添加して中和抗体に結合させた後，マトリックス干渉アッセイを行う．この場合，あらかじめ試験サンプルに添加する治療用タンパク質は，その中に含まれる抗体のすべてに結合し，治療用タンパク質の量を横軸にその治療用タンパク質が及ぼす作用の強さを縦軸に取った用量反応曲線において，最大の反応性が得られる量を用いる必要がある．この条件において干渉効果が得られる場合は，中和抗体以外のマトリックス成分によることが示される．なお，用量反応曲線において低い反応性を示す量の治療用タンパク質を用いる場合は，干渉効果がみられた場合に，その原因が中和抗体あるいは他のマトリックス成分のどちらであるか特定することが困難であり，実験条件として適切ではない．

2.3 カットオフポイントの設定

2.3.1 ブランクドナー血清

カットオフポイントの設定には，治療用タンパク質を投与していない健常人あるいは当該疾患の患者の血清を用いる．カットオフポイントは適切なパラメトリック又は非パラメトリック統計アプローチを用い，片側95%予測区間の上限を用いて設定できる．これら血清サンプルで値の変動が大きい場合，アッセイの感度が確保される範囲内でサンプルを希釈して用いることが望まれる．これらの処置によっても値の変動が大きい場合は，棄却検定により異常値を除去し，偽陽性及び偽陰性の数を減らすように適切なカットオフポイントを設定する必要がある．

2.3.2 抗体スパイク有無のブランクドナー血清の比

先に述べた血清サンプルの希釈によっても，ドナー間で値が変動する場合は以下のような方法を用いることができる．1.5から2倍の濃度で治療用タンパク質による反応の抑制が見られる濃度の陽性コントロール抗体を添加したドナー血清と添加していない血清で中和抗体アッセイを行う．スパイク前後の比の片側95%予測区間の低い限界値をカットオフポイントとして用いることができる．

3. 治療用タンパク質に対する患者血清抗体価の測定例

本項では患者において産生が誘導された治療用タンパク質に対する抗体を，様々な結合アッセイ及び中和抗体アッセイにより測定した代表的な例として，IFN-β，エリスロポエチン，パニツムマブに対する抗体について概説する．その他の例については成書など[14,37~42]を参考にされたい．

3.1 IFN-βに対する抗体の結合アッセイ及び中和抗体のアッセイ

IFN-βは再発寛解型多発性硬化症の治療に広く用いられている[43]．しかし，長期間治療を受けた患者の一部でIFN-βの生物活性を妨害する中和抗体が出現する[44,45]．その結果，IFN-βの活性の低下によりIFN-βの有効性が低下し，その後病状が悪化する[46~49]．そのため，抗体の存在を患者で定期的にモニタリングすることが重要であり，様々な結合アッセイ及び中和抗体アッセイが開発されている．

3.1.1 IFN-βに対する抗体の結合アッセイ

IFN-βに対する結合抗体のアッセイとして，ウェスタンブロット法，直接ELISA，放射免疫沈降法，ブリッジELISAが検討された．その結果，ウェスタンブロット法と直接ELISAは偽陰性及び偽陽性の起きる率が高いため，欧州神経学会により不適当と判断された[50]．放射免疫沈降法は，後述するA549細胞を用いたIFN-βによるMyxovirus resistant protein A（MxA）の誘導を用いた中和抗体アッセイよりも感度が高く，より多くの陽性を検出できた[51]．直接ELISAでは，後述する細胞変性アッセイを用いた中和抗体アッセイで陽性と判断された58サンプルのうち10サンプルが陰性と判定されたのに対し，ブリッジELISAではすべて陽性と判定された[52]．また，ブリッジELISAで高いかあるいは非常に高い値を示すサンプルのみが細胞変性アッセイで陽性と判定された．このような妥当性により，ブリッジELISAと放射免疫沈降法は中和抗体を測定する前のIFN-βの抗体スクリーニングとして推奨されている．

3.1.2 IFN-βに対する抗体の中和抗体アッセイ

3.1.2.1 細胞変性アッセイ

IFN-βに対する中和抗体を検出する代表的な標準法は細胞変性アッセイであり，ウイルスによる細胞変性をIFN-βが抑制する原理に基づいている．本法はWHOにより推奨されており[53]，現在最も共通に用いられている[54]．WHOはヒト肺がん細胞（A549細胞株）と脳心筋炎ウイルスの使用を推奨している[53]．一方，Wistar Institute Susan Hayflic（WISH）細胞株とシンドビスウイルスの組み合わせ[55]及びWISHと水疱性口内炎ウイルスの組み合わせ[48,56~59]も使用されている．細胞変性アッセイでは，IFN-βの濃度を一定にして血清を希釈して試験するように推奨されている[50]．一方，血清を例えば，20倍のように一定に希釈し，IFN-βの濃度を変動させて試験を行うことにより，感度を10から20倍増加できるという報告もある[60]．細胞変性アッセイの長所は非常に感度が高いことである．しかし，以下に述べるように様々

な問題もあり，次項で述べるバイオマーカーの定量を用いたアッセイ系の研究が精力的に行われている．①ウイルスを取り扱うため実験者に対して安全性の懸念が生じる．②IFN-βに特異的ではないので，血清中にIL-6あるいはIFN-γのような抗ウイルス活性を示す物質の存在により，ウイルスによる細胞変性効果が抑制される．③血清サンプル中に可溶性IFN-β受容体が高濃度存在する場合，IFN-βと結合し，中和抗体が存在する場合と同様にIFN-βによるウイルスによる細胞変性効果の抑制が低下する[61]．④血清サンプル中の中和抗体により抗IFN-β活性は用いる細胞及びウイルスの種類，インキュベーション時間，洗浄ステップにより変動するため，施設間における値の変動は大きく，その比較は困難である[62]．⑤操作が煩雑で測定まで4日を要する．⑥中和抗体のタイター（抗体価）とIFN-βの有効性の低下との関連については不明の点が多い[63,64]．

3.1.2.2　バイオマーカーの定量

Neopterin, 2'-5' oligoadenylate synthetase, β-2 microglobulin, MxAのようなIFN-βに誘導される遺伝子とそのタンパク質は，IFN-βで治療した患者における抗体の中和抗体アッセイの測定指標として用いられている[65~69]．特にMxAは1型IFNであるIFN-α及びIFN-βにより特異的に誘導されるため，多くの検討が行われている．

培養細胞におけるIFN-βによる通常24時間後のMxAタンパク質の誘導の，中和抗体による抑制がELISAにより測定されている[70~72]．本法における結果は細胞変性アッセイの結果と相関があり[71,72]，細胞変性アッセイよりも感度が高く，検出できる抗体のタイターの範囲は約7倍広い[72]．MxA以外にIFN-βにより誘導される遺伝子あるいはその遺伝子のプロモーターを用いたレポーターアッセイが中和抗体アッセイとして用いられている．A549細胞においてIFN-βにより誘導される6～16タンパクmRNAレベルの中和抗体による抑制が調べられた[73]．その結果，6～16 mRNAのIFN-βによる誘導は4時間で測定可能であり，中和抗体のタイターは細胞変性アッセイの結果と良く一致した．6～16遺伝子のプロモーターをルシフェラーゼ遺伝子に連結させたプラスミドをヒト線維肉腫細胞株HT1080細胞株に導入し，IFN-βによるルシフェラーゼ遺伝子発現の中和抗体による抑制が細胞変性アッセイと比較された[74]．その結果，ルシフェラーゼアッセイにより測定した中和抗体のタイターは細胞変性アッセイにより測定したタイターと良く一致した．

患者におけるIFN-βの投与によるMxAタンパク質の誘導が，末梢血液単核球あるいは血液サンプルを用いてフローサイトメトリーあるいはELISAで測定されている[58,75]．フローサイトメトリーを用いたアッセイでは，細胞変性アッセイで中和抗体陽性の患者9人のうち7人でMxAタンパク質が誘導されず，細胞変性アッセイによる中和抗体のタイターとMxAタンパク質発現レベルには負の相関関係があった[58]．ELISAとフローサイトメトリーを用いたアッセイでは，患者51人のうち13人が中和抗体陽性と判定され，そのうち9人でMxAタンパク質が誘導されなかった[75]．MxAタンパク質は細胞において非常に安定で，IFN-β投与後24～48時間後にそのタンパク質レベルは最大に達する[76]．したがって，本法では偽陰性の生じる可能性が低く，血液サンプリングもIFN-β投与前と投与後一日あるいは二日後の適当な時間に行うことができる．したがって，患者の通院に対する時間的な制約が少なくてすむ．

患者におけるIFN-βの投与によるMxA mRNAの誘導が，患者の末梢血液単核球あるいは血液サンプルを用いてリアルタイムRT-PCRで測定されている[63,77~79]．患者にIFN-βを投与12時間後に血液単核球あるいは血液からRNAを抽出しMxA mRNAの発現が調べられた[77]．その結果，54人の患者のうち7人でMxA mRNAの発現がIFN-βを投与していない健常者と同じレベルであり，IFN-βに不応答性と判断された．また，これらの患者の結合抗体及び中和抗体のタイターは高かった．同様な検討が様々なタイターの中和抗体の患者で行われた[78]．その結果，両者の値の比較により中和抗体のレベルは，IFN-βに応答性，応答性が顕著に低下，不応答性の三つに分けられることが示された．患者においてIFN-βによるMxA mRNAの発現誘導及び細胞変性アッセイによる中和抗体が測定され，その後の無再発生存率と死亡時期が比較された[80]．MxA mRNA発現陰性の患者は陽性の患者に比べて無再発生存率が低かった．同様な結果が中和抗体においても見られたが，死亡時期はMxA mRNA発現陰性の患者のほうが1か月早く，MxA mRNAのほうが予測に若干優れていることが示された．IFN-β投与後MxA mRNAレベルは13時間でピークに達しその後低下するため[81]，上記の知見は同様な時間の測定により得られている．

