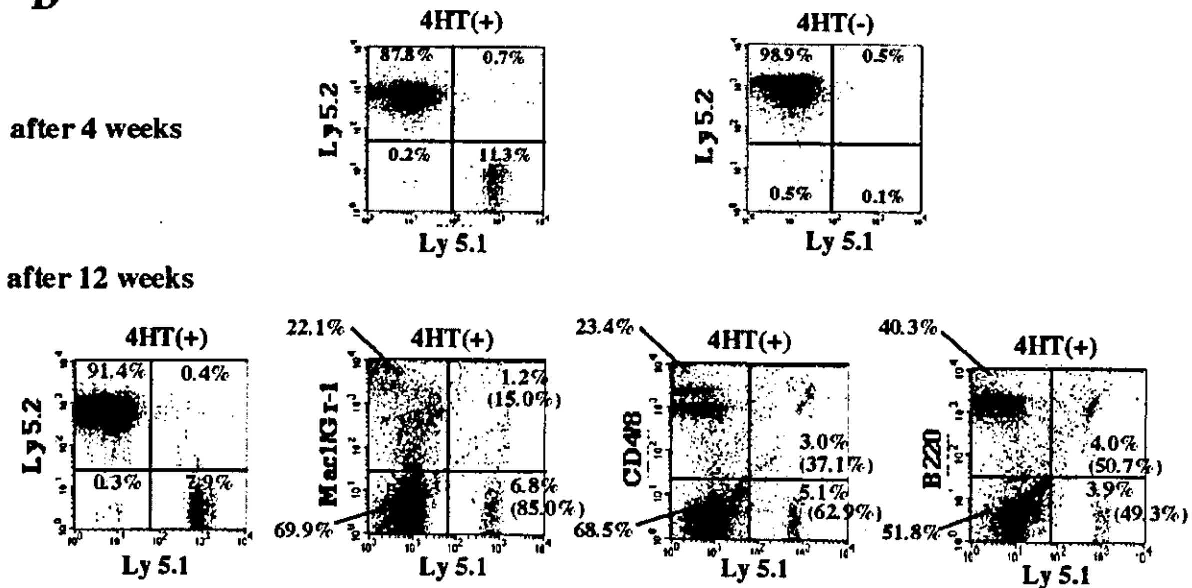


FIG. 4. **Characterization of the cells expanded and maintained by c-Myc for 28 days.** *A*, the surface phenotype of Myc/ERT-transduced cells was examined by flow cytometric analyses before and after the 28-day culture with 4-HT in the presence of SCF or SCF + FL + IL-6. The antilineage Abs recognize Gr-1, B220, CD3e, Mac1 and Ter119. *B*, Myc/ERT-transduced murine Lin$^-$Sca-1$^+$ cells were cultured with G418 for 3 days, and the living cells were seeded into methylcellulose media (containing IL-3, IL-6, SCF, and erythropoietin). The number of the indicated

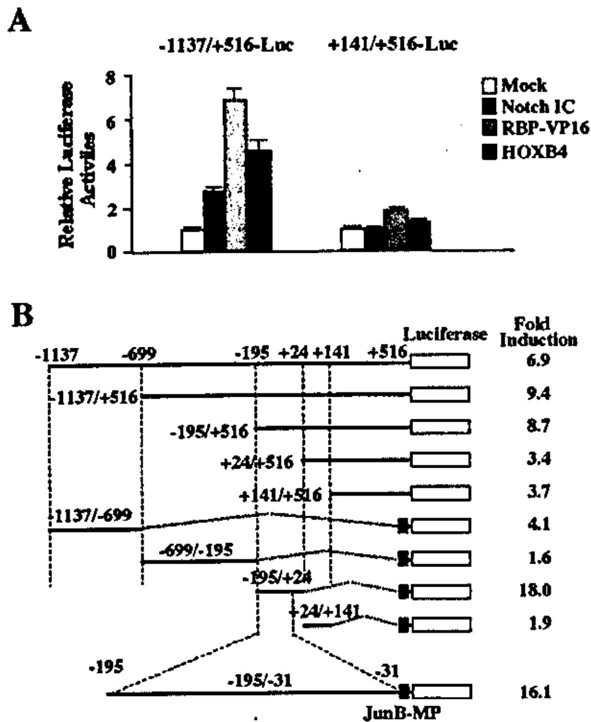


FIG. 5. The effects of Notch signaling and HOXB4 on the activity of the c-myc promoter. *A*, 293T cells seeded in 60-mm dish were transfected with 2 μg of the appropriate effector genes and reporter gene together with 5 ng of pRL-CMV-Rluc by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and cultured without serum for 24 h and then subjected to luciferase assays. After normalizing transfection efficiencies according to the *renilla* luciferase activities, the relative firefly luciferase activities were calculated. The results are shown as the mean ± S.D. of triplicate cultures. *B*, structures of c-*myc* luciferase reporter genes are indicated in the *left panel*, and the effect of RBP-VP16 on the respective reporter gene is indicated as fold induction in the *right panel*.

