このように、mKirreの抗体は通常の手法では作成することが困難であり、この原因としては次の２点が考えられる。（１）mKirreの細胞外領域は極めて抗原性が低いこと、及び（２）免疫グロブリン様領域という多くのタンパク質に存在するモチーフをmKirreが有しており、これに反応する抗体ができるとmKirre特異性が失われる。

そこで、免疫グロブリン様領域を避け、抗原性の高いペプチド配列部分を想定する方法を検討した。免疫グロブリン様領域を避け、抗原性の高いペプチド配列部分を想定するためにコンピュータソフトウェアを用いた。用いたソフトウェアはマックベクター（MacVector）、ペプツール（Peptool）、及びスキャンプロサイト（Scan Procite）の３種類である。その結果、最終的にタンパク質から切り離されるシグナル配列（１－１７）、及び免疫グロブリン様領域を除外した細胞外領域においては、アミノ酸配列で３２－４８番目の（ＧＹＭＡＫＤＫＦＲＲＭＮＥＧＱＶＹ）領域を想定することができた。この領域の平均疎水性値は－１３．３で、電荷密度（Charge Density）は０．５３であった。この値は、検討した配列の中で最良の結果を示すものであった。

このアミノ酸配列をヒトの配列と比較すると、１００％一致していたが、免疫動物であるラットと比較するとアミノ酸配列３２－３３において異なっており（マウスＧＹ、ラットＡＴ）、抗原としても有利であると考えられた。

そこで、このアミノ酸配列（ＧＹＭＡＫＤＫＦＲＲＭＮＥＧＱＶＹ）よりペプチドを合成し、ＫＬＨキャリアタンパク質に結合させ、ラビットに免疫してポリクローナル抗体を作製、抗原カラムにて精製し、一部はFITC標識した。この結果得られた抗体はウェスタン解析にて極めてバックグラウンドが低くmKirreを認識し、またmKirreを高発現したCHO-k1細胞をflowcytemetryにて認識した。

そこで次の段階として同じ抗原を用い、モノクローナル抗体の作製を試みた。７週齢のラット♀)のフットパッドに上記抗原を計３回免疫した（初回免疫後、３日目、７日目）。初回免疫後９日目にラットの鼠経リンパ節を採取し、リンパ球をPEG法にてマウスミエローマ細胞株Ｐ３Ｕ１と融合させて、融合細胞を作製した。ＨＡＴ培地中で培養し、ハイブリドーマを選択した。ペルオキシダーゼ標識抗ラットＩｇＧ（Ｈ＋Ｌ）によるＥＬＩＳＡ反応にて陽性細胞集団を選択した。

これら陽性細胞集団から最終的に単クローンにて抗原ペプチドとの反応性を示すモノクローナル抗体を安定的に産生するハイブリドーマを３クローン（１Ａ８、４Ａ８、及び４Ｂ１０）樹立することができた。

得られたモノクローナル抗体の各クローンについて抗原とした合成ペプチドによるＥＬＩＳＡ反応を測定した。結果を次の表２に示す。

【表２】

クローン名	測定値
4B10	1.642
1A8	1.218
4A8	1.172
Negative control	0.091

これらのクローンは全て限界希釈法にて一個の細胞から樹立されたものであり、また、ミエローマ細胞はマウス由来であり、このELIZA反応がラット特異的２次抗体を使用してい

ることから、得られたモノクローナル抗体が、ラット由来であり、且つmKirre ペプチドを認識していることが証明された。

また、抗原として用いたペプチド配列（GYMAKDKFRRMNEGQVY　マウス mKirre のアミノ酸配列の３２－４８番目）は、ヒトホモログであるKIAA１８６７のアミノ酸配列３２－４８と完全に一致しており、本研究のモノクローナル抗体は、マウス由来ではあるが、ヒトの Kirre をも認識することができるものである。

これらの３クローンをウェスタン解析及び免疫染色に用いてみたところ、ウェスタン解析にはいずれも用いることができなかったが、4B10 のみマウス成体骨髄の凍結切片を染色する事が判明し、骨髄の辺縁部で骨組織に接している部分に多くの陽性細胞が観察された。現在、より詳細に検討中である。

単離された膜型タンパク質の分泌型フォーム

以前の本研究において、ストローマ細胞株 OP9 よりシグナル配列単離法にて単離された 205 クローンの内、その発現組織部位、増殖因子 LIF 刺激による発現の変化、構造を指標に７クローンの未知遺伝子の全長 cDNA を単離し SST1-7 とした。これらの cDNA のカルボキシル末端に FLAG タグを付加した全長 cDNA を一過性に COS7 細胞に発現させると、一部の cDNA(SST1, SST4 (mKirre) , SST5)において、全長タンパク質の他に 20-30 kDa の短いタンパク質の発現が確認された。この際、ウェスタン解析はカルボキシル末端に付加した FLAG タグに対するモノクローナル抗体で行ったこと、SST1, SST4, SST5 はいずれも構造上 Ia 型の膜型タンパク質であること、これらの短いバンドの大きさがそれらのタンパク質の膜貫通領域および細胞内領域の合計に相当する事などから、これらのタンパク質において、細胞外領域がプロテアーゼによって切断されることが予想された。

そこで、COS7 細胞に一過性に発現ベクターを遺伝子導入した後、メチオニン標識し、培養上清中の標識タンパク質を検出した。この際、COS7 細胞自身も多くの分泌タンパク質を産生しているが、empty vector のみを遺伝子導入した control の COS7 細胞の培養上清と比較する事により、遺伝導入した cDNA 由来の細胞外分泌タンパク質を全て検出する事ができる。この実験において、SST1, SST4, SST5 にて培養上清への蛋白質分泌が検出された。これらの遺伝子は構造的に膜型蛋白質であること、分泌蛋白質の分子量がウェスタン解析で検出された成熟型蛋白質よりも約 20kDa 小さくなっていることから、これらの分泌型蛋白質はカルボキシル末端の膜貫通領域が

切断されたものと考えられる。カルボキシル末端の膜貫通領域が切断されて分泌されるタイプの増殖因子としてSCFがある。一方で、構造上分泌型のタンパク質であるSST3およびSST7においては、メチオニン標識された分泌タンパク質が検出されなかった。これらについては細胞外マトリックスタンパク質であり、培養上清中ではなく、大半が細胞外マトリックスの複合体内にトラップされる可能性や、細胞膜に付加した形で存在する可能性などを考えている。

上記のSST1, SST4, SST5が、細胞膜貫通領域直上にて切断される事により細胞外領域のみが培養上清中に分泌されるという仮説を証明するために、それぞれの細胞外領域を抗原とした、ポリクローナル抗体を作成した。COS7細胞に一過性にこれらの発現ベクターを遺伝子導入した後、無血清培地に置換し、得られた培養上清を濃縮してウェスタン解析を行ったところ、SST1およびSST5においては、メチオニン標識の実験にて確認されたバンドと同じ大きさのタンパク質の存在が確認された。しかしSST4については、メチオニン標識の実験の際に確認されたタンパク質と同じ大きさのタンパク質は検出できなかった。切断後の高次構造の変化、糖鎖付加による抗原性の変化などの可能性も考えられる。

mKirreの細胞外領域に結合するリガンド／受容体の探索

SST4(mKirre)はOP9細胞に高発現させた場合、マウス造血幹細胞の支持機能を高める事が明らかとなっているが、その機序は不明である。最も単純なものとしては、mKirreがリガンドとして造血幹細胞に発現されている未知の受容体に作用する可能性、あるいは逆に造血細胞が発現する膜／分泌型タンパク質の受容体となり、ストローマ細胞の造血細胞支持機能を間接的に増強しているという可能性があげられる。これらのモデルではmKirreの細胞外領域に結合する受容体あるいはリガンドが存在すると仮定され、これらを同定する事でmKirreの機能解析が大きく前進することになる。そこで、mKirreの細胞外領域全長を含み細胞膜貫通領域以下を欠失した細胞外分泌型cDNAをヒト免疫グロブリンIgG1のFc領域との融合タンパク質の形で作成し、CHO-k1細胞に安定的遺伝子導入してその培養上清から精製した。このリコンビナントタンパク質をFluorescein標識しFACS解析にていくつかのヒト白血病細胞株の細胞膜上にその受容体／リガンドが存在するかどうかを検索した。この際、作成したリコンビナントタンパク質はマウスの配列であり、入手可能な白血病