このように，IFN-β投与12時間後に採血のため患者が再度通院することは患者にとって負担となる．そこでIFN-β投与4時間で同様な検討が行われた[79]．その結果，中和抗体のタイターとMxA mRNAの発現レベルに良い相関関係がみられた．更に，MxA mRNAの発現をIFN-β投与前後で測定して誘導率で示すと，投与後のみの測定及び細胞変性アッセイで陰性と判定された患者2人が陽性と判定され，偽陰性の数を減らすことができた．

このように，患者にIFN-βを投与後血液におけるMxA

mRNAあるいはタンパク質を測定することにより，患者において投与したIFN-βの生物活性を直接測定できる．したがって，中和抗体が患者におけるIFN-βの生物活性に及ぼす影響について，細胞変性アッセイあるいは他の細胞を用いたアッセイよりもより正確に測定できる可能性がある．今後，本法は中和抗体によりIFN-βに対する応答性が顕著に低下した患者の判定に特に有用となるかもしれない．

3.2 エリスロポエチンに対する抗体の結合アッセイ及び中和抗体のアッセイ

エリスロポエチンは腎性貧血治療患者の治療などに幅広く使用されているが，それに対する中和抗体の出現が原因で生じる赤芽球癆は非常にまれな合併症であった[82,83]．しかし，ある特定のエポエチンアルファ製剤であるEprex®で治療した慢性腎不全の患者で赤芽球癆の例数が劇的に増加した[7,84]．そこで，赤芽球癆を併発した患者におけるエリスロポエチンに対する抗体が以下に示すように様々な方法で測定されている．

3.2.1 エリスロポエチンに対する結合抗体のアッセイ

赤芽球癆患者血清における結合抗体がブリッジングELISA法，放射免疫沈降法，表面プラズモン共鳴法により調べられた[85]．その結果，調べた患者8人のすべてで放射免疫沈降法及び表面プラズモン共鳴では陽性であったが，2人はブリッジングELISA法では陰性であった．他の施設で同血清サンプルを含む赤芽球癆患者13人の血清サンプルが放射免疫沈降法により調べられた結果，すべて陽性であった[7]．また，プロテインAを用いた放射免疫沈降法と表面プラズモン共鳴で，抗体の相対濃度が良く一致した．これらの結果，患者の臨床所見及び後述する細胞を用いた中和抗体のアッセイの結果[7,85]から，ブリッジングELISA法における陰性は偽陰性と考えられる．ブリッジングELISA法で陰性を示した理由については明らかではないが，親和性が低いため操作中の洗浄の過程で除かれた可能性が考えられる．表面プラズモン共鳴法で抗体のアイソタイプを調べると，IgG_1とIgG_4が主でIgG_3の存在量は少なく，IgMは検出されなかった[85]．この抗体のアイソタイプの測定結果は以下の点で興味深い．まず，IgMが検出されなかったことは，サンプルを採取した時点で患者の免疫応答が成熟化しており，赤芽球癆が抗体によるエリスロポエチンの中和反応により起こったことを示す．次に，IgG_3の存在量が少なくIgMが検出されなかったことは，先に述べたように放射免疫沈降法で測定できない抗体が，血清にはほとんど存在していないことを示している．したがって，放射免疫沈降法と表面プラズモン共鳴法では，ほぼ同じアイソタイプの抗体が検出されることが予想され，両測定法で抗体の相対濃度が良い相関を示したことと一致する．赤芽球癆患者血清における結合抗体がフローサイトメトリーによる結合アッセイにより測定された[17]．その結果，コントロールビーズを比較に用いた解析から，抗体はエリスロポエチンに特異的に結合し，抗体の相対濃度は表面プラズモン共鳴における値と相関することが示された．

3.2.2 エリスロポエチンに対する中和抗体のアッセイ

エリスロポエチンに対する中和抗体のアッセイに用いる細胞としては，主に健常人の骨髄由来赤血球前駆細胞[7]及びヒトエリスロポエチン受容体を導入したIL-3依存的なマウスの32D細胞株[86]が用いられている．前者の細胞を用いたアッセイでは，エリスロポエチン依存的な赤血球コロニー形成促進の抗体による阻害を測定する．後者の細胞を用いたアッセイでは，エリスロポエチン依存的な増殖促進の抗体による阻害を測定する．先に示した赤芽球癆患者13人で前者の細胞を用いた中和抗体のアッセイの結果と放射免疫沈降法の結果が比較された[7]．その結果，中和抗体のアッセイの結果に基づき，中和抗体13検体を完全阻害と50%以下の阻害の二つに分類すると，放射免疫沈降法の値は，完全阻害では8検体で11～86 U/mL，各1検体で6 U/mL及び4 U/mLであったのに対し，50%以下の阻害では2検体で4 U/mL，1検体で3 U/mLであり，中和抗体の量と阻害の強さが全体的に良く一致した．先に示した放射免疫沈降法及び表面プラズモン共鳴により陽性と判定された八つのサンプルで中和抗体のアッセイが行われた[85]．その結果，相対抗体濃度は中和抗体のアッセイと結合抗体アッセイとで全体的に良く一致したが，一つのサンプルで中和抗体の値が低かった[85]．抗体はポリクローナルな集団であるため，このサンプルでは中和活性を持たない結合抗体の比率が高い可能性が考えられる．

3.3 パニツムマブに対する抗体の結合アッセイ及び中和抗体のアッセイ

パニツムマブはヒトepidermal growth factor receptor（EGFR）に対する高親和性完全ヒトIgG_2モノクローナル抗体である．パニツムマブは標準的な抗癌剤治療実施後に転移した結腸・直腸癌の治療薬として有効であることが認められ[87]，2009年米国，2010年日本で承認された．

臨床試験においてパニツムマブによる抗体産生の誘導を評価するため，結合アッセイ及び中和抗体のアッセイが開発されている[28]．結合アッセイとしてはブリッジELISAと表面プラズモン共鳴が開発された．ブリッジELISAでは抗体と結合したパニツムマブの干渉を防ぐために酸解離法が用いられている．また，中和抗体のバ

イオアッセイとしては，A431細胞を用いたパニツムマブによるEGF依存的なEGFRリン酸化の促進の阻害に対する抗体の抑制作用を測定する．患者612名のうち2名がブリッジELISAで抗体陽性と判定され，その中で1名が中和抗体のバイオアッセイで陽性であった．同様に表面プラズモン共鳴では25名が陽性と判定され，その内8名が中和抗体のバイオアッセイで陽性と判定された．なお，中和抗体のバイオアッセイで陽性と判定された患者では，治療効果の低下は認められず，中和抗体の力価と治療効果の低下との関連は示されなかった．

抗体陽性と判定された患者の数がブリッジELISAより表面プラズモン共鳴のほうが多い理由として，ブリッジELISAでは低親和性の抗体が検出されなかった可能性が考えられる．そこで，パニツムマブに対する親和性の異なるマウスモノクローナル抗体が作成され，その段階希釈により，ブリッジELISAの検出感度に及ぼす影響が調べられた．その結果，親和性の低い抗体ほど感度が低下することが示された．なお陽性コントロールを用いて最小検出感度を測定した結果，ブリッジELISAでは10 ng/mL，表面プラズモン共鳴では1 μg/mLと，ブリッジELISAの方が高かった．

一方，抗体治療薬は比較的大量に治療に用いられ，血清における半減期は長いことから，血清中に残存する抗体治療薬がその治療薬に対する抗体の検出を妨害することが懸念される．そこで，先の抗パニツムマブマウスモノクローナル抗体に異なる濃度のパニツムマブをスパイクし，酸解離法を用いてブリッジELISAにおける抗体の検出に及ぼす影響が調べられた．その結果，親和性が高い抗体ほど干渉を起こしにくく，場合によっては最大380倍過剰量パニツムマブを添加しても干渉を起こさないことが示された．この理由については，以下のように考えられる．高親和性の抗体はウエルに固定したパニツムマブと溶液中の標識パニツムマブとより効果的にブリッジを形成する．一方，親和性の低い抗体は固定したパニツムマブと解離しやすく，遊離された抗体は溶液中の標識パニツムマブ及び残存するパニツムマブとより結合しやすくなる．その結果，感度の低下とパニツムマブの干渉を受けやすくなる．表面プラズモン共鳴でも酸解離法を用いないで，同様の検討が行われた．その結果，添加パニツムマブにより干渉を受けやすく，抗体よりも2倍高いモル濃度添加するだけで抗体の80%が干渉を受けた．この点は，血清中に高濃度の治療薬の存在が疑われる場合は，表面プラズモン共鳴において考慮すべき点かもしれない．