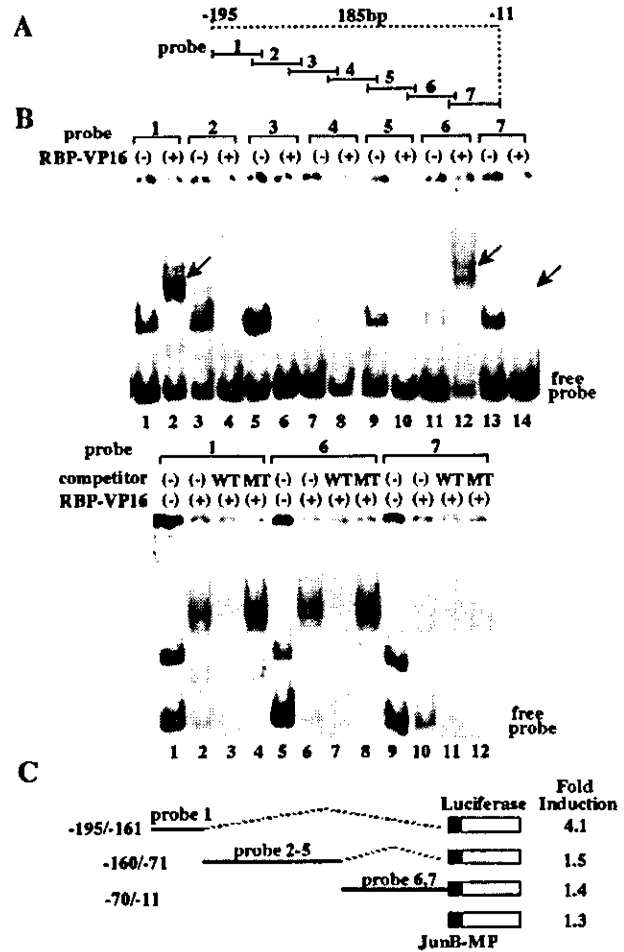


FIG. 6. Analysis of the responsive element to RBP-VP16 in the c-myc promoter. *A*, EMSA was performed with seven overlapping probes spanning between −195 bp and −11 bp in the c-*myc* promoter. The sequences of their probes are as follows: *probe 1*, 5′-ACTGGACO-GCGGGCTGAGGCTCCTCCTCCTCTTTC-3′; *probe 2*, 5′-CTCCTCTTT-CCCCGGCTCCCCACTAGCCCCCTCCC-3′; *probe 3*, 5′-GCCCCCTCC-CGAGTTCCCAAAGCAGAGGGCGGGGA-3′; *probe 4*, 5′-AGGGCGGG-GAAACGAGAGGAAGGAAAAAAATAGAG-3′; *probe 5*, 5′-AAAAATAG-AGAGAGGTGGGGAAGGGAGAAAGAGAG-3′; *probe 6*, 5′-AGAAAGA-GAGGTTCTCTGGCTAATCCCCGCCCACC-3′; *probe 7*, 5′-CCCGCCC-ACCCGCCCTTTATATTCCGGGGGTCTGC-3′. *B*, nuclear extracts were isolated from 293T cells transfected with FLAG-RBP-VP16 or a mock vector and subjected to EMSA. In competition assays, a 1000-fold molar excess of unlabeled wild-type or mutant competitor oligonucleotide was added to the binding mixture. *C*, 293T cells were transfected with the indicated reporter gene together with FLAG-RBP-VP16 as an effector gene. After 12 h, the cells were washed and cultured without serum for 24 h and then subjected to luciferase assays. The results are shown as the mean ± S.D. of triplicate cultures.