細胞株の大半がヒト由来であるため、マウスの mKirre がヒトの受容体／リガンドに結合しない可能性も考えられたが、ヒトの mKirre の配列も判明しており、細胞外領域においてはアミノ酸レベルにてマウス mKirre と数個の違いしかないことから、使用可能と判断した。

FACS 解析の結果

BALL1	1.97%
BalmI	0.23%
Daudi	0.01%
HA	0.26%
Hel	1.47%
IM9	0.08%
JoskI	52.02%
(FcBlock 1.55%)	
Jurkat	0,66%
K562	3.12%
(FcBlock 2.87%)	
P30OHK	0.28%
Ramos	0.96%
THP1	57.4%
(FcBlock 1.73%)	

JoksI, THPI に関しては、単球系の細胞株であり、FcBlock にてその陽性細胞が劇的に減っていることから、ヒト免疫グロブリン Fc 部分を Fc 受容体が認識している擬陽性と考えられた。K562 に関しては、FcBlock にてもほとんど陽性率が変化しなかったが、その陽性率は低く受容体／リガンド分子を単離する有望な材料とは考えられなかった。また、もし mKirre の細胞外領域に結合する分子が分泌型の分子であった場合、この方法で高発現細胞を選別するのは困難である可能性も考えられる。

SST7 の脂肪細胞分化に対する効果

SST7 は構造的には非常に短い分泌型のタンパク質と考えられるが、既存の遺伝子と相同性が認められない事、メチオニン標識実験にて培養上清中に分泌が認められなかった事から、解析が進んでいなかった。しかし、OP9 細胞を LIF で刺激した際に一過性の発現低下が認められる事から、ストローマ細胞において、造血支持機能に関与する何らかの役割を担っている可能性も考えられた。しかし、その造血支持機能を調べるために SST7 をレトロウィルス発現ベクターpMX-puro を用いて OP9 細胞に高発現させたところ、約 4 週間の培養の後、OP9 細胞を高率に脂肪細胞に分化させる効果があることが判明した。この遺伝子が、造血幹細胞よりもむしろ間葉系の細胞に作用して脂肪細胞への分化に関与している可能性が考えられた。

D. 考察

mKirre モノクローナル抗体の応用

今回作製した抗 mKirre モノクローナル抗体は、現在詳細に検討中であるが、抗原としてはマウス、ヒトと共通配列部位を用いており、ヒト Kirre タンパク質を特異的に認識することを期待している。mKirre の検出、同定だけでなく、mKirre 発現細胞の分離・純化、mKirre シグナル伝達阻害実験など、骨髄造血微小環境による造血の制御機構の解析のための応用を検討中である。

こうした研究の延長線上に造血支持細胞を分離・純化し、それらと造血幹細胞を共培養することで in vitro で造血幹細胞の増幅をめざす。また、多くの白血病細胞は in vitro での増殖には限界があるにも関わらず、骨髄内では効率よく無制限に増殖できる事が知られている。これは、白血病細胞が正常の造血幹・前駆細胞の性質を一部保持しており、造血微小環境を占拠することで正常の造血を抑制すると同時にストローマ細胞からのシグナルを受けて増殖しているためと考えられている。mKirre のシグナルはストローマ細胞の造血幹細胞支持能において重要であることから、本研究の抗 mKirre モノクローナル抗体が mKirre を介して伝達されるシグナルを阻害し、白血病など骨髄内で増殖する悪性腫瘍に対する抗腫瘍効果があるか検討したい。

mKirre 分泌型フォームの存在

シグナル配列単離法によって得られた遺伝子の大半は膜型タンパク質であり、また構造的には分泌型であっても細胞外マトリックスの場合には基本的にこれら遺伝子が発現する細胞に接着した細胞に対してのみ作用を持ちうると考えられる。しかし、造血幹細胞を造血支持細胞とともに培養してその切片を電子顕微鏡によって観察すると、造血幹細胞の細胞膜は必ずしも造血支持細胞と直接は接着しておらず、造血支持細胞の作る閉鎖空間内に浮遊しているか、造血支持細胞の突起に限局的に接触している像が観察される。この事は造血支持細胞の産生するタンパク質の内、造血幹細胞に作用しうる遺伝子産物としては膜型タンパク質よりも分泌型タンパク質の方が有利である事を示唆している。実際、これまで、単離された造血因子の多くは分泌型のタンパク質である。一方で、SCF は膜型と分泌型の両方のフォームを持っており、膜型分子は接着分子的役割も担っている事が報告されている。今回私たちが単離した未知遺伝子の７個のうち３個までもが細胞外領域が切断されることで分泌される事は興味深い事実であ

る。もちろん増殖因子であれば、造血支持細胞と直接接触しなくても遠隔的に造血細胞に作用しうる事になり、一方で接着分子としても、その切断によって接着の強さの調整などに関与している可能性も考えられる。

造血における細胞外マトリックスタンパク質の役割

今回のシグナル配列単離法によって多くの既知細胞外マトリックスタンパク質が単離され、また未知遺伝子のなかでも構造上 SST3 は細胞外マトリックスタンパク質である可能性が高い。造血支持細胞は多くの細胞外マトリックスタンパク質を産生しており、造血支持細胞自身の形態維持、造血微小環境 (niche) の立体的構築に重要な役割を担っている事はもちろんであるが、一方で、前述の電子顕微鏡的観察からも、造血幹細胞の増殖、生存、ホーミングに重要な役割を果たしている事が予想される。実際、(1) fibronectin のノックアウトマウスは造血障害を起こす事が知られている事、(2) その受容体のひとつである Integlin が造血幹細胞の骨髄へのホーミングに重要である事が報告されている事、(3) Integlin などマトリックスタンパク質受容体の細胞内情報伝達経路も詳細に解析されており、そのシグナル伝達経路が受容体型チロシンキナーゼおよびサイトカイン受容体と、一部共通である事、などの事実から、マトリックスタンパク質も広い意味での造血因子と考えられる。今後の研究において、ストローマ細胞の産生するマトリックス蛋白質の機能に関しても興味深い。

mKirre の造血支持機序について

前述の様に mKirre がストローマ細胞の造血支持機能を増強する機序は不明であり、我々はいくつかの仮説を立てて解析を進めている。(1) リガンドとして造血幹細胞に発現されている未知の受容体に作用する可能性、(2) 造血細胞が発現する膜／分泌型タンパク質の受容体となり、ストローマ細胞の造血細胞支持機能を間接的に増強しているという可能性、(3) ストローマ細胞自身が受容体／リガンドを発現しており、autocrine に造血支持機能を増強している可能性、などが考えられる。また、Eph, Eph-ligand の様に互いの細胞内情報伝達経路に作用して双方向に受容体− リガンド関係を担っている可能性も考えられる。これまで得られた知見では、mKirre はストローマ細胞存在下においてのみ造血幹細胞を支持しており、少なくともその機能を発揮するためにはその他の多くの造血関連分子との協調が必要であると考えられる。

SST7と脂肪細胞分化

成体骨髄内には造血組織だけでなく、間葉系幹細胞、骨芽細胞、破骨細胞、脂肪細胞などの多くの種類の細胞が存在し様々な機能を担っている。造血支持細胞であるストローマ細胞は間葉系幹細胞に由来していると考えられるが、近年の報告により、造血微小環境を構成し、造血支持機能を有している細胞は骨芽細胞であるという報告がなされている。今回の研究においても、OP9細胞自身がある一定の条件下においては脂肪細胞に分化したり、OP9 細胞から単離された遺伝子の中に osteopontin など骨代謝関連遺伝子が含まれているなど、それらの密接な関係を示唆する知見が得られている。こうした背景の元、ストローマ細胞から単離された遺伝子の中に、造血関連遺伝子だけでなく、骨代謝や脂肪細胞制御に関連する遺伝子群が含まれている事は充分予想できる。実際現段階では非常に preliminary な知見であるが、SST7 がストローマ細胞の脂肪細胞分化に関与している事を示唆する結果が得られた事は大変興味深い。