おわりに

治療用タンパク質に対する抗体の産生は，規制当局，製薬会社，医師，患者にとって有効性及び安全性における大きな懸念である．したがって，患者における抗体の産生を適切に検出し，抗体が有効性及び安全性に及ぼす影響を評価することが重要である．中和抗体アッセイを行う前のスクリーニングとして用いる結合アッセイには様々な方法があり，それぞれ短所及び長所がある．操作性，感度，大量処理能の観点から，最初の選択としてはELISAと表面プラズモン共鳴を併用することが一般的かもしれない．特に表面プラズモン共鳴は低親和性抗体を検出できるだけでなく，各種の抗体の特性解析に有用であり，他の結合アッセイとの併用が有用である．次に行う中和抗体アッセイは個々のタンパク質治療薬の生物活性に依存して異なり，適切な測定指標を設定する必要がある．これら抗体の結合アッセイ及び中和抗体アッセイの妥当性評価において特に注意すべき点は，血清中のマトリックス成分により測定結果が影響を受ける可能性である．したがって，マトリックスの影響を適切に評価し，マトリックスが測定結果に影響を与えないことを示す必要がある．更に，重要なカットオフポイントの設定については，適切なブランクサンプル及び統計手法を用いて設定し，真の陽性患者の見逃しを極力回避することが重要である．最終的に，結合アッセイ特に中和アッセイにより抗体陽性と判定された場合は，有効性及び安全性との関連で評価し，必要に応じて適切な処置を取る必要がある．その関連をより共通に評価するには，個々のタンパク質治療薬について，対象とするタンパク質治療薬の生物活性に基づいて設定された中和抗体のアッセイ方法，陽性コントロール並びにカットオフポイントの設定方法等を標準化する必要がある．更に，有効性及び安全性に影響を及ぼす中和抗体のタイターの下限値が設定可能ならば，治療を継続すべきかどうかの判断に有用となる．次回は，本稿も含めた免疫原性に関する一連の総説[8,12]のまとめとして，リスクに基づいた治療用タンパク質に対する抗体の評価の戦略について概説する．

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Development of Defective and Persistent Sendai Virus Vector

A UNIQUE GENE DELIVERY/EXPRESSION SYSTEM IDEAL FOR CELL REPROGRAMMING[*] [S]

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The ectopic expression of transcription factors can reprogram differentiated tissue cells into induced pluripotent stem cells. However, this is a slow and inefficient process, depending on the simultaneous delivery of multiple genes encoding essential reprogramming factors and on their sustained expression in target cells. Moreover, once cell reprogramming is accomplished, these exogenous reprogramming factors should be replaced with their endogenous counterparts for establishing autoregulated pluripotency. Complete and designed removal of the exogenous genes from the reprogrammed cells would be an ideal option for satisfying this latter requisite as well as for minimizing the risk of malignant cell transformation. However, no single gene delivery/expression system has ever been equipped with these contradictory characteristics. Here we report the development of a novel replication-defective and persistent Sendai virus (SeVdp) vector based on a noncytopathic variant virus, which fulfills all of these requirements for cell reprogramming. The SeVdp vector could accommodate up to four exogenous genes, deliver them efficiently into various mammalian cells (including primary tissue cells and human hematopoietic stem cells) and express them stably in the cytoplasm at a prefixed balance. Furthermore, interfering with viral transcription/replication using siRNA could erase the genomic RNA of SeVdp vector from the target cells quickly and thoroughly. A SeVdp vector installed with *Oct4/Sox2/Klf4/c-Myc* could reprogram mouse primary fibroblasts quite efficiently; ~1% of the cells were reprogrammed to Nanog-positive induced pluripotent stem cells without chromosomal gene integration. Thus, this SeVdp vector has potential as a tool for advanced cell reprogramming and for stem cell research.

The generation of induced pluripotent stem (iPS)[3] cells by reprogramming tissue cells with defined factors opened the door for realizing the medical application of patient-derived engineered stem cells (1). iPS cells were established originally by the ectopic expression of multiple transcription factors (*e.g.* Oct3/4, Sox2, Klf4, and c-Myc) using a retroviral vector (1). Since then, researchers have established iPS cells by several different approaches (and by their combination), including gene transfer, protein transduction, and treatment with chemical compounds (2). However, because of superior reproducibility and efficacy, ectopic expression of reprogramming factors by gene transfer is still the primary method of choice.

Various lines of evidence indicate that efficient cell reprogramming requires the sustained and simultaneous expression of several (usually 4) exogenous factors for at least 10–20 days (3). On the other hand, after reprogramming has been completed, these exogenous factors should be replaced promptly with their endogenous counterparts if the cells are to acquire autoregulated pluripotency (3). For this reason, retroviral and lentiviral vectors have been used preferentially; chromosomal insertion of the vector genome allows for stable gene expression, whereas epigenetic modification of the viral promoter shuts off the vector-mediated gene expression after cell reprogramming has been accomplished. Nevertheless, cell reprogramming with these insertional vectors has a crucial disadvantage in that silencing and reactivation of the inte-

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[3] The abbreviations used are: iPS, induced pluripotent stem; ES, embryonic stem; SeV, Sendai virus; SeVdp, defective and persistent Sendai virus; Bs, blasticidin S; Zeo, zeocin; *Zeo*, phleomycin-binding protein gene; *Bsr*, blasticidin S deaminase gene; *Hyg*, hygromycin B phosphotransferase gene; EGFP, enhanced green fluorescent protein; KO, Kusabira Orange; KR, Keima Red; MEF, mouse embryonic fibroblast; NP, nucleocapsid protein; CFU, colony-forming unit.

grated reprogramming genes is often unmanageable, which might affect the differentiation potency of iPS cells and the safety of the iPS-derived cells. Thus, investigators have focused on generating iPS cells carrying no exogenous genetic materials either by repetitive transient gene expression (4, 5), by passive elimination of stable episomal DNA (6), or by recombinase-mediated excision of integrated genes from the chromosome (Refs. 7 and 8; for review, see Ref. 9). However, all of these approaches are not only inefficient but also laborious in practice, and development of a simpler gene delivery/expression system suitable for cell reprogramming is needed.

Sendai virus (SeV) is a nonsegmented negative-strand RNA virus belonging to the *Paramyxoviridae* (10). As SeV can infect various animal cells with an exceptionally broad host range and is not pathogenic to humans, various applications have been explored for SeV as a recombinant viral vector capable of transient but strong gene expression (11). We have demonstrated the potential of SeV as a tool for stable gene expression through an analysis of the Cl.151 strain (12). This unique variant was originally isolated as a mutant capable of persistent infection at a nonpermissive temperature (38 °C) (13). We cloned the entire genome of SeV Cl.151 and determined that more than two genetic elements were responsible independently for the establishment of stable persistent infections (12). We also demonstrated that SeV Cl.151 installed with a single exogenous gene could express it stably without chromosomal insertion (12). As this characteristic is advantageous for cell reprogramming, we planned to optimize this gene delivery/expression system through a more extensive analysis of SeV-mediated stable gene expression.

Here we describe the replication-defective and persistent Sendai virus (SeVdp) vector, a novel gene transfer/expression system based on SeV Cl.151, with the following characteristics, 1) efficient, harmless, and simultaneous delivery of up to four exogenous genes installed on a single vector, 2) stable and reproducible expression of installed genes at a pre-fixed balance without chromosomal integration, and 3) quick and complete erasure of the vector genome by interfering with viral RNA-dependent RNA polymerase using siRNA. We also demonstrated that an SeVdp vector installed with *Oct4*/*Sox2*/*Klf4*/*c-Myc* could reprogram mouse primary fibroblasts efficiently. These characteristics should make SeVdp a universal tool for stem cell research, especially for advanced cell reprogramming.

EXPERIMENTAL PROCEDURES

Reconstitution of SeVdp Vector by Reverse Genetics—All recombinant DNA experiments were performed according to our institutional guidelines and under the permission of the institutional recombinant DNA experiment committee of the National Institute of Advanced Industrial Science and Technology and of the National Institutes of Health Sciences. Replication-competent SeV was reconstituted as described (12). Full-length SeVdp vector genomic cDNA for SeV (Cl.151 strain and Nagoya strain) and for installed genes was constructed on the lambda Dash II vector as described in supplemental Fig. S1. In brief, the *M*, *F*, and *HN* genes were replaced with exogenous genes cloned between KasI and MluI restriction sites (for *M*), between BglII sites (for *F*), and between NheI and SphI sites (for *HN*). Additional extra genes were inserted into an NheI/NotI site created between the *P*/*C*/*V* and *M* genes. cDNAs encoding blasticidin S deaminase (*Bsr*), phleomycin-binding protein (*Zeo*), enhanced green fluorescent protein (*EGFP*), *Cypridina noctiluca* luciferase (*CLuc*), humanized Kusabira Orange (*KO*), and human gp91phox (*CYBB*) were amplified by polymerase chain reaction using pCX4-bsr (14), pUT58 (15), pEGFP-1 (Takara Bio, Otsu, Japan), pCLm (ATTO, Tokyo, Japan), phKO1-MN1 (Medical & Biological Laboratories, Nagoya, Japan), and gp91phox-pCI-neo as templates, respectively. cDNA encoding humanized Keima Red (*KR*) was synthesized by GenScript (Piscataway, NJ), according to a published peptide sequence (16).