nism through which Notch1 and HOXB4 induce the c-*myc* mRNA expression. For this purpose, we performed luciferase assays with a reporter gene −1137/+516-Luc containing the murine c-*myc* promoter (numbered from the transcription initiation site) with NotchIC (an activated form of Notch1), RBP-VP16 (a transcriptionally active derivative of RBP-J), and HOXB4 as effectors. In 293T cells, NotchIC, RBP-VP16, and HOXB4 activated −1137/+516-Luc by 2.9-, 6.9-, and 4.7-fold, respectively, whereas these effector genes hardly stimulated its backbone vector +141/+516-Luc (Fig. 5*A*). Similar results were obtained from a murine fibroblast cell line NIH3T3 (data not shown). To determine which region is responsive to Notch in the c-*myc* promoter, we generated several reporter genes and performed luciferase assays with RBP-VP16 as an effector gene. As shown in Fig. 5*B*, −195/+24-Luc was most responsive to RBP-VP16 (18-fold induction). Also, the similar level (16.1-fold) of activation was induced by RBP-VP16 in −195/−31-Luc. To further identify which element is responsive to RBP-VP16, we isolated nuclear extracts from 293T cells transfected with FLAG-RBP-VP16 or a mock vector and performed EMSA with seven overlapping probes spanning between −195 and −11 of the c-*myc* promoter (Fig. 6*A*). As compared with DNA binding patterns formed from mock-transfected cells, nuclear extracts isolated from FLAG-RBP-VP16-transfected cells bound to the

colonies was counted after 12 days. The results are shown as mean ± S.D. of quintupled cultures. *BFU-E*, burst-forming unit-E. *CFU-GM*, colony forming units-granulocyte/macrophage. *C*, after 7-day cultures, the cultured cells were subjected to the stretch PCR method for the evaluation of telomerase activities. *Lane 1*, Myc/ERT-transduced cells cultured without 4-HT; *lane 2*, Myc/ERT-transduced cells cultured with 4-HT; *lane 3*, the heat-treated sample of *lane 2*; *lane 4*, negative control (PCR was performed without any template.); *lane 5*, Hela cells as a positive control. *D*, reconstitution assays using the cells expanded by Myc/ERT. MycERT-transduced Ly5.1 cells (4×10^6 cells) cultured with (*upper left panel*) or without (*upper right panel*) 4-HT were transplanted into lethally irradiated mice in combination with 1×10^5 normal bone marrow cells with Ly5.2 phenotype. The contribution of Myc/ERT-transduced Ly5.1 cells in the peripheral blood of the recipient mice was examined by flow cytometric analyses after 4 (*upper two panels*) and 12 weeks (*lower four panels*) from transplantation. The lineage distribution of Ly5.1 cells was examined with the indicated Abs. The relative frequency of the each fraction in the Ly5.1$^+$ cells is shown in the parenthesis.

probes 1, 6, and 7 (Fig. 6*B*, *upper panel*, *lanes 2*, *12*, and *14*, DNA-binding complexes are indicated by *arrows*), whereas a significant difference was not detected with the other probes. These binding complexes were abolished by non-labeled wild-type competitors (Fig. 6*B*, *lower panel*, *lanes 3*, *7*, and *11*) but not by mutated competitors (Fig. 6*B*, *lower panel*, *lanes 4*, *8*, and *12*), indicating that these complexes were formed in a sequence-specific manner. To further characterize the role of these sites in Notch signals, we performed luciferase assays with reporter genes each containing the sequence of the probe 1 (−195/−161-Luc) and probes 6–7 (−70/−11-Luc). As shown in Fig. 6*C*, RBP-VP16 activated −195/−161-Luc by 4.1-fold, whereas it was hardly effective on −70/−11-Luc, implying that the protein that binds to the probe 1 would contribute to the activation of the *c-myc* promoter by Notch signaling. However, this complex reacted neither to the anti-FLAG Ab nor to the anti-RBP-J Ab (data not shown). Also, the probe 1 did not contain the typical RBP-J-binding sequence. These results suggest that RBP-VP16 would activate this element indirectly. In addition, since the reactivity of −195/−161-Luc was not as prominent as that of −195/−31-Luc, it was speculated that an additional element would be necessary for the *c-myc* promoter to achieve the maximal response to Notch signals.