E. 研究発表

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F. 知的所有権の取得状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

1) 造血幹細胞増殖調整因子及びそれをコードするポリヌクレオチド

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2) 抗 mKirre 抗体
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以上出願中

研究成果の刊行に関する一覧表

書籍

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Retrovirus-mediated gene transfer and expression cloning: Powerful tools in functional genomics

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Most of the human genome has now been sequenced and about 30,000 potential open reading frames have been identified, indicating that we use these 30,000 genes to functionally organize our biologic activities. However, functions of many genes are still unknown despite intensive efforts using bioinformatics as well as transgenic and knockout mice. Retrovirus-mediated gene transfer is a powerful tool that can be used to understand gene functions. We have developed a variety of retrovirus vectors and efficient packaging cell lines that have facilitated the development of efficient functional expression cloning methods. In this review, we describe retrovirus-mediated strategies used for investigation of gene functions and function-based screening strategies. **© 2003 International Society for Experimental Hematology. Published by Elsevier Inc.**

Function-based gene cloning

It was only 30 years ago that recombinant DNA technology was initiated [1]. The globin gene was cloned as the first mammalian gene in 1976 that was reverse transcribed from the purified mRNA for globin [2]. For less abundant mRNAs, the cDNAs were frequently isolated based on the amino acid sequences of purified proteins. In late 1970s, a method called hybrid selection was developed. The principle of this method was to detect proteins translated from the mRNA hybridized to a particular pool of subdivided cDNA library fixed on nitrocellulose membranes, thereby identifying a pool that contains a cDNA of interest. Xenopus oocyte was used for production of proteins. This type of experiment is called "expression cloning," which means "cloning of cDNA by detection of proteins expressed from cDNA libraries." This strategy is suitable for isolation of rare cDNAs, such as cDNAs for cytokines and cytokine receptors. Levels of the protein expression are low, but a small amount of protein is enough to exert biologic functions.

A variety of expression cloning strategies has been established and utilized for cloning of cDNAs based on the biologic functions of their protein products. One of the early expression cloning methods used the *Escherichia coli* expression system for expression of cDNAs followed by detection by antibodies. In the early 1980s, the hybrid selection method was used for identification of cDNAs for cytokines using growth stimulation as a screening method. This method was later modified to directly transcribe cDNAs in Xenopus oocyte using the SP6 promoter. Alternatively, genomic DNAs were used to functionally clone cDNAs. The famous oncogene screening method, the focus-forming assay, using NIH3T3 cells falls into this category. It was notable that the invention of COS cells, in which plasmids can be amplified for the first time in mammalian cells, enabled expression cloning using mammalian cells [3]. A variety of cDNAs were isolated by the COS cells-based functional cloning method. However, this strategy depended on specific cells such as COS7 cells where the SV40 large T antigens are expressed to enable amplification of SV40 origin-bearing plasmids [4]. Therefore, only transient assays can be used for the screening.

To overcome the limitations of the conventional expression cloning system using COS cells, we and others turned to the idea of harnessing the power of retrovirus gene transfer to develop function-based screening of cDNAs.

Retrovirus-mediated expression screening; rationale and application

Retrovirus-mediated expression cloning was developed in mid 1990s [5–8]. Construction of a cDNA library in a retrovirus vector is not different from that in a plasmid vector [9]. One can generate either uni-directional or bi-directional

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cDNA libraries depending on the intended application. Complimentary DNAs are generated using either oligo-dT primers or random hexamer primers. The library is kept as DNA solution, and is converted to retroviruses by using packaging cell lines. To generate retroviruses that represent and cover a high complexity of cDNA libraries, it is recommended to use 293-based packaging cell lines that are efficient in transient packaging [10,11]. The virus stock containing high-titer retroviruses is used to infect target cells, and the infected cells are selected for the phenotype of interest. The integrated cDNA then is recovered by genomic polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR to determine which cDNA is responsible for the phenotype and is subjected to the sequence.

The retrovirus-mediated expression cloning method is efficient because the number of the provirus integrations in each cell is limited. Therefore, it is not necessary to recover and reintroduce the plasmid from, and into, the cells repeatedly, as in the conventional method using COS7 cells. In retrovirus-mediated expression cloning, the infection efficiencies should be controlled between 10% and 30% to avoid multiple integration in a cell as much as possible. Alternatively, one can recover the integrated retroviruses by transfecting a helper construct harboring *gag-pol* and *env* genes into the isolated clone that has acquired a phenotype of interest after transduction of the cDNA library. In this case, the recovered retroviruses are infected to the target cells to determine which integration was responsible for the phenotype.

The most important advantage over the conventional method is that any functional assay can be applied to identify cDNAs by their functions because, once integrated, the expression of the retrovirally transduced cDNA usually is stable.

Retrovirus-mediated expression cloning: some examples

A variety of functional assays can be utilized in retrovirus-mediated expression cloning. For instance, cellular receptors for various viruses were identified based on infectability of the viruses. Infection-resistant cells transduced with the library derived from infectible cells are screened by infection of the virus vector harboring a reporter gene such as GFP. The cDNA recovered from reporter gene-positive cell (i.e., infectible cell) should encode a receptor for a virus of interest. Co-receptors for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) were identified in this way from cDNA libraries derived from human T cells [12,13]. A receptor for polytropic and xenotropic retrovirus, which had been searched for, was identified using the same approach [14].

Tumor necrosis factor (TNF) and Fas induce apoptosis through activation of downstream signaling pathways. After introduction of cDNA library into the cells, some cells may become resistant to TNF and Fas stimulation by expressing a retrovirally introduced cDNA. Using this strategy, a novel transcription factor BSAC and an adhesion molecule ICAM-2 have been identified as inhibitors of TNF-induced and TNF- and Fas-induced apoptosis, respectively [15,16]. Intriguingly, ICAM-2 was found to activate PI3K, PDK-1, and Akt, leading to inhibition of apoptosis [16]. Thus, identification of proteins based on their functions sometimes leads to unexpected and important findings. Another example of the unexpected result was encountered in our study. Mouse leukemic M1 cells differentiate into macrophages and undergo apoptosis upon interleukin-6 (IL-6) stimulation. Starr et al. [17] identified an inhibitor of the cytokine signal, SOCS-1, by isolating an IL-6–resistant M1 clone after transducing a cDNA library to M1 cells via retrovirus infection. By the same strategy, we identified A1, which is an anti-apoptotic protein of the bcl-2 family and protected M1 cells from IL-6–induced apoptosis [18]. We also discovered a novel GAP MgcRacGAP in the anti-sense orientation from an IL-6–resistant clone; expression of anti-sense MgcRacGAP protected M1 cells from IL-6–induced differentiation and apoptosis. On the other hand, overexpression of MgcRacGAP induced differentiation into macrophages in HL60 cells. An unexpected finding brought by the subsequent study was that MgcRacGAP is required for cell division, especially for cytokinesis [19,20]. It would be interesting to investigate the link between cytokinesis and cell differentiation. Thus, functional identification of protein is a powerful tool in cell biology.

Structure/function analysis using retrovirus-mediated screening combined with PCR-driven random mutagenesis

In addition to applications for a variety of expression cloning strategies, the retrovirus-mediated expression system can be used to identify a mutant molecule with altered functions. For instance, we identified constitutively active forms of a cytokine receptor MPL [21] and a transcription factor STAT5 [22]. In brief, we introduced random mutations into MPL and STAT5 by PCR. The PCR products then were ligated into the retrovirus vector pMX [9], and the ligated DNA was amplified in *E. coli*. Thus, the resulting plasmid DNA represented a mutation library of the protein of interest. This library was transiently transfected into a 293-based packaging cell to generate the retrovirus stock representing the mutation library of a particular molecule. It then was infected to target cells, and the infected cells were selected for a phenotype of interest, followed by retrieving and sequencing the integrated retrovirus of the selected clones. In our experiments, we used mouse IL-3–dependent Ba/F3 cells as targets, and selected the library-transduced Ba/F3 cells in the absence of IL-3 to isolate factor-independent clones. In that manner, we were able to identify constitutively active

mutants of MPL [21] and STAT5A [22] that induced factor-independent growth in Ba/F3 cells as well as other IL-3–dependent cell lines. The constitutively active mutants of various signaling molecules will be useful for analyzing signaling pathways. In addition to isolation of active mutants of various molecules, this strategy will be applicable in generating various mutants with acquired functions. For instance, one may want to generate restriction enzymes that recognize altered restriction sites, or cytokines with stronger activities or with less side effect in vivo.