The reconstructed cDNA plasmids (2 μg) and the expression vector plasmids for SeV nucleocapsid protein (NP), P/C, and L proteins (1 μg each) and pSRD-HN-Fmut (17) (2 μg) were transfected into BHK/T7/151M(SE) cells using Lipofectamine LTX Plus reagent (Invitrogen). BHK/T7/151M(SE) cells were established by expressing humanized T7 RNA polymerase and the M protein of the SeV Cl.151 strain stably in BHK-21 cells and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The SeVdp vector was reconstituted in cells from positive-strand antigenome RNA transcribed from this cDNA and the SeV NP, P/C, L, Fmut, HN, and M (Cl.151) proteins. The vector-packaging cells harboring the SeVdp vector were established by selecting with antibiotics (blasticidin S at 10 μg/ml, zeocin at 500 μg/ml, or hygromycin B at 200 μg/ml) except for SeVdp(*c-Myc*/*Klf4*/*Oct4*/*Sox2*). The SeVdp vector was rescued by transient expression of the SeV *Fmut*, *HN*, and *M* (Cl.151) genes (driven by the SRα promoter derived from pcDL-SRα) (18) in the packaging cells as described above and recovered into the culture supernatant after incubation at 32 °C for 4 days. *Fmut*, a modified F gene for expressing the protease-susceptible SeV F protein, was generated as described (17). The supernatant was filtered through 0.45-μm cellulose acetate filters and stored in small aliquots at −80 °C. Titers of SeVdp vectors were determined by examining $LLCMK_2$ cells infected with a diluted SeVdp vector suspension using indirect immunofluorescence microscopy with an anti-NP rabbit polyclonal antibody.

Cell Culture, Fluorescence Microscopy, and Flow Cytometry—Long term stability of gene expression mediated by the SeVdp vectors was examined in $LLCMK_2$ cells and in human primary fibroblasts (TIG3) cultured in Eagle's minimum essential medium supplemented with 10% FCS. For examining stability under antibiotic selection, the cells were incubated in the presence of blasticidin S (Bs) (5 μg/ml), hygromycin B (200 μg/ml), or a mixture of blasticidin S (5 μg/ml) and zeocin (Zeo) (100 μg/ml). For examining stability without selection, the cells were preselected with antibiotics and then cultured without selection. The presence of SeVdp was determined by detecting SeV NP antigen with indirect immunofluorescence microscopy, counterstained with DAPI. EGFP, KO, and KR were detected by fluorescence microscopy (Zeiss, Oberkochen, Germany) using specific filters customized for these proteins. Flow cytometry was performed using a

FACSCalibur (BD Biosciences; see Figs. 2*B* and 3, *E* and *F*) and with FISHMAN R (On-chip Biotechnologies, Tokyo, Japan; Fig. 3, *G* and *H*) according to the standard procedures provided by the manufacturers.

Gene Delivery to Human Hematopoietic Stem Cells—All experiments using human resources were performed according to National Institute of Advanced Industrial Science and Technology and National Institutes of Health Sciences guidelines. OP9 cells (19) (provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan) were cultured with minimal essential medium-α containing 20% FCS and 4 mM l-glutamine. Human umbilical cord blood was collected after a normal pregnancy and delivery after obtaining informed consent from the mothers. Human mononuclear cells were isolated from the cord blood using Lymphoprep (Axis-Shield, Oslo, Norway) according to the protocol provided by the manufacturer. CD133/1(+) cells were prepared from mononuclear cells using CD133 microbead kits and an AutoMACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) with a purity greater than 90%, confirmed by flow cytometry using a FACSCalibur.

The purified CD133/1(+) cells were infected with SeVdp(*Bsr*/Δ*F*/*KO*) at a multiplicity of infection of 4 at 37 °C for 2 h. For examining the efficiency of gene delivery, the infected cells were cultured for 10 days in Iscove's modified Dulbecco's medium supplemented with 20% FCS, 8 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 ng/ml human stem cell factor, 5 ng/ml interleukin-6 (IL-6), 5 ng/ml IL-3, 25 ng/ml flt-3 ligand (all from PeproTech, Rocky Hill, NJ), and 50 ng/ml human thrombopoietin (Kirin, Tokyo, Japan), and the fraction of KO-positive cells was determined by flow cytometry (Fig. 2*B*). For long term culture-initiating cell assays (20), 20- 200 SeVdp-infected cells were seeded on 1.25×10^4 γ-ray-irradiated OP9 cells in 96-well plates in Iscove's modified Dulbecco's medium with 12.5% FCS, 12.5% horse serum, 8 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1 μM hydrocortisone for 5 weeks; half of the medium was exchanged every week. Then whole cells in a well were recovered and cultured in 300 μl of semisolid colony-forming cell assay medium (MethoCult GF+ H4433, StemCell Technologies, Vancouver, Canada) in 48-well plates. After culturing for 2 weeks, the numbers of KO-positive colonies were determined.

RNA Interference Analysis—The sequences of siRNAs against SeV *NP*, *P*, and *L* mRNAs (designed and synthesized by iGENE Therapeutics, Tokyo, Japan) used in this study are listed in supplemental Table S1. For examining the effect of siRNAs on the infection of replication-competent SeV vectors (supplemental Fig. S4*A*), 2×10^4 HeLa cells were seeded in 48-well plates with DMEM containing 10% FCS on day 0. On day 1, the cells were treated with siRNAs (100 nM) mixed with Lipofectamine 2000 (Invitrogen) for 6 h, then infected with SeV Cl.151(*EGFP*) at a multiplicity of infection of 100. The medium was replaced on day 2, and the cells were examined using fluorescence microscopy on day 4. For examining the effect of siRNAs on the removal of SeVdp vector from BHK-21 cells expressing the T7 RNA polymerase and F protein of the SeV Nagoya strain constitutively (BHK/T7/NaF cells) (supplemental Fig. S4*B*), 1×10^4 cells harboring SeVdp(*M*/*EGFP*/*Bsr*) were seeded in 48-well plates on day 0. On day 1 the cells were treated with siRNA (100 nM) mixed with Lipofectamine 2000 as described above. On day 5, the cells were examined using fluorescence microscopy. In both of these experiments, siRNA against firefly luciferase (21) was used as a control.

For examining the effect of siRNA on the removal of SeVdp vector using luciferase activity as a quantitative index (Fig. 4), 1.5×10^5 (Fig. 4*B*) or 3×10^4 (Fig. 4*C*) HeLa cells harboring SeVdp(*KO*/*Hyg*/*EGFP*/*Luc2CP*) were seeded with siRNAs (40 nM) mixed with Lipofectamine RNAiMAX (Invitrogen) as described above on day 0 in a 6-well plate (Fig. 4*B*) or in a 24-well plate (Fig. 4*C*), respectively. siRNA against *Renilla reniformis* luciferase (22) was used as a control. The cells were passaged with fresh siRNA on days 3 and 7, and the culture medium was replaced on the next day at each point. Firefly luciferase activity in the cell extract was determined on the indicated day using a luciferase assay system (Promega, Madison, WI). Specific luciferase activity was determined by normalizing against the amount of protein, determined using a Bradford protein assay kit (Bio-Rad). The cell lysates prepared on days 3, 7, and 12 were also analyzed by Western blotting using affinity-purified anti-SeV L protein rabbit antibody (2 μg/ml). For certifying complete removal of the SeVdp genome, the cells harboring SeVdp(*KO*/*Hyg*/*EGFP*/*Luc2CP*) and treated with siRNA as described above were cultured in the absence of siRNA for 4 weeks. Then, 1×10^4 of the cells were seeded in a 6-well plate and cultured in the presence of hygromycin B (100 μg/ml) for 10 days. The surviving cells were fixed, then stained with 0.01% crystal violet.

Biochemical Assays—SDS-PAGE and protein blotting were performed as described (23) using SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Waltham, MA). For filter trap assays to detect the NP antigen (Table 1), culture supernatants of the cells harboring SeVdp vectors were passed through 0.45-μm cellulose acetate filters, trapped onto supported nitrocellulose membranes (0.2 μm, Bio-Rad) by vacuum filtration, and probed with an anti-SeV NP monoclonal mouse antibody.

Cell Reprogramming—Isolation and culture of mouse embryonic fibroblasts (MEFs) from a Nanog/GFP knock-in mouse (provided by the Riken BioResource Center) (MEF/Nanog-GFP), reprogramming with retroviral vectors and culture of mouse iPS cells were performed as described previously (24). Retroviral vectors installed separately with *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* (RvMX4) were prepared as described (1) using template DNA obtained from Addgene (Cambridge, MA). For reprogramming with SeVdp vectors installed with *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*, 1.25×10^5 of MEF/Nanog-GFP-expressing cells were infected with SeVdp vectors at 32 °C for 14 h. Then 1.0×10^3 of infected cells were seeded onto the feeder cells in 6-well plates and cultured as indicated in the legend to Fig. 5. The numbers of iPS colonies expressing GFP were determined using fluorescent microscopy. At 10 days after SeVdp infection, GFP-positive clones were isolated and treated with siRNA L527 as described above.