DISCUSSION

With regard to the roles for Notch and HOXB4 in cell cycle regulation, Notch1 activation was reported to shorten the G_1 phase of cell cycle, thereby delaying the differentiation of human HL-60 cells and primary $CD34^+$ cells (47). Also, Ronchini and Capobianco (48) showed that the intracellular domain of Notch directly activated cyclin D1 promoter in a human kidney cell line, 293T. In addition, Krosl and Sauvageau (49) reported that overexpression of HOXB4 led to the induction of Jun-B and Fra-1 expression and subsequent up-regulated expression of cyclin D1 in Rat-1 fibroblast. To identify a critical target molecule in Notch- and HOXB4-induced self-renewal of HSCs, we analyzed their effects on the expression of cell cycle regulatory molecules and found that both molecules up-regulated the expression of *c-myc*, cyclin D2, cyclin D3, cyclin E, and E2F1. However, neither Notch 1 nor HOXB4 up-regulated the expression of cyclin D1, whereas its expression was up-regulated by these molecules in other cell types (as described above). These findings were consistent with the previous report that cyclin D1 was not detected in the proliferating normal hematopoietic stem/progenitor cells (50). Although cyclin D1 expression did not accompany the proliferation of normal hematopoietic stem/progenitor cells, our results suggest that G_1/S transition in self-renewal of HSCs is not so unique but rather common to other cell types. In addition, we found that Myc/ERT efficiently induced the expression of cyclin D2, cyclin D3, and cyclin E and promoted the growth of HSCs. In addition, previous reports suggested that c-Myc would transcriptionally repress the expression of $p21^{WAF1}$, which plays a crucial role in the quiescence of HSCs (19, 51), and of $p15^{INK4b}$, which is induced by transforming growth factor-β signaling (52, 53). Together, these results imply that the *c-myc* could be a master regulator of the cell cycle in both Notch1- and HOXB4-induced self-renewal of HSCs.

In this study, promoter assays revealed that both Notch signaling and HOXB4 activated the *c-myc* promoter. However, the element most responsive to RBP-VP16 in the *c-myc* promoter did not contain the putative RBP-J-binding sequence. Also, we confirmed that the nuclear protein, which bound to this element, did not contain RBP-VP16 itself in EMSA with supershift assays, suggesting RBP-VP16 could activate the *c-myc* promoter indirectly. To understand the mechanism of Notch-induced self-renewal of HSCs, we are now trying to purify this molecule using nuclear extracts from RBP-VP16-transfected 293T cells.

In this study, reconstitution assays revealed that cells expanded by Myc/ERT could contribute to hematopoiesis for more than 6 months, indicating that these cells still possess characteristics of HSCs in terms of long term reconstitution. In addition, these cells developed Mac1/Gr-1^+ cells, CD4/8^+ cells, and $B220^+$ cells, implying that the cells expanded by Myc/ERT have a multilineage reconstitution ability. When we compare the reconstitution efficiency of Myc/ERT-expanded cells with that of Notch1-expanded cells (43), Myc/ERT-expanded cells showed similar or a little more potent reconstitution abilities than Notch-expanded cells. (120-fold excess of Notch1-expanded cells (*i.e.* transplantation of 120×10^5 Notch1-expanded cells in combination with 1×10^5 normal BM cells) contributed to hematopoiesis in about 23% of BM cells after transplantation *versus* 40-fold excess of Myc/ERT-expanded cells (*i.e.* transplantation of 40×10^5 Myc/ERT-expanded cells in combination with 1×10^5 normal BM cells) participated in hematopoiesis in about 10% of the peripheral blood cells.) However, even if sufficient numbers (1×10^7) of Myc/ERT-expanded cells were transplanted, co-injection of normal supporting BM cells was required for radioprotection in the recipient mice (data not shown). These results indicate that the cells expanded by Myc/ERT lack some ability to fully or rapidly reconstitute hematopoiesis *in vivo*. The similar defect was observed in HSCs expanded by Notch1 (43). Like Myc/ERT-expanded cells, Notch1-expanded cells alone could not reconstitute hematopoiesis in lethally irradiated mice, whereas these cells could participate in hematopoiesis for more than 6 months in combination with normal supporting BM cells. One possible explanation is that the *ex vivo* culture of HSCs or modulation of cell cycle by Notch1 or c-Myc might result in the reduced homing abilities after intravenous transplantation. For example, Szilvassy *et al.* (54) showed that HSCs expanded by growth factors *in vitro* expressed little or no β_1 integrin, and this change was associated with a failure of radioprotection. Alternatively, HSCs induced to proliferate by Notch or c-Myc *in vitro* might not be able to rapidly undergo terminal differentiation after transplantation, resulting in the failure to supply a sufficient number of mature cells required for radioprotection.