SST-REX, an efficient signal sequence trap based on retrovirus-mediated gene transfer

Sorting of the protein between the cellular components is regulated by various sorting signals in the proteins, such as the nuclear localization signal and the mitochondrial targeting sequence. Signal sequence is one such sorting signal found in type I transmembrane proteins and secreted proteins. Tashiro et al. [23] developed an elegant method called signal sequence trap (SST) by which signal sequence-harboring cDNAs are specifically isolated. The rationale of the method is to search for a cDNA fragment that contains a signal sequence and directs a signal sequence-defective CD25 to the cell surface by the fusion. This method, however, is time consuming and leads to frequent isolation of false-positive clones. Klein et al. [24] invented a modified SST method using growth of a yeast mutant YT455 as a screening method. We applied retrovirus-mediated gene transfer and developed an efficient and accurate method SST-REX (*s*ignal *s*equence *t*rap–*r*etrovirus-mediated *ex*pression screening) using mammalian cells [25]. We used the constitutively active MPL [21] in developing SST-REX, as illustrated in Figure 1. In brief, we construct a cDNA library in a retrovirus vector in which cDNA fragments are fused to an extracellular deletion mutant of the constitutively active MPL. The fusion library then is transduced into IL-3–dependent Ba/F3 cells via retrovirus infection. When the inserted cDNA fragment contains a signal sequence, it directs the mutant MPL on the cell surface and confers Ba/F3 cells factor independence. We then recover the integrated cDNA from the factor-independent Ba/F3 clones. Ten milliliters of the virus supernatant gives rise to isolation of about 1000 to 2000 signal sequences with 95 to 100% accuracy. Whereas previous methods collected shorter cDNA fragments for SST, we use cDNA fragments in the range from 0.5 to 6 kbp. In the results, the average length of cDNA fragments isolated in SST-REX is about 1 kbp. In SST, generally speaking, shorter cDNA fragments (shorter than 100–200 bp) will give false-positive results more frequently (unpublished results), and we believe this is a reason why SST-REX achieves higher accuracy than other methods.

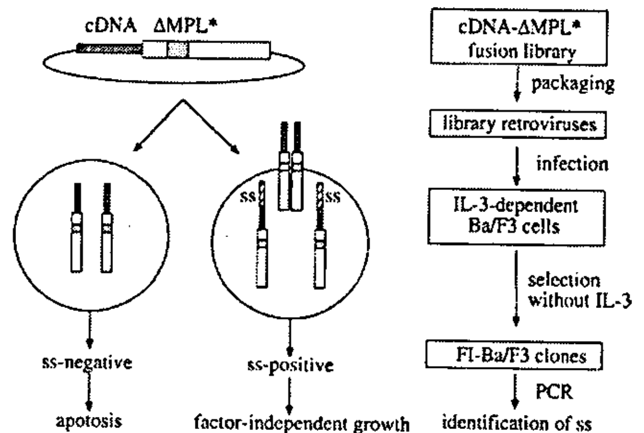

Figure 1. Retrovirus-mediated signal sequence trap method. SST-REX. The signal sequence and a part of the extracellular domain were deleted from the constitutively active mutant of MPL (ΔMPL*). When a cDNA fragment fused to this mutant contains the signal sequence (ss positive), it directs surface expression of cDNA-ΔMPL* fusion product, thereby inducing factor independence in Ba/F3 cells.

FL-REX, a method by which cDNA is identified based on subcellular localization of its protein product

We also developed a novel expression screening method (FL-REX: *f*luorescence *l*ocalization–*r*etrovirus-mediated *ex*pression screening) in which a cDNA can be isolated based on subcellular localization of the protein [26]. Briefly, we express cDNAs as GFP-fusion proteins in NIH3T3 cells via a retrovirus vector, and GFP-fused proteins are identified by subcellular localization through fluorescence microscopy. With this method, it is possible to clone cDNAs for proteins that specifically localize in the nucleus, nucleoli, Golgi apparatus, cell surface, and mitochondria. If computerized fluorescence microscopy is combined with FL-REX, it also would be possible to isolate cDNAs whose protein products shuttle between different cellular compartments in response to various stimuli, such as cytokines, ultraviolet irradiation, and heat shock.

Genetic approaches in a mammalian system

Genetic approaches using complementation is another field for retrovirus-mediated expression cloning. It is possible to complement the deficiency of the mutant cells from patients with genetic disorders by introducing cDNA libraries from normal cells. If efficient assay systems were available, it would be easy to isolate the causative genes of various genetic diseases using retrovirus-mediated expression cloning.

It also is possible to first establish the mutant by chemical mutagenesis or irradiation and then complement the defect of the mutant using a cDNA library derived from normal cells to search for a missing gene in the mutant. In that way, one can identify a series of molecules responsible for a

particular function. For example, Yamaoka et al. [27] identified an important subunit of IKK complex NEMO by complementing the mutant cells in which the NFκB pathway was disrupted by chemical mutagenesis. Stoeclin et al. [28] identified BRF1 as a regulator essential for ARE (AU-rich element)-dependent mRNA decay. In this experiment, a mutagenized cell line (slowC) that failed to degrade cytokine mRNA was used. When a GFP reporter construct with ARE was introduced into slowC mutant, unlike in normal cells, mRNA for GFP was not degraded because of a missing factor that was responsible for the ARE-dependent degradation of the mRNA, thereby maintaining the high GFP expression. A cDNA library was introduced into the slowC mutant to identify the revertant based on the reduced expression of GFP, and BRF1 was found to be a regulator that degraded GFP mRNA in an ARE-dependent manner.

We also used a genetic approach combined with retrovirus-mediated expression cloning [29] to search for bone marrow stroma cell-derived growth factors (Fig. 2). We used the Ba/F3 cell line, which is unique because of its dependence on only mouse IL-3 and not other cytokines. Most bone marrow stroma cell lines do not produce IL-3 and therefore cannot maintain the growth of Ba/F3 cells. Our strategy was to establish stroma-dependent Ba/F3 mutants. Most of such mutants were found to be dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF) or stem cell factor (SCF), thus indicating that they ectopically expressed receptors for GM-CSF and SCF through chemical mutagenesis. Some mutants did not respond to known factors, and we chose one such clone (S21), which can grow on ST2 stroma cells but not on other several lines such as MS10. Subdivided pools of an ST2-derived cDNA library are transduced via retrovirus infection into MS10 cells to identify the pool that contains a cDNA encoding the ST2-derived factor responsible for induction of S21 growth. After isolation of a pool expected to contain such a cDNA, the pool is further divided to eventually identify a single clone (sibling). In this way, we were able to identify a membrane integral protein with six transmembrane domains. This molecule had been originally identified as a soluble factor called immune suppressor factor (ISF), but curiously it turned out to be a subunit of the vacuolar-type ATP-associated pump. This experiment never would have been successful by the conventional expression cloning method utilizing COS7 cells because cell–cell interaction was required for the induction of cell growth by ISF. Intriguingly, when ISF was overexpressed in MS10 cells, the S21 Ba/F3 mutant as well as bone marrow progenitor cells proliferated on and underneath the stroma cells, and formed a cobblestone-like area, which is a hallmark of proliferation of hemopoietic progenitor cells.