Characterization of iPS Cells—Semiquantitative RT-PCR Assays were performed using GoTaq qPCR Master Mix (Promega) and the primer sets listed in supplemental Table S2. Template cDNA was synthesized with random primers using SuperScript III reverse transcriptase (Invitrogen) from 2 μg of total cellular RNA isolated using ISOGEN (Nippon Gene, Tokyo, Japan). Bisulfite sequencing analysis was performed using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) with primer sets listed in supplemental Table S2. The PCR DNA fragments were cloned into pCR2.1 vector (Invitrogen), and sequenced by TAKARA BIO INC. (Shiga, Japan). Telomerase activity was determined using the Quantitative Telomerase Detection kit (Allied Biotech, Vallejo, CA). Teratoma formation was performed by subcutaneous injection of 1×10^6 SeVdp-iPS cells (clone #13) into SCID mice. Tumors recovered at necropsy after 6 weeks were processed for fixing and paraffin wax embedding, sectioned (4 μm), and stained with hematoxylin and eosin. Histological findings were evaluated using a DM3000 microscope (Leica, Wetzlar, Germany). Chimera animals were generated by microinjection of iPS cells into eight-cell or morula stage embryos. The embryos were collected in Medium 2 (Millipore, Billerica, MA) from oviduct and uterus of ICR female mice 2.5 days post-coitum. These embryos were transferred into potassium simplex optimized medium with amino acids (KSOM-AA, Millipore) and cultured for 1–2 h. iPS cells were trypsinized and suspended in iPS cell culture medium. A piezo-driven micro-manipulator (Prime Tech, Tokyo, Japan) was used to drill zona pellucida under the microscope, and 10–15 iPS cells were introduced into the subzonal space of individual 8-cell or morula-stage embryos. After injection, embryos underwent follow-up culture in KSOM-AA for 24 h (until blastomere stage) and then were transferred into the uteri of pseudopregnant recipient ICR female mice.

RESULTS

Basic Design of the SeVdp Vector—The SeV genome consists of six independent cistrons (NP, P/C/V, M, F, HN, and L), encoding eight proteins (10). Each cistron is preceded by a gene-start signal (3′-UCCCNNUUUC) and is followed by a gene-end signal (3′-AUUCUUUUU), which are the only essential *cis*-elements for transcription (25). This simple structure of each cistron makes it easier to design a defective viral vector by gene replacement. The *NP*, *P*, and *L* genes of SeV encode a major NP and two subunits of RNA-dependent RNA polymerase (P and L), respectively. All of these are indispensable for viral transcription and replication (10). We revealed previously that the *L* gene of the SeV Cl.151 strain with four missense mutations (V981I, S1088A, C1207S, and V1618L) contributes to long term persistence by providing the mechanism to escape from interferon β (IFNβ) induction (12). Among these mutations, V1618L is most critical; SeV with a mutant L protein (V1618L) is defective in IFNβ induction as with the Cl.151 strain.[4] We also found that uncapped read-through transcripts synthesized in an early stage of infection with wild-type SeV were only barely detectable in cells infected with SeV/L (V1618L).[4] We hypothesized that the defect in IFNβ induction might be correlated with the altered transcription of uncapped read-through RNA by the mutant SeV RNA polymerase.

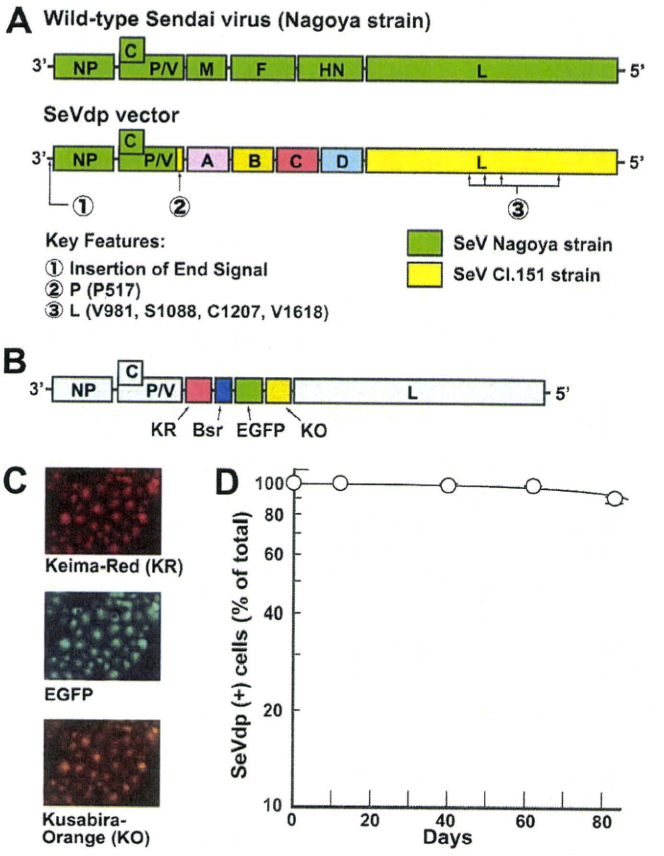


FIGURE 1. **Design and characteristics of the SeVdp vectors.** *A*, genome structure of the Sendai virus and an SeVdp vector is shown. Exogenous genes installed on the SeVdp vector are indicated as *A–D*. *B*, genome structure of SeVdp(*KR/Bsr/EGFP/KO*) is shown. *C*, expression of the fluorescent marker genes installed on the SeVdp vector is shown. LLCMK$_2$ cells were infected with SeVdp(*KR/Bsr/EGFP/KO*) at a multiplicity of infection of 0.1, selected with blasticidin S (5 μg/ml), and examined by fluorescence microscopy with dye-specific filters. *D*, stability of gene expression induced by the SeVdp vector is shown. LLCMK$_2$ cells were infected with SeVdp(*KR/Bsr/EGFP/KO*) and selected with blasticidin S as described in *C*. The cells were then cultured for the indicated period in the absence of blasticidin S. The ratio of SeV NP antigen-positive cells in the total cells was determined by fluorescence microscopy, as described under "Experimental Procedures."

In addition, we identified a missense mutation of the *P* gene (P517H) that is also essential for establishing long term persistency (Fig. 1*A*, supplemental Fig. S2*A*). The P protein makes a complex with the NP and L proteins, and the C terminus of P protein (amino acids, 479–568) has been assigned as a binding region for the NP (26). However, the precise role of this alteration in long term persistency remains to be determined. On the other hand, the 3′-distal region (nucleotides 1–2870) of the genome of SeV Cl.151 consists of the whole *NP* gene and part of the *P/C/V* genes but does not contribute to viral persistency (12) (Fig. 1*A*, supplemental Fig. S2*A*). Rather, we found that replacement of this 3′-distal region with that of the wild-type Nagoya strain significantly improved the recovery of the recombinant SeV from full-length genomic cDNA (supplemental Fig. S2*B*). We also inserted a

[4] K. Nishimura and M. Nakanishi, unpublished information.

gene-end signal just upstream of the gene-start signal of the *NP* gene (Fig. 1*A*, supplemental Fig. S2*C*). This modification further stabilized SeV-mediated gene expression through more stringent control of IFNβ induction by forced termination of uncapped read-through transcripts.[4]

We then planned to expand the capacity of the SeV Cl.151-based vector by deleting all the viral genes dispensable for stable gene expression. We have revealed previously that mutations within the central region (nucleotides 2871–9594) of the SeV Cl.151 genome contributed to viral persistence independently from the altered *L* gene (12). The region consists of *M*, *F*, and *HN* genes, which encode a matrix protein underlining the viral envelope (M) and envelope glycoproteins (F and HN), respectively. These structural genes are essential for production of infectious virions but are dispensable for viral transcription/replication, as SeV vectors with all of the *M*, *F*, and *HN* genes deleted could be generated successfully (27). However, as these defective vectors could not support stable gene expression, the role of these structural genes in long term persistency remains obscure.

We found previously that cells infected with SeV Cl.151 expressed large quantities of F and HN proteins on their surface (28). This observation suggested that the accumulation of the structural gene products (proteins and/or mRNAs) might interfere with the lytic infection cycle by a negative feedback mechanism, as proposed previously for the M protein (29). To examine this premise directly, we characterized recombinant SeVs with a dysfunction in each of the *M*, *F*, or *HN* genes either by deletion or by nonsense mutation. A selective marker gene (*Bsr*) conferring resistance to blasticidin S was used for estimating persistency rapidly (supplemental Fig. S3*A*). We found that all of these single-gene defective viruses established stable Bs-resistant colonies (supplemental Fig. S3*A*). Moreover, any two of these structural genes could be replaced with exogenous genes without affecting the persistent phenotype (supplemental Fig. S3*A*). To examine the role(s) of the M, F, and HN proteins further, we coexpressed these proteins directly from the cloned cDNAs and found that those derived from wild-type SeV strains induced much stronger cytopathic effects than did those derived from the SeV Cl.151 strain (supplemental Fig. S3*B*). From these results, we conclude that all of the *M*, *F*, and *HN* genes of SeV Cl.151 are dispensable and can be replaced with exogenous genes without disturbing viral persistency. We also succeeded in expanding the capacity of the vector by inserting an extra gene cassette between the *P*/*C*/*V* genes and the *M* gene without affecting viral persistency (Fig. 1*A*).