In summary, our results indicate that c-Myc is, at least in part, capable of supporting self-renewal of HSCs as a downstream mediator of Notch and HOXB4. Further analyses on cell cycle regulation would undoubtedly provide more informative findings to expand HSCs *in vitro*.

REFERENCES

1. Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000) *J. Exp. Med.* **192,** 1281–1288
2. Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996) *Science* **273,** 243–245
3. Cheshier, S. H., Morrison, S. J., Liao, X., and Weissman, I. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3120–3125
4. Nakauchi, H., Sudo, K., and Ema, H. (2001) *Ann. N. Y. Acad. Sci.* **938,** 18–24
5. Petzer, A. L., Zandstra, P. W., Piret, J. M., and Eaves, C. J. (1996) *J. Exp. Med.* **183,** 2551–2558
6. Sitnicka, E., Lin, N., Priestley, G. V., Fox, N., Broudy, V. C., Wolf, N. S., and Kaushansky, K. (1996) *Blood* **87,** 4998–5005
7. Ueda, T., Tsuji, K., Yoshino, H. Ebihara, Y. Yagasaki, H. Hisakawa, H., Mitsui, T., Manabe, A., Tanaka, R., Kobayashi, K., Ito, M., Yasukawa, K., and Nakahata, T. (2000) *J. Clin. Invest.* **105,** 1013–1021
8. Massague, J., Blain, S. W., and Lo, R. S. (2000) *Cell* **103,** 295–309
9. Fortunel, N. O., Hatzfeld, A., and Hatzfeld, J. A. (2000) *Blood* **96,** 2022–2036
10. Clark, B. R., and Keating, A. (1995) *Ann. N. Y. Acad. Sci.* **770,** 70–78
11. Yokota, T., Oritani, K., Mitsui, H., Aoyama, K., Ishikawa, J., Sugahara, H., Matsumura, I., Tsai, S., Tomiyama, Y., Kanakura, Y., and Matsuzawa, Y. (1998) *Blood* **91,** 3263–3272
12. Varnum-Finney, B., Purton, L. E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K. A., Le Roux, I., Mann, R., Gray, G., Artavanis-Tsakonas, S., and Bernstein, I. D. (1998) *Blood* **91,** 4084–4091
13. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., Jr., and Potter, S. S. (1991) *Cell* **65,** 677–689
14. Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt,