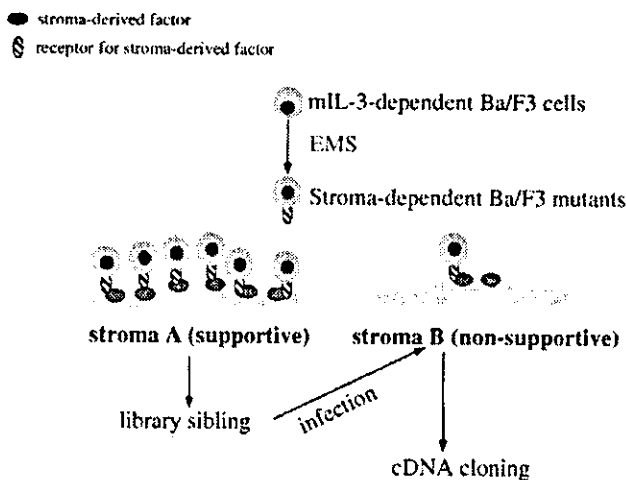

Figure 2. Genetic approach for isolation of stroma-derived factors that stimulate cell growth. Chemical mutagenesis is combined with functional complementation based on retrovirus-mediated gene transfer. Theoretically, all of the stroma-derived factors that support cell growth can be identified by this method.

Advantages of retrovirus-mediated gene transfer in investigating gene functions

In addition to the function-based screening of cDNA libraries, efficient retrovirus-mediated gene transfer is useful to investigate functions of genes, particularly those with inhibitory functions in the control of cell growth because it is difficult to establish stable transformants that express such genes. One may want to use inducible expression systems including the tet-repressor system. However, it turned out to be difficult to establish stable transformants expected to inducibly express genes such as SOCS-1, probably because even leaky expression of such genes hampers establishment of a stable transformant (unpublished results).

If the infection efficiency is more than 30 to 50% in a particular cell type, we should be able to readily see the gene function by observing the effect of gene expression in a bulk culture transduced with the gene of interest via retrovirus infection. It also is possible to use a bicistronic retrovirus vector that harbors the internal ribosomal entry site (IRES) and simultaneously expresses a gene of interest and a reporter gene from one mRNA. Alternatively, retrovirus vectors can be designed to express a dominant-negative form of a particular protein, the anti-sense cDNA, or the RNAi construct to investigate functions of a particular gene in a particular cell type. Using our efficient retrovirus-mediated gene transfer system described in the following, we are able to investigate gene functions even in primary cultured cells such as T cells and mast cells.

Improvement of retrovirus-mediated gene transfer

Improved retroviral systems are being developed to facilitate applications to expression cloning and functional genomics.

Our own efforts to enhance both packaging and vector components are briefly reviewed in the following to illustrate possible approaches.

Improvement of retrovirus packaging cells
Retrovirus vectors lack viral structural genes and require packaging cells to generate viral particles [30,31]. Conventionally, NIH3T3 cell-based packaging cells were used to establish stable producers of recombinant retroviruses. This strategy was time consuming. In 1993, Pear et al. [10] established the unique packaging cell line BOSC23; they stably introduced *gag-pol* and *env* expression constructs into 293T cells well known for high efficiency in transient transfection. Establishment of BOSC23 brought a revolution in retrovirus-mediated gene transfer. High-titer retroviruses (~10^6 IU/mL) can be readily prepared in a couple of days by transiently transfecting retrovirus vectors into BOSC23 cells. However, it was difficult to maintain the potential of the BOSC23 cells to produce high-titer retroviruses by transient transfection during long-term culture.

To generate more stable and efficient packaging cell lines, we developed unique packaging constructs to express *gag-pol* and *env* genes (Fig. 3). The packaging constructs used the EF1α promoter for efficient expression in 293T cells, the IRES to simultaneously express viral structural genes and drug selection markers from one mRNA, and the Kozak sequence for efficient translation. These packaging constructs were stably transfected into 293T cells, and an efficient packaging cell line PLAT-E was obtained [11]. PLAT-E cells produce 1×10^7 IU/mL by transient transfection of pMX even after long-term culture if the cells are maintained in the presence of blasticidin and puromycin. We also used *env* genes derived from amphotropic retrovirus (4070A) and feline endogenous retrovirus (RD114) to generate PLAT-A and PLAT-F cell lines. The titers of retroviruses produced from PLAT-A and PLAT-F are 1×10^6 IU/mL, one tenth of those produced from PLAT-E (unpublished results). The RD114 Env derived from feline endogenous viruses was reported to facilitate gene transfer into human hematopoietic stem cells [32]. Another packaging cell line PLAT-gp expressing only the *gag-pol* gene is available for generating pseudotype retroviruses with differing envelop proteins such as VSV-G.

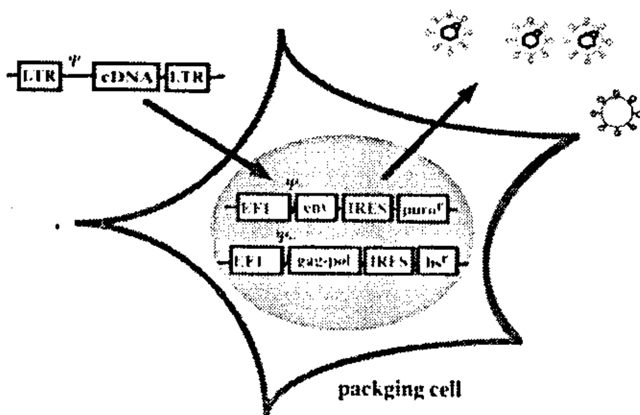

Figure 3. Efficient packaging of retroviruses by PLAT-E cells. The following features have been incorporated to the packaging constructs. 1) The EF1α promoter was used to express *gag-pol* and *env* because it was the strongest promoter among the seven tested in 293T cells. The EF1α promoter was 100-fold stronger than the MMLV LTR used in most packaging cell lines, including BOSC23. 2) The IRES was used to simultaneously express viral structural genes and drug selection markers from one mRNA, which provides the stable expression of *gag-pol* and *env* products under drug selection. 3) Only the coding regions of *gag-pol* and *env* genes were used in order to avoid incorporation of the retrovirus sequence as much as possible, thereby decreasing the probability of production of replication-competent retroviruses by recombination. 4) To increase the translation efficiency, the Kozak sequence was placed before the ATG start codon of *gag* and *env*.

New series of retrovirus vectors that avoid expression of Gag and Gag-fusion proteins
A variety of retrovirus vectors are available for many different purposes. Comparison of these vectors is not the purpose of this review. Here we introduce mainly our retrovirus vectors designed for expression cloning and efficient gene transfer. We have been using the pMX vector [9] and its derivatives that were derived from the MFG vector [33]. The pMX vector harbors 5′ long terminal repeat (LTR) and the extended packaging signal derived from MFG followed by a multi-cloning site (MSC) suitable for cDNA library construction and 3′ LTR of MMLV. The resulting vector pMX in combination with PLAT-E cells produces, on average, 1×10^7 IU/mL. With high-titer retroviruses, one can efficiently introduce the gene of interest into most mouse cell lines as well as primary cultured cells such as T cells, mast cells, and neuronal cells with an infection efficiency ranging from 20 to 60% [34,35]. To further increase infection efficiency, either Retronectin (Takara, Kyoto, Japan) or concentration of retroviruses by medium-speed centrifuge (8000*g* at 4° C for 16 hours) can be applied, and the infection efficiencies in these cells can reach 80% [34].