Dysfunction of structural genes is also essential for preventing self-replication of the vector. As the vectors used for cell reprogramming are installed with tumorigenic genes, such as *c-Myc* and *LIN28*, avoiding the production of secondary infectious particles from the gene-transferred cells is important not only to observe the regulation of recombinant DNA experiments but also to guarantee the safety of the vector in any therapeutic application. In the case of SeV, cultured cells infected with SeV variants defective in single structural genes produced significant amounts of virus-like particles (30, 31), suggesting that a dysfunction in single genes is insufficient for the complete blockage of self-replication. To reexamine this phenomenon, we determined the numbers of infectious particles and the amount of NP antigen in the culture supernatant of cells infected with various SeV Cl.151-derived defective viruses carrying the *Bsr* gene (Table 1). We found that cells infected with SeVs bearing a defect in one of the *F*, *HN*, or *M* genes produced significant amounts of NP antigen as well as infectious particles capable of transmitting Bs resistance to naïve cells (Table 1). This phenomenon was not observed when the viruses carried defects in at least two of the structural genes (Table 1). Therefore, we conclude that all three structural genes should be eliminated for maximizing the safety of the SeVdp vector through abolishing self-replication and for maximizing the vector capacity for installing exogenous genes.

TABLE 1

Determination of infectious virions and the NP protein in the culture supernatant of the cells harboring SeV Cl.151-based vectors

All the vectors were installed with the *Bsr* gene encoding blasticidine S deaminase. Aliquots of 10^6 of $LLCMK_2$ cells harboring each SeVdp vector were seeded in 90-mm wells with 8 ml of medium. After culturing for 3 days, culture supernatant was recovered and filtered through 0.45-μm cellulose acetate membranes. NP protein was determined by blotting 0.04–20 μl of the supernatant on nitrocellulose membranes as described under "Experimental Procedures." The supernatant was also incubated with 10^6 uninfected $LLCMK_2$ cells for 14 h and then cultured in the presence of Bs (10 μg/ml) for 7 days. The numbers of cell colonies resistant to Bs were determined by staining with crystal violet.

Structural genes			NP protein	Number of Bs^r colonies
			ng/day/10^5 cells	
M[a]	F[a]	HN[a]	8.75	$> 10^6$
–[b]	F	HN	5.36	366
M	–[b]	HN	7.62	2
M	F	–[b]	72.05	10
M	–[b]	–[b]	2.51[c]	0
–[b]	F	–[b]	2.76[c]	0
–[b]	–[b]	HN	2.61[c]	0
–[b]	–[b]	–[b]	2.56[c]	0

[a] Replication-competent vector.
[b] Corresponding genes were deleted or replaced with exogenous genes.
[c] Background caused by spontaneous cell lysis.

In summary, we have designed the basic genome structure of the SeVdp vector. This consists of three separate genetic elements (Fig. 1*A*) as follows. 1) The 3′-terminal structure comprises the *NP* and *P*/*C*/*V* genes derived from the Nagoya strain with an alteration for supporting stable gene expression. 2) Internal gene cassettes capable of installing up to four exogenous genes, created by deletion/replacement/insertion of *M*, *F*, and *HN* genes. 3) The *L* gene and the 5′-terminal structure derived from the Cl.151 strain with four missense mutations necessary for stable gene expression and for escaping from IFNβ induction.

Characterization of SeVdp Vector-mediated Gene Expression—We then prepared the SeVdp vectors installed with four exogenous genes and characterized vector-mediated gene expression. We first constructed SeVdp(*KR/Bsr/EGFP/KO*) installed with *Bsr* and three marker genes encoding KR, EGFP, and KO (Fig. 1*B*). All the cells infected with this vector expressed the three marker genes stably after selection with Bs (Fig. 1*C*). Furthermore, even in the absence of selection, 98.2% of cells retained the vectors for 62 days (Fig. 1*D*). Stability of gene expression induced by SeVdp vectors was solely dependent on the vector backbone described above and was not affected either by the installed genes or by the characteris-

TABLE 2

Stability of gene expression induced by SeVdp vectors

Cells harboring the SeVdp vectors were cultured in the presence of Bs (10 μg/ml) to certify that 100% of the cells were SeVdp (+). On day 0 the cells were set up in the medium either with Bs (Bs(+)) or without Bs (Bs(−)). Expression of SeV NP antigen was recorded periodically, and the day on which 100% ($T_{100\%}$), 95% ($T_{95\%}$), or 80% ($T_{80\%}$) of the cells expressed NP is indicated. The gene cassette no. corresponds to those shown in Fig. 1*A*. *Bsr*, blasticidin S deaminase; *KO*, Kusabira Orange; *EGFP*, enhanced green fluorescent protein; *CLuc*, *Cypridina noctiluca* luciferase; *CYBB*, gp91 phox; a*Gal*, human a-galactosidase; *KR*, Keima Red; ND, not determined because of the limited lifespan of the cells.

Gene cassette no.				Bs (−)		Bs (+)
A	B	C	D	$T_{95\%}$	$T_{80\%}$	$T_{100\%}$
					Days	
−[a]	*Bsr*	−[a]	*KO*	80[b]	205[b]	>180[b]
−[a]	*Bsr*	−[a]	*KO*	>80[c]	ND[c]	ND[c]
−[a]	*Bsr*	*EGFP*	*CLuc*	65[b]	105[b]	>180[b]
−[a]	*Bsr*	*EGFP*	*CYBB*	65[b]	170[b]	>180[b]
−[a]	*Bsr*	*EGFP*	*α-Gal*	60[b]	112[b]	>180[b]
KR	*Bsr*	*EGFP*	*KO*	70[b]	195[b]	>180[b]

[a] No exogenous gene was installed.
[b] Determined in $LLCMK_2$ cells.
[c] Determined in normal human fibroblasts.

tics of host cells (Table 2). Under selection with antibiotics, nearly 100% of cells could retain the expression of all the marker genes for at least 6 months (Table 2). Reflecting the characteristics of its parental virus, the SeVdp vector could deliver and express the installed genes stably in various host cells, including cell lines derived from the mouse (NIH3T3), hamster (CHO, BHK-21), monkey ($LLCMK_2$, CV-1, COS-7), and human (HeLa, U937) as well as human and mouse primary fibroblasts (12). Thus, we proved that the SeVdp vectors had preserved the same characteristics of the parental SeV Cl.151 to establish stable persistent infection after it had been modified with four exogenous genes.

We then examined the feasibility of using the SeVdp vectors in stem cell research, focusing on their biological inertness. Most gene delivery/expression systems using either recombinant viruses or physical/chemical means often trigger cellular defense systems against pathogenic microbes (32). The stimulated cells secrete various cytokines, which affect the proliferation, differentiation, and survival of stem cells. Wild-type SeV and conventional SeV vectors based on the wild-type Z strain powerfully induced the production of IFNβ, INFγ, TNFα, IL-1β, IL-6, IL-8, and many other cytokines (33–35), resulting in the apoptotic death of target cells. On the other hand, we revealed previously that SeV Cl.151 has a defect in inducing these inflammatory cytokines (12), suggesting strongly that the SeV Cl.151-based vector is biologically inert. To verify this under more stringent experimental conditions, we examined the effect of SeVdp-mediated gene transfer/expression on human hematopoietic stem cells (HSCs) by long term culture-initiating cell assays (20).

We isolated a CD133 (+) HSC-enriched fraction from human cord blood, infected it with the SeVdp vector bearing the *KO* gene (SeVdp(*Bsr*/Δ*F*/*KO*)) on day 0, and cultured it further in standard conditions. More than 90% of the cells in the HSC-enriched fraction were susceptible to the SeVdp vector under this infection protocol and sustained strong KO expression on days 3 (Fig. 2*A*) and 10 (Fig. 2*B*). Seven weeks after culturing on OP9 stromal cells, all kinds of myeloid lineage colonies derived from human HSCs, including colony-forming unit (CFU)-granulocytes (*CFU-G*), CFU-macrophages (*CFU-M*), CFU-granulocyte-macrophage , and burst-forming unit-erythroid (*BFU-E*) cells were readily detectable (Fig. 2*D–F*). Most importantly, 92% of these colonies still expressed KO very strongly on the seventh week (Fig. 2*C*), indicating that the SeVdp vector can deliver the gene quite efficiently into HSCs and is inert enough to sustain gene expression without affecting the differentiation of multipotent HSCs.