F. W., and Orkin, S. H. (1994) *Nature* **371**, 221–226
15. Heyworth, C., Gale, K., Dexter, M., May, G., and Enver, T. (1999) *Genes Dev.* **13**, 1847–1860
16. Persons, D. A., Allay, J. A., Allay, E. R., Ashmun, R. A., Orlic, D., Jane, S. M., Cunningham, J. M., and Nienhuis, A. W. (1999) *Blood* **93**, 488–499
17. Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002) *Cell* **109**, 39–45
18. Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., and Humphries, R. K. (1995) *Genes Dev.* **9**, 1753–1765
19. Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D. T. (2000) *Science* **287**, 1804–1808
20. Cheng, T., Shen, H., Rodorigues, N., Stier, S., and Scadden, D. T. (2001) *Blood* **98**, 3643–3649
21. Ogawa, S., Hangaishi, A., Miyawaki, S., Hirosawa, S., Miura, Y., Takeyama, K., Kamada, N., Ohtake, S., Uike, N., and Shimazaki, C. (1995) *Blood* **86**, 1548–1556
22. Takeuchi, S., Bartram, C. R., Seriu, T., Miller, C. W., Tobler, A., Janssen, J. W., Reiter, A., Ludwig, W. D., Zimmermann, M., and Schwaller, J. (1995) *Blood* **86**, 755–760
23. Dang, C. V. (1999) *Mol. Cell. Biol.* **19**, 1–11
24. Dang, C. V., Resar, L. M., Emison, E., Kim, S., Li, Q., Prescott, J. E., Wonsey, D., and Zeller, K. (1999) *Exp. Cell Res.* **253**, 63–77
25. Obaya, A. J., Mateyak, M. K., and Sedivy, J. M. (1999) *Oncogene* **18**, 2934–2941
26. Davis, A. C., Wilms, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993) *Genes Dev.* **7**, 671–682
27. Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N., and Weintraub, H. (1990) *Science* **250**, 1149–1151
28. Blackwood, E. M., and Eisenman, R. N. (1991) *Science* **251**, 1211–1217
29. Galaktionov, K., Chen, X., and Beach, D. (1996) *Nature* **382**, 511–517
30. Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J. F., Obaya, A. J., O'Connell, B. C., Mateyak, M. K., Tam, W., Kohlhuber, F., Dang, C. V., Sedivy, J. M., Eick, D., Vogelstein, B., and Kinzler, K. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2229–2234
31. Lasorella, A., Noseda, M., Beyna, M., Yokota, Y., and Iavarone, A. (2000) *Nature* **407**, 592–598
32. Mateyak, M. K., Obaya, A. J., Adachi, S., and Sedivy, J. M. (1997) *Cell Growth & Differ.* **8**, 1039–1048
33. Mateyak, M. K., Obaya, A. J., and Sedivy, J. M. (1999) *Mol. Cell. Biol.* **19**, 4672–4683
34. O'Hagan, R. C., Ohh, M., David, G. de Alboran, I. M., Alt, F. W., Kaelin, W. G., Jr., and DePinho, R. A. (2000) *Genes Dev.* **14**, 2185–2191
35. Wagner, A. J., Meyers, C., Laimins, L. A., and Hay, N. (1993) *Cell Growth & Differ.* **4**, 879–883
36. Berns, K., Hijmans, E. M., and Bernards, R. (1997) *Oncogene* **15**, 1347–1356
37. Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. (1997) *Nature* **387**, 422–426
38. Muller, D., Bouchard, C., Rudolph, B., Steiner, P., Stuckmann, I., Saffrich, R., Ansorge, W., Huttner, W., and Eilers, M. (1997) *Oncogene* **15**, 2561–2576
39. Kaczmarek, L., Hyland, J. K., Watt, R., Rosenberg, M., and Baserga, R. (1985) *Science* **228**, 1313–1315
40. Eilers, M., Schirm, S., and Bishop, J. M. (1991) *EMBO J.* **10**, 133–141
41. Schroeder, T., and Just, U. (2000) *EMBO J.* **19**, 2558–2568
42. Ezoe, S., Matsumura, I., Nakata, S., Gale, K., Ishihara, K., Minegishi, N., Machii, T., Kitamura, T., Yamamoto, M., Enver, T., and Kanakura, Y. (2002) *Blood* **100**, 3512–3520
43. Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S., and Bernstein, I. D. (2000) *Nat. Med.* **6**, 1278–1281
44. Counter, C. M., Gupta, J., Harley, C. B., Leber, B., and Bacchetti, S. (1995) *Blood* **85**, 2315–2320
45. Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., and Yamakido, M. (1995) *J. Immunol.* **155**, 3711–3715
46. Chiu, C. P., Dragowska, W., Kim, N. W., Vaziri, H., Yui, J., Thomas, T. E., Harley, C. B., and Lansdorp, P. M. (1996) *Stem Cells (Miamisburg)* **14**, 239–248
47. Carlesso, N., Aster, J. C., Sklar, J., and Scadden, D. T. (1999) *Blood* **93**, 838–848
48. Ronchini, C., and Capobianco, A. J. (2001) *Mol. Cell. Biol.* **21**, 5925–5934
49. Krosl, J. Sauvageau, G. (2000) *Oncogene* **19**, 5134–5141
50. Ragione, F. D., Borriello, A., Mastropietro, S., Pietra, V. D., Monno, F., Gabutti, V., Locatelli, F., Bonsi, L., Bagnara, G. P., and Iolascon, A. (1997) *Biochem. Biophys. Res. Commun.* **231**, 73–77
51. Claassen, G. F., and Hann, S. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9498–9503
52. Dao, M., Hwa, J., and Nolta, J. A. (2002) *Blood* **99**, 499–506
53. Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., and Massague, J. (2001) *Nat. Cell Biol.* **3**, 400–408
54. Szilvassy, S. J., Meyerrose, T. E., Ragland, P. L., and Grimes, B. (2001) *Exp. Hematol.* **29**, 1494–1502

Journal of Biological Sciences 5 (1): 50-60, 2005
ISSN 1727-3048
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Cell Cycle Regulation in Hematopoietic Stem/progenitor Cells