Unlike most other retrovirus vectors such as LXSN and MSCV, the pMX vector harbors the splicing donor and acceptor sites and produces two types of transcripts, the full-length genome RNA and the subgenomic RNA (Fig. 4A). Therefore, in addition to the correct proteins produced from the subgenomic RNA, Gag-fusion proteins could be expressed from the full-length genome RNA if the reading frame of *gag* and that of the inserted gene match and there is no stop codon between them. To generate improved vectors free of the Gag and the Gag-fusion proteins, we disrupted the ATG start codon of *gag*, inserted a stop codon downstream of the CTG start codon of glyco *gag*, and inserted triple stop codons in three different reading frames just before MCS. The improved vectors are termed pMXs. A variety of pMXs series are depicted in Fig. 4B, including

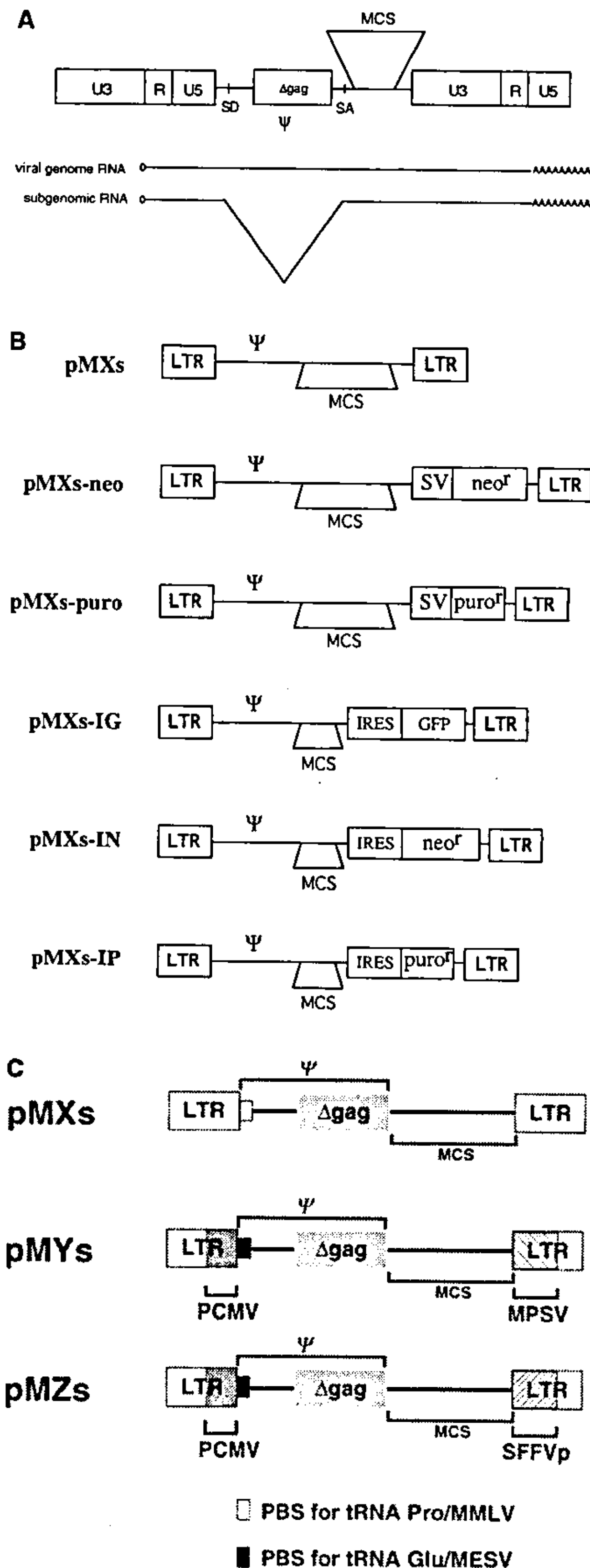

Figure 4. Structures of retrovirus vectors. (A) Basic structure of retrovirus vectors and two transcripts from the vector. In the replication competent MMLV, the Gag-Pol and the Env proteins are translated from the genomic RNA and the subgenomic RNA, respectively. The subgenomic RNA is a spliced form RNA, and the splicing occurs from the splice donor site (SD) to the splice acceptor site (SA). Both 5′ and 3′ LTRs consist of U3, R, and U5 regions. Ψ = packaging signal; Δgag = truncated *gag* sequence. (B) Structures of versatile pMXs-derived vectors. LTR = long terminal repeat; Ψ = packaging signal; MCS = multi-cloning site; IRES = internal ribosomal entry site; GFP = green fluorescent protein' neo^r = neomycin-resistant gene; puro^r = puromycin-resistant gene. (C) Structures of pMXs, pMYs, and pMZs vectors. The 5′ LTR, primer binding site (PBS), and the extended packaging signal of pMXs are derived from the MFG vector. PBS used in pMYs and pMZs are derived from MESV (murine embryonic stem cell virus) and binds tRNA-glu instead of tRNA-pro. MCS is designed for cDNA library construction and is preceded by triple stop codons (not shown). White box = MMLV LTR; gray box = PCMV LTR; hatched box = MPSV LTR or SFFVp LTR. MCS = multi-cloning site.

pMXs-puro, pMXs-neo, pMXs-IG (IRES-GFP), pMXs-IN (IRES-neo), and pMXs-IP (IRES-puro).

MMLV-based vectors usually are silenced in immature cells, including embryonic carcinoma (EC) cells and embryonic stem (ES) cells, and possibly hematopoietic stem cells. Indeed, pMX vectors are quickly silenced in EC and ES cells. MESV and PCMV are mutants of MMLV and can stably express genes in immature cells [31]. To enable expression of genes in immature cells, vectors that incorporated the PCMV LTR and MESV primer binding site (PBS) were developed by Ostertag's group in Germany [36]. These include FMEV (a hybrid between FMCF and MESV) and MPEV (a hybrid between MPSV and MESV) vectors, which can express genes in EC and ES cells. These vectors were modified by Hawley's group in Canada [37], and the resulting vector was called MSCV (mouse stem cell virus). However, these vectors will not give optimum titers in transient packaging because the U3 region of 5′ LTR lacks one of the 75-bp direct repeats in the enhancer region. In the transient transfection, the promoter of 5′ LTR drives expression of transfected vectors, thus determining the retrovirus titers. To increase the promoter activity in transient transfection, we replaced the U3 region of 5′ LTR of MPEV and FMEV with that of MMLV using the KpnI restriction site (Fig. 4C) and inserted the pMXs-derived extended packaging signal and MCS before 3′ LTR. We named these vectors pMYs and pMZs. The pMYs vector is based on MPEV, and its 3′ LTR consists of U3 of MPSV and U5 of MMLV. The pMZs vector is based on FMEV, and its 3′ LTR consists of U3 of SFFV and U5 of MMLV. As expected, pMYs and pMZs vectors produced high-titer retroviruses in transient transfection, and these viruses can efficiently express genes in EC cells, most ES cells, and hemopoietic progenitor cells.

We also have developed pMCs vector that uses PCMV LTRs for both 5′ and 3′ ends like MSCV (T. Mizutani, H. Iba, and T. Kitamura, unpublished data). The U3 region of

5′ LTR has been replaced by MuLV LTR to increase the efficiency of transient transfection. Thus, the pMCs vector produces proviruses similar to those produced by MSCV. However, pMCs is supposed to produce higher titers of retroviruses because of its higher efficiency in transient transfection and is expected to produce higher amounts of proteins because of the presence of the splice donor and acceptor sites in pMCs that was derived from MFG vector and is present in pMXs, pMYs, and pMZs.

Closing remarks

In the present review, we describe the efficient retrovirus expression system and its applications in a variety of experiments. Although we mainly introduced our works, there are almost infinite other potential applications for retrovirus-mediated expression screening. For example, short peptide libraries can be screened using the retrovirus expression system [38]. The enhancer trap and poly A trap methods also were developed using retrovirus-mediated gene transfer [39,40]. It would be useful to perform two-hybrid screening in mammalian cells, in which molecular interactions will be more physiologic. In the past, insertional mutagenesis using retrovirus vectors was performed extensively; however, it was difficult to identify the responsive gene induced by the retrovirus because retrovirus integration could alter transcriptional activities of the genes within 20 to 30 kbp from the integration site. Recent genome information and expression profiling using gene chips and DNA microarrays have made it much easier to identify the responsive gene in insertional mutagenesis. Recently, even in vivo mammalian genetic screenings have been performed successfully using retrovirus-mediated insertional mutagenesis in the mouse [41,42].

Most of the human genomes have now been sequenced, and about 30,000 genes have been identified. However, there is still much to learn about the human genome. We believe that retrovirus-mediated gene transfer and expression cloning will continue to be important tools to understanding the genome. As we introduced some examples in the present review, isolation of proteins based on their functions sometimes opens up insights into cell biology. In addition to expression cloning, retrovirus-mediated gene transfer also is useful for investigating gene functions and can be applied in high-throughput analysis of gene product in a variety of cells. We sincerely hope that this review will invite more researchers to retrovirus-based technologies.