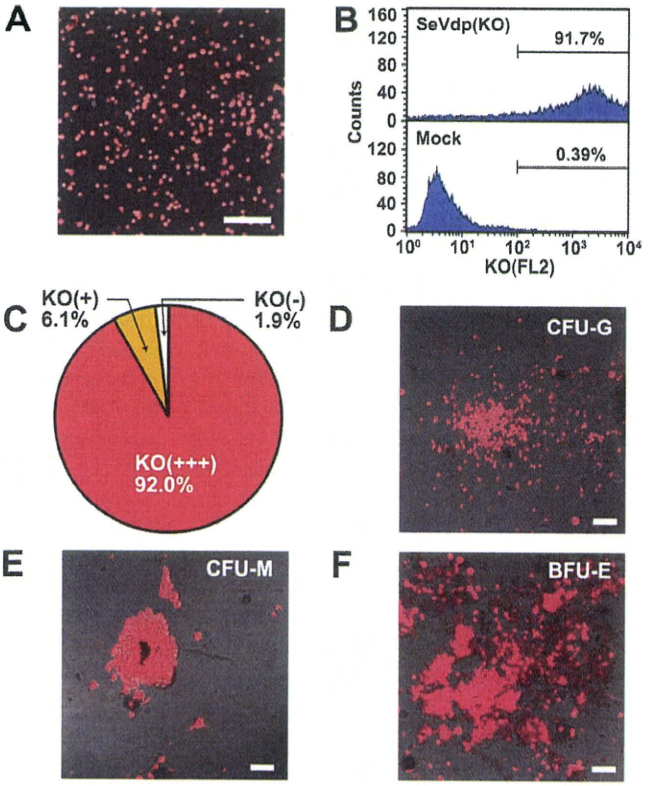


FIGURE 2. **Expression of KO in human hematopoietic stem cells and in their descendant cells.** *A* and *B*, expression of KO in CD133 (+) cord blood cells is shown. CD133 (+) cells were purified with magnetic beads conjugated with anti-CD133 antibody (Miltenyi Biotech). The cells were infected with SeVdp(*Bsr*/Δ*F*/*KO*) at a multiplicity of infection of 4 at 37 °C for 2 h. The cells were then cultured for 3 days (*A*) and 10 days (*B*) and examined using fluorescence and phase-contrast microscopy (*A*) and with flow cytometry using a FACSCalibur (BD Biosciences) (*B*), respectively. *C–F*, expression of KO in descendant colonies differentiated *in vitro* is shown. Cells infected with SeVdp(*Bsr*/Δ*F*/*KO*) as described above were cultured on OP9 cells in a 96-well plate for 5 weeks for lineage commitment. The cells in each well were then harvested, cultured in semisolid medium for 2 weeks, and examined for the expression of KO using fluorescence microscopy. *C*, the ratio of KO-positive colonies; 2931 differentiated colonies were examined. KO (+++), colonies expressing KO strongly; KO (+), colonies expressing KO weakly or heterogeneously; KO (−), colonies with no detectable KO expression. *D–F*, fluorescence and phase-contrast micrographs of typical colonies representative of each lineage. *D*, *CFU-G*, CFU-granulocytes. *E*, *CFU-M*, CFU-macrophages. *F*, *BFU-E*, burst-forming unit-erythroid cells. *Scale bar*, 100 μm.

For efficient and reproducible cell reprogramming, it is also important to express the various reprogramming genes at a fixed balance in each target cell (36–39). The SeVdp vector installed with four exogenous genes could deliver these genes simultaneously, so is theoretically superior to those systems delivering the genes separately. To clarify this issue further, we prepared an SeVdp vector installed with *KO* and *EGFP* together (SeVdp(*KO*/*Hyg*/*EGFP*/*Luc2CP*)) (Fig. 3*A*) and two

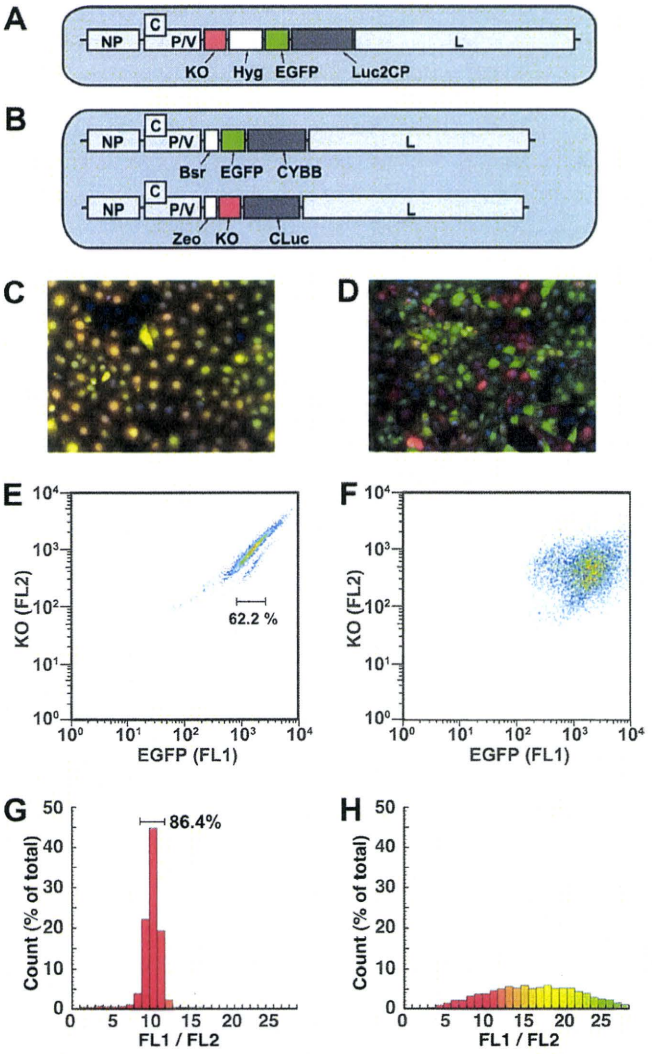


FIGURE 3. **Compatibility of two independent SeVdp vectors in a single cell.** *A*, genome structure of SeVdp(*KO/Hyg/EGFP/Luc2CP*) is shown. *B*, genome structure of SeVdp(*Bsr/EGFP/CYBB*) and SeVdp(*Zeo/KO/CLuc*), coexisting in a single cell is shown. *C* and *D*, fluorescence and phase-contrast micrographs of the cells described in schema (*C*) and in schema *B* (*D*) are shown. $LLCMK_2$ cells were infected with the SeVdp vectors as described in Fig. 1*C* and selected with hygromycin B (200 μg/ml) (*C*) or with zeocin (100 μg/ml) and blasticidin S (5 μg/ml) (*D*). Fluorescence images of KO and of EGFP were obtained separately with specific filter sets, converted to artificial color (*green* for EGFP and red for KO), and merged using iVision software (BioVision Technologies, Exton, PA). *E–H*, quantitative analysis of EGFP and KO expression by flow cytometry is shown. The cells shown in *C* (*E* and *G*) or in *D* (*F* and *H*) were harvested as single-cell suspensions with trypsin, and the fluorescent signals were analyzed using a FACSCalibur (BD Biosciences) for quantifying the signals of EGFP (FL1, 515–545 nm) and KO (FL2, 564–606 nm) after compensation (*E* and *F*) and analyzed with FISHMAN R (On-tip Biotechnologies) for determining the ratio of the signals of EGFP and KO in each cell as a histogram (*G* and *H*).

others installed with *KO* and *EGFP* separately on different SeVdp vectors (SeVdp(*Bsr/EGFP/CYBB*) and SeVdp(*Zeo/KO/CLuc*)) (Fig. 3*B*). We then characterized the expression levels of KO and EGFP induced by a single infection with SeVdp(*KO/Hyg/EGFP/Luc2CP*) and by coinfection with SeVdp(*Bsr/EGFP/CYBB*) and SeVdp(*Zeo/KO/CLuc*) (Fig. 3). When the cells were infected solely with SeVdp(*KO/Hyg/EGFP/Luc2CP*) (Fig. 3*A*), they expressed both KO (*red*) and EGFP (*green*) at a constant balance, shown by a uniform yellow color in merged microscopy images (Fig. 3*C*). In contrast, the cells coinfected with SeVdp(*Bsr/EGFP/CYBB*) and SeVdp(*Zeo/KO/CLuc*) (Fig. 3*B*) expressed KO and EGFP at a significantly different balance even after selection under Zeo plus Bs conditions (Fig. 3*D*). We then examined these cells quantitatively by flow cytometry (Fig. 3, *E–H*). When coinfected with SeVdp(*Bsr/EGFP/CYBB*) and SeVdp(*Zeo/KO/CLuc*), nearly 100% of the infected cells expressed both KO and EGFP after antibiotic selection (Fig. 3*F*), but the balance of expression varied widely (Fig. 3, *F* and *H*). In contrast, 86.4% of the cells infected with SeVdp(*KO/Hyg/EGFP/Luc2CP*) expressed KO and EGFP at a fixed balance (Fig. 3, *E* and *G*) and at a constant level (62.2% of the cells expressed EGFP and KO within a 3-fold range) (Fig. 3*E*). From these results, we conclude that only the SeVdp vector installed with all the genes required to be expressed from a single genome can express these genes reproducibly at a fixed balance, thus providing a significant advantage for cell reprogramming.

Elimination of SeVdp Vector with siRNA—The last hurdle for efficient cell reprogramming is to establish a method for eliminating the vector genome from those cells harboring it stably. Although the viral family *Paramyxoviridae* includes major human pathogens (*e.g.* measles virus and respiratory syncytial virus), there is no specific small-molecule antiviral drug available. Instead, siRNAs against viral genes have been investigated with the aim of interfering with viral replication (40). However, the effects of siRNAs on stable persistent infections such as the SeVdp system have not been established. Therefore, we examined the effect of knocking down the viral replication machinery on the stability of the SeVdp genome using specific siRNAs. We designed siRNAs against each of the *NP*, *P*, and *L* genes (Fig. 4*A*) and examined their effects on the infection of a replication-competent SeV Cl.151 installed with the *EGFP* gene (SeV Cl.151(*EGFP*)). When the cells were treated with these siRNAs just before infection, the replication of SeV Cl.151(*EGFP*) was blocked almost completely (supplemental Fig. S4*A*). However, the effects of these siRNAs on the cells already harboring an SeVdp vector stably were quite different; siRNA against the *L* gene was most effective, and that against the *NP* gene showed almost no effect (supplemental Fig. S4*B*). This phenomenon might simply reflect the relative abundance of the target gene products; *NP* mRNA is about 34 times more abundant than *L* mRNA (41). Otherwise, suppression of a catalytic subunit of RNA polymerase (L protein) might interfere with the replication of the SeVdp genome more profoundly.