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Abstract: Hematopoietic Stem Cells (HSCs) are characterized by two distinct abilities, that is, self-renewal ability and multipotency. To keep the homeostasis of hematopoiesis and protect the exhaustion of HSCs throughout the life, most of HSCs are kept quiescent and only a limited number of HSCs enter cell cycle to supply mature blood cells. Cell cycle state of HSCs is crucially regulated by external factors such as cytokines, Notch ligands and Wnt signals in the Bone Marrow (BM) microenvironment, so called hematopoietic niche. In addition, the intrinsic factors expressed in HSCs such as c-Myb, GATA-2, HOX family proteins and Bmi-1 also control their growth through the gene transcription. Cell cycle regulation in HSCs is not so unique but rather common to other cell types. However, the specific function of each cell cycle regulatory molecule in HSCs has been clarified during the last few years. Especially, $p21^{WAF1}$ and $p18^{INK4C}$ keep the quiescence of HSCs and $p27^{CIP1}$ keeps that of progenitor cells, respectively, thereby governing their pool sizes and/or preventing their exhaustion. On the other hand, the inactivation or deletion of $p16^{INK4A}$ and $p15^{INK4B}$ genes is supposed to contribute to malignant transformation of hematopoietic cells. These results imply that appropriate cell cycle control at the stage of stem/progenitor cells in the BM is required for maintaining normal hematopoiesis.

Key words: Hematopoietic stem/progenitor cell, cell cycle

INTRODUCTION

HSCs are characterized by two distinct abilities; self-renewal ability and multipotency. With these activities, HSCs are capable of maintaining a life-long supply of all lineages of hematopoietic cells according to systemic needs. The durability of the output potential of HSCs is believed to be dependent on their ability to execute self-renewal divisions; that is, an ability to proliferate without activation of a latent readiness to differentiate along restricted lineages. To maintain the homeostasis of hematopoiesis and protect the exhaustion of HSC population, most of HSCs are kept quiescent and only a limited number of cells enter cell cycle to supply mature blood cells. During this cell division, HSCs are obliged to undergo self-renewal, differentiation, or apoptosis. This step is controlled by external stimuli transmitted from the Bone Marrow (BM) microenvironment, including cytokines, Notch ligands, Wnt signals and sonic hedgehog (Shh) signals. Also, intrinsic factors expressed in HSCs, such as transcription regulators and cell cycle regulatory molecules, are crucially involved in this regulation (Fig. 1).

During the last decade, a number of cell cycle regulatory molecules such as cyclins, Cyclin-dependent Kinases (CDKs) and CDK inhibitors (CKIs) have been identified and their roles and regulation have been well characterized in various types of cells[1-3]. Cell cycle is positively regulated by CDKs associated with cyclins and their activities are negatively regulated by CKIs also included in these complexes at the same time. CKIs are classified into two families based on their structures and CDK targets. One class of inhibitors including $p21^{WAF1}$ (hereafter indicated as p21), $p27^{KIP1}$ (p27) and $p57^{KIP2}$ share a CDK2-binding motif in the N-terminus and inhibit the activities of cyclinD-, E- and A-dependent kinases. The other class of inhibitors also known as the INK4 family, including $p16^{INK4A}$ (p16), $p15^{INK4B}$ (p15), $p18^{INK4C}$ (p18) and $p19^{INK4D}$, contain fourfold ankyrin repeats and specifically inhibit CDK4 and CDK6. Members of both families are important for executing cell cycle arrest in response to a variety of stimuli such as DNA damage, contact inhibition and transforming growth factor-β1 (TGF-β1) treatment.

Molecular mechanisms governing the stemness of HSCs from a viewpoint of cell cycle regulation are presented in this study.

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Characteristic of HSCs: The procedure for the purification of HSCs has made great progress along with the identification of molecular markers that characterize the cells having reconstitution activities in transplanted mice. The most primitive HSCs are considered to be with the CD34$^{low/-}$c-Kit$^+$Sca-1$^+$Lin$^-$ (CD34-KSL) phenotype, since a single cell with this phenotype could reconstitute whole hematopoiesis *in vivo* with high probability[4]. In addition to the specific surface phenotype, HSCs present in steady-state adult mouse BM are functionally characterized by their ability to efflux Rhodamine-123 and Hoechst 33342[5,6]. When adult mouse BM cells are stained with Hoechst 33342, exposed to the UV light and examined at 2 emission wavelengths simultaneously, HSCs are found in the rare Side Population (SP) with the dim fluorescence because of this ability[7]. The low fluorescence of HSCs after staining with Rhodamine-123 and Hoechst 33342 is attributed to their selective expression of different ABC transporters, P-glycoprotein and bcrp-1, respectively[8-10]. In addition, a more recent study proved that the cells having the strongest dye efflux capacity (Tip-SP cells) with the CD34-KSL phenotype are the most primitive HSCs, which can reconstitute long-term hematopoiesis with almost 100% probability after the single cell transplantation[11]. The cells in the SP fraction is considered to be in the G0 phase and this state is supposed to be restrictedly regulated by "hematopoietic niche" in the BM as described later.