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A stromal cell–derived membrane protein that supports hematopoietic stem cells

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Hematopoietic stem cells cannot be maintained *in vitro* without stromal cells, even if they are provided with growth factors, and it is likely that supportive cells in the bone marrow express membrane or secreted proteins that maintain hematopoiesis. Here we show that *mKirre*, a mammalian homolog of the gene *kirre* of *Drosophila melanogaster*, encodes a type Ia membrane protein that is involved in the hematopoietic supportive capacity of OP9 mouse stromal cells. Repressing *mKirre* expression with a short interfering RNA significantly reduced this supportive capacity. Our data suggest that mKirre is cleaved by metalloproteinases and that the extracellular domain of mKirre is responsible for supporting hematopoietic stem cells. These results contribute to our understanding of the mechanisms by which the hematopoietic microenvironment regulates hematopoiesis.

In bone marrow, hematopoiesis-supporting cells form a hematopoietic microenvironment called a 'niche' and, by producing growth factors, adhesion molecules and matrix proteins, govern the homing, growth, survival and differentiation of hematopoietic stem cells (HSCs). Growth factors expressed by hematopoiesis-supporting cells include cytokines such as interleukin 3 (IL-3) and IL-6 (ref. 1), ligands of receptor tyrosine kinase such as stem cell factor (SCF)[2] and Flt-3 ligand[3], Notch ligands[4], bone morphogenetic protein 4 (BMP4)[5] and sonic hedgehog[6]. But even if these known growth factors are supplied in combination, HSCs cannot be maintained *in vitro* without stromal cells.

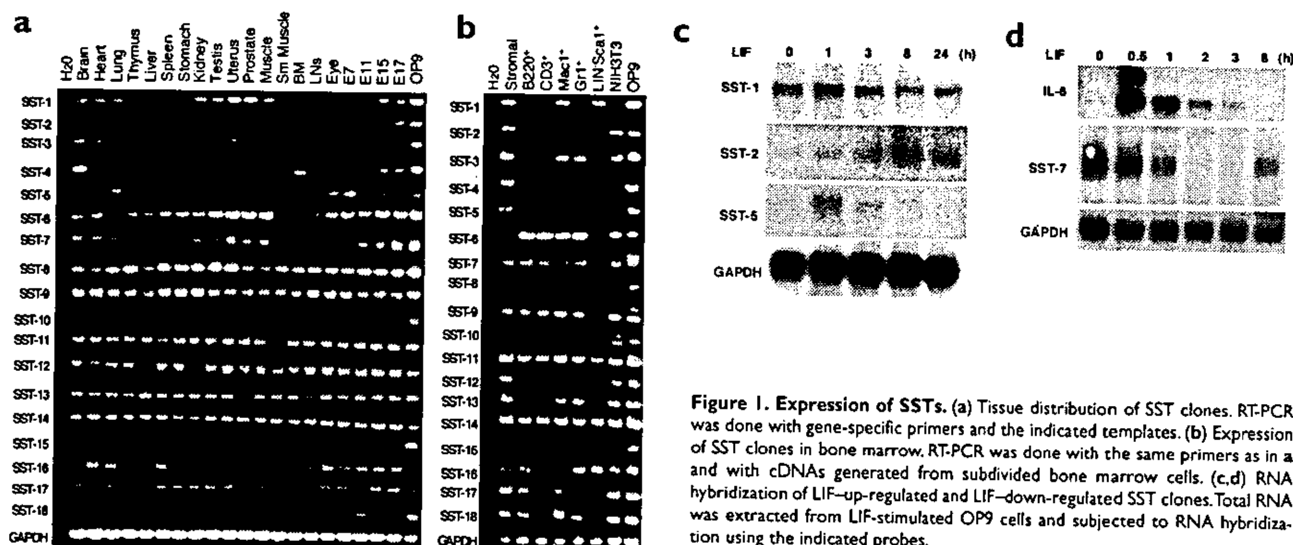

Figure 1. Expression of SSTs. (a) Tissue distribution of SST clones. RT-PCR was done with gene-specific primers and the indicated templates. (b) Expression of SST clones in bone marrow. RT-PCR was done with the same primers as in a and with cDNAs generated from subdivided bone marrow cells. (c,d) RNA hybridization of LIF–up-regulated and LIF–down-regulated SST clones. Total RNA was extracted from LIF-stimulated OP9 cells and subjected to RNA hybridization using the indicated probes.

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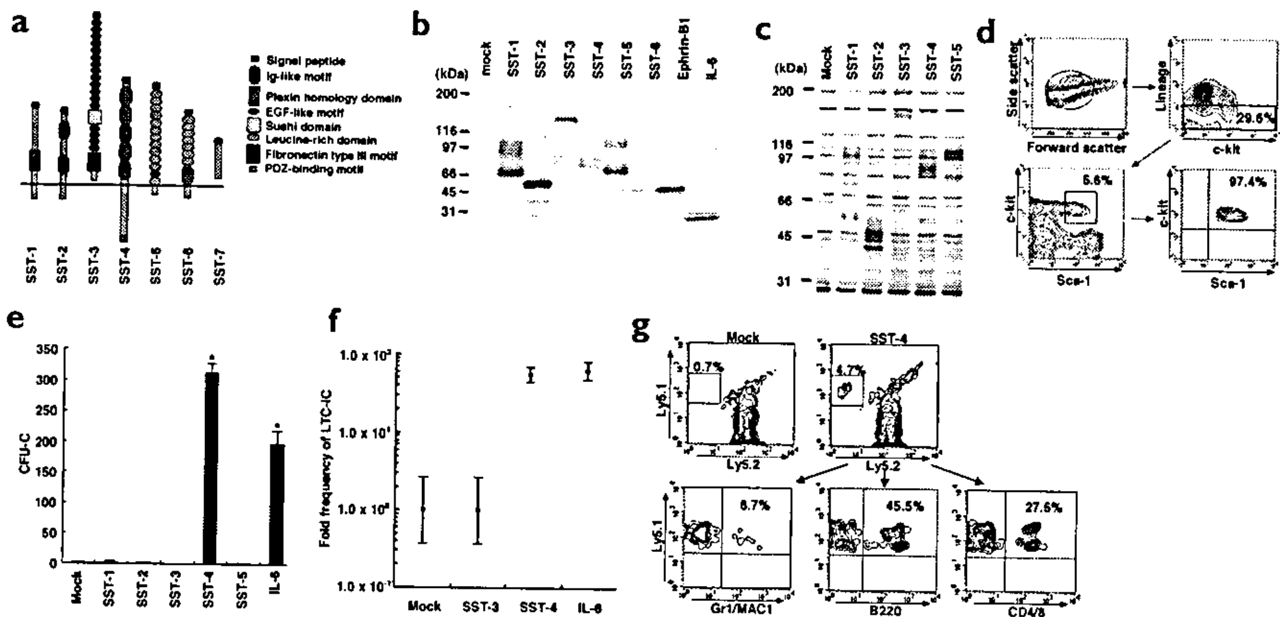

Figure 2. The hematopoiesis-supporting ability of SST proteins. (a) Structural representation of SST-1 to SST-7. Full-length cDNAs were isolated and functional domains were predicted from the deduced amino acid sequence. (b) Expression of Flag-tagged SST genes. The indicated cDNAs were transiently expressed in COS7 cells and total cell lysates were subjected to immunoblot analysis with anti-Flag. (c) Stable expression of SST genes in OP9 cells. Total cell lysates were extracted from the indicated OP9 clones and subjected to immunoblot analysis with anti-Flag. (d) Purification of KSL cells from the bone marrow of C57BL/6 mice by FACSVantage. (e) Bulk LTC-IC analysis of SST genes expressed in OP9 cells. The numbers of CFU-C are indicated. Error bars indicate the s.e.m. *$P < 0.01$ versus mock transfected cells. (f) LDA-based LTC-IC analysis. The fold values relative to mock-transfected OP9 cells are reported. Error bars indicate the s.e.m. (g) SST-4 can sustain HSCs that have bone marrow-reconstituting ability. Mock-transfected or SST-4-transfected OP9 cells were analyzed by a bone marrow reconstitution assay (Methods). Peripheral blood cells from recipient mice were subjected to FACS analysis with indicated antibodies. Experiments were done twice and the engrafted donor-derived cells in peripheral blood were 0.5% (mean of four mice) for mock-transfected OP9 cells and 4.2% (mean of four mice) for SST-4-transfected OP9 cells. Representative FACS analyses are shown.