We then examined the time course with which an SeVdp vector would be eliminated by siRNA against the *L* gene (Fig. 4). To monitor elimination quantitatively, we used a cell line harboring the SeVdp vector installed with a destabilized firefly luciferase gene (SeVdp(*KO/Hyg/EGFP/Luc2CP*)) and determined luciferase activity as a faithful marker of gene expression from the SeVdp vector. As shown in Fig. 4*B*, the siRNA blocked expression of the L protein quite efficiently after day 3. In parallel with this suppression, the SeVdp was eliminated at a half-life of 17.5 h after a short time lag: the luciferase activity fell below the detection limit after day 8 (Fig. 4*C*). This elimination was irreversible; when the cells

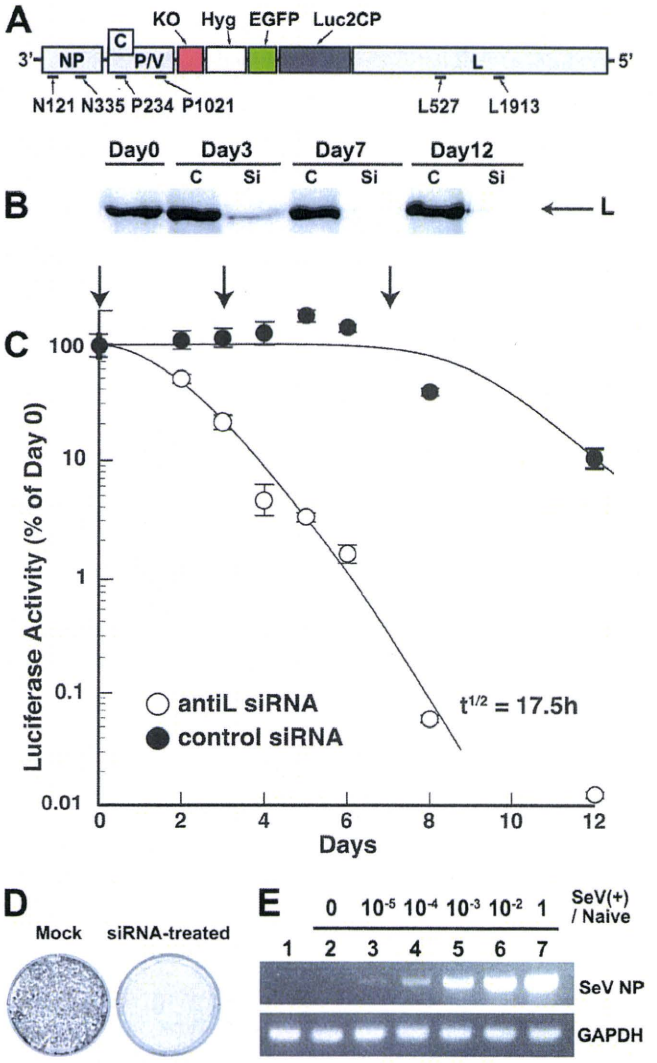
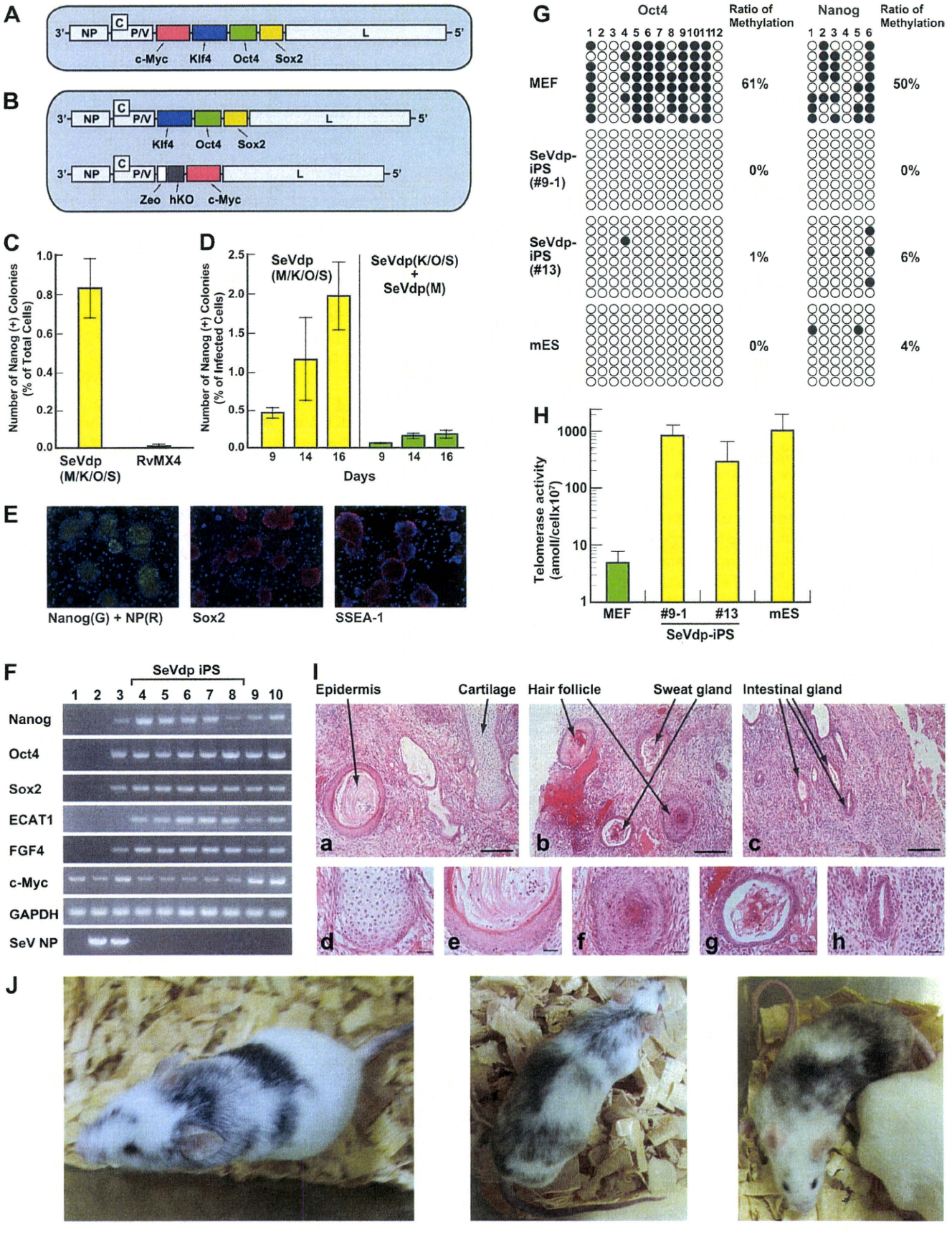


FIGURE 4. **Elimination of SeVdp vectors from the cells with specific siRNAs.** *A*, the genome structure of SeVdp(*KO/Hyg/EGFP/Luc2CP*) and target sites of siRNAs is shown. *B*, quantitative analysis of L protein by Western blotting is shown. HeLa cells carrying SeVdp(*KO/Hyg/EGFP/Luc2CP*) were cultured in the absence of hygromycin B and treated with siL527 (*Si*) or with control siRNA (*C*) complexed with Lipofectamine RNAiMAX (Invitrogen) on days 0, 3, and 7. The cells were harvested periodically as indicated, and 50-μg aliquots of cell extracts were separated on SDS-PAGE. The amount of L protein was determined by Western blotting probed with an anti-SeV L protein rabbit polyclonal antibody. *C*, quantitative analysis of SeVdp-mediated gene expression is shown. The HeLa cells carrying SeVdp(*KO/Hyg/EGFP/Luc2CP*) were treated with siRNA as described in *B*. The cells were harvested periodically as indicated, and the specific firefly luciferase activity was determined as described under "Experimental Procedures." *Open circles*, treated with siL527; *closed circles*, treated with control siRNA; *vertical arrows*, the day of siRNA treatment. *D*, detection of the cells carrying the SeVdp vector after treatment with siL527 is shown. HeLa cells carrying SeVdp(*KO/Hyg/EGFP/Luc2CP*) and treated with siL527 for 8 days as described in *C* were further cultured for 4 weeks in the absence of the siRNA and of hygromycin B. Aliquots of 1×10^4 cells were then seeded into 6-well plates with medium containing hygromycin B (100 μg/ml) and cultured for 10 days. The cells were fixed then stained with 0.01% crystal violet. *E*, detection of SeVdp by semiquantitative RT-PCR is shown. cDNAs were prepared by using 2-μg aliquots of total cellular RNAs as indicated, and the cDNA corresponding to 10^4 cells was