Cytokines involved in cell cycle regulation in HSCs: A number of cytokines regulate growth, differentiation and survival of HSCs both positively and negatively. Among these, stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3) and IL-6 are known to promote the growth of HSCs *in vitro*[12-14]. In fact, Sl/Sl and W/W mice each having homozygous defect in the SCF gene and its receptor c-kit gene reveal severe anemia[15]. Also, total number of HSCs was reduced in the BM of c-mpl (TPO receptor)-null mice[16]. In addition, HSCs obtained from c-mpl$^{-/-}$ mice reveled severely decreased activities in reconstitution assays. These lines of evidence indicate that cytokine signals are required for the growth and survival of HSCs *in vivo* as well as *in vitro*[17].

During the last few years, the number of cases with hematologic malignancies receiving cord blood transplantation has increased more and more. However, the insufficient number of HSCs in each cord blood and the delayed recovery of hematopoiesis have limited their applicability to transplantation for adults. So, it has been of particular interest to expand hematopoietic cells *ex vivo*. Regarding the effects of cytokines in the *ex vivo* expansion of HSCs, a number of cytokine combinations were employed and their effects were evaluated by long-term reconstitution assays in transplanted mice. Among these, the combination of SCF, FL, TPO and IL-6/soluble IL-6 receptor seems to induce the *ex vivo* expansion of HSCs most efficiently with 4.2-fold increase of SRC (SCID-repopulating cells)[18].

TGF-β1 is a 25 kd protein produced by the stromal cells and by the hematopoietic progenitors, which induces the growth arrest in HSCs in autocrine and/or paracrine manners[19-23]. Using antisense oligonucleotides, it was demonstrated that the inhibition of TGF-β1 production could release HSCs in the umbilical cord blood or BM from quiescence[22,24,25]. Furthermore, the inhibition of the TGF-β1 signaling pathways in human HSCs using blocking antibodies against TGF-β1 or its receptor allowed quiescent cells to enter the cell cycle[26]. TGF-β1 has been supposed to induce cell-cycle arrest through p21 and p27 in various cell types including HSCs[27-33]. However, a recent paper provided evidence that TGF-β1 induces growth arrest independently of p21 or p27 by demonstrating that TGF-β1 can suppress the growth of HSCs and progenitor cells lacking both p21 and p27[34]. As for the other possible mechanisms of TGF-β1-induced growth arrest, TGF-β1 was reported to transcriptionally induce the expression of p15[35,36] and to down regulate the expression of c-Kit, FLT3 andIL-6 receptor on HSCs, thereby disrupting cytokine-dependent growth signals[37,38].

In contrast, another TGF-β super family protein, bone morphogenetic protein-4 (BMP-4) was recently reported to induce self-renewal of HSCs[39].

Effects of the BM microenvironment "hematopoietic niche" on cell cycle regulation in HSCs: HSCs receive critical signals for proliferation and differentiation from the BM microenvironment consisting of stromal cells and the extracellular matrix (ECM)[40-42]. ECM is composed of a variety of molecules such as fibronectin (FN), collagens, laminin and proteoglycans. ECM in the BM is not merely an inert framework but mediates specialized functions[43-45]. Some components of ECM have been shown to bind to growth factors produced by stromal cells and to immobilize them around cells, resulting in giving spaces where hematopoietic cells and growth factors colocalize. In addition, ECM can bind to glycoproteins expressed on HSCs. FN, collagens and laminin are ligands for integrins that not only control anchorage, spreading and migration of HSCs but also activate signal transduction pathways in these cells[43,44,46,47].

As were the cases with the niches for gut and certain skin stem cells[48-50], it has been supposed that HSCs also receive critical signals for proliferation and differentiation from the BM microenvironment "hematopoietic niche". However, it has been unknown where the hematopoietic niche is located in the BM or what types of cells