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Direct contact between stromal cells and HSCs is also crucial for keeping HSCs in a primitive state[7]. These findings suggest that supportive cells in the bone marrow express unidentified membrane molecules that regulate hematopoiesis.

Bone marrow stromal cell lines are histologically heterogeneous[8], and it is probable that many types of cells participate in supporting hematopoiesis in bone marrow. But some stromal cell lines, such as M2-10B4 (ref. 9), MS-5 (ref. 10), AFT204 (ref. 11) and S17 (ref. 12), can support HSCs *in vitro*, and it is expected that gene transcripts involved in supporting hematopoiesis are expressed in these cell lines.

Here we have used OP9 mouse stromal cells to identify molecules that can maintain HSCs *in vitro*. Because OP9 cells can differentiate embryonic stem (ES) cells into all lineages of hematopoietic cell[13,14], we considered that these cells must express the genes necessary for regulating hematopoiesis. We assumed that such genes encode secreted or membrane proteins because they have to transmit signals from supportive cells to HSCs. Several studies have reported that leukemia inhibitory factor (LIF) up-regulates the hematopoietic supportive capacity of stromal cells[15–17]. OP9 cells express both the α chain of the LIF receptor and gp130, and they also express SCF, IL-3, IL-6 and IL-11 after stimulation with LIF (data now shown). We therefore thought that we might identify genes important for hematopoiesis by selecting for genes that show altered expression in OP9 cells after treatment with LIF.

We analyzed genes expressed in OP9 cells and genes induced by LIF treatment by DNA microarray. Known genes that showed high expression on LIF treatment included those encoding growth factors such as SCF and connective tissue growth factor (CTGF), as well as phosphatases, transcription factors and regulators of the cell cycle (data not shown). Because secreted and membrane proteins represent only a small proportion of the genes that are expressed in mammals[18], we selected for such proteins by a retrovirus-based signal sequence trap (SST) method[19]. We first screened for secreted and membrane proteins in OP9 cells and then selected candidate genes according to the tissue distribution of their expression, change of expression on LIF activation and structures of partial protein fragments identified by SST. We isolated the full-length complementary DNAs of candidate genes and examined the hematopoiesis-supporting activity of these genes.

We show that *mKirre*, which encodes a type Ia membrane protein, is involved in the hematopoietic supportive capacity of stromal cells. Our results suggest that mKirre is cleaved by metalloproteinases and that its secreted extracellular domain directly sustains HSCs and maintains them in an undifferentiated state.

Results

Identification of SST cDNA candidates

We constructed a cDNA library from OP9 cells and screened for genes expressing proteins with a signal peptide at the N terminus. We used the SST-REX method[19], in which proteins carrying signal peptides translocate a constitutively active variant of c-Mpl lacking its extracellular domain to the plasma membrane, which renders Ba/F3 cells, an IL-3–dependent pro-B cell line, IL-3–independent.

We obtained 216 IL-3–independent Ba/F3 clones, from which we isolated 205 cDNAs by genomic polymerase chain reaction (PCR; see **Supplementary Table 1** online). Of these, 170 were known genes and 35 were unknown genes. The unknown genes included molecules whose nucleotide sequence have been reported but not functionally analyzed. The

known genes encoded 49 growth factors, 25 receptors, 71 adhesion and matrix proteins, and 38 other types of protein. Some of the unknown genes overlapped, which left 18 independent clones. We designated these clones SST-1 to SST-18.

We predicted partial amino acid sequences from the cDNA fragments and searched for known protein motifs by InterProScan (http://www.ebi.ac.uk/interpro/; **Supplementary Table 1 online**). Although the full-length cDNA of SST-6 had been reported to encode the protein immunoglobulin superfamily containing leucine-rich repeat (ISLR)[20], its function was unclear and we therefore included it in further analyses.

Expression of SST cDNAs

We first examined the tissue distribution of the 18 SST clones and verified their expression in bone marrow cells by PCR with reverse transcription (RT-PCR). Because OP9 is an immortalized cell line[13], genes that are expressed may have changed epigenetically; thus, it was possible that some of the cDNAs that we isolated were not expressed in normal bone marrow stromal cells. We therefore examined expression of the SST clones in primary bone marrow stromal cells, as well as in NIH3T3 cells, a mouse fibroblast cell line that lacks hematopoietic supportive capacity, as a negative control.

As expected, all SST clones were expressed in OP9 cells; however, two clones were not expressed in primary stromal cells (SST-10 and SST-15). Expression of SST-4 was restricted to adult brain, bone marrow stromal cells and whole embryo and was not seen in NIH3T3 cells (**Fig. 1a,b**). These results suggest that SST-4 has a specified role in bone marrow stromal cells.

We next selected the SST clones for LIF-induced expression by RNA hybridization. We found that treatment with LIF resulted in up-regulation of SST-1, SST-2 and SST-5 but down-regulation of SST-7 in OP9 cells (**Fig. 1c,d**). Expression of SST-3, SST-4 and SST-6 was not affected by LIF treatment (data not shown).

From these results, we selected the seven unknown or uncharacterized genes, SST-1 to SST-7, isolated their full-length cDNAs and used PsortII (http://psort.ims.u-tokyo.ac.jp/form2.html) and InterProScan to predict the structures of the proteins encoded by these genes (**Fig. 2a**). Although the full-length murine genes of clones SST-1, SST-2 and SST-5 (ref. 21) and a human homolog of clone SST-4 (ref. 22) had been reported, the functions of these genes were unknown. We therefore examined whether any of these clones was involved in hematopoiesis.

Hematopoiesis-supporting ability of SST cDNAs

The cDNAs of SST-1 to SST-7 were transiently transfected into COS7 cells and their expression was confirmed by immunoblot analysis (**Fig. 2b**). We also established stable transfectants of OP9 cells (**Fig. 2c**). Because we could not establish OP9 cells stably overexpressing SST-6 and SST-7, we did not include these two clones in subsequent analyses.

To examine whether the products of the SST clones could affect the hematopoiesis-supportive capacity of bone marrow stromal cells, we carried out a long-term culture–initiating cell (LTC-IC) assay by culturing the stable SST transfectants with the c-kit^{+}Sca-1^{+}, lineage-negative (KSL) fraction of bone marrow cells (**Fig. 2d**). Of the clones tested, SST-4 significantly up-regulated the LTC-IC frequency (**Fig. 2e**, $P < 0.01$). We verified this result by a limiting dilution analysis (LDA)-based LTC-IC assay (**Fig. 2f**) and by *in vivo* bone marrow reconstitution analysis (**Fig. 2g**), in which we showed that the engraftment of multilineage hematopoietic cells was enhanced by overexpressing SST-4. Taken together, these results indicate that SST-4 can enhance the ability of stromal cells to support HSCs and to keep them undifferentiated.

Figure 3. Sequence of *mKirre*. (a) Amino acid sequence of mKirre (clone SST-4). The signal peptide is boxed and the arrow indicates the predicted processing site. Shaded boxes indicate putative *N*-linked glycosylation sites. Five immunoglobulin domains are underlined. The transmembrane domain is underlined twice. Broken line indicates the peptide sequence used to produce an mKirre polyclonal antibody. Bold letters indicate binding motifs for PDZ domains. (b) Comparison of the amino acid sequence spanning the five immunoglobulin domains of mKirre, KIAA1867, Kirre and Rst. Identical amino acids are indicated by black background shading and related amino acids by blue background shading. (c) Conserved PDZ-binding motifs at the C terminus of mKirre, KIAA1867, Kirre and Rst. The X(T/S)X(V/L) motifs are highlighted by green background shading.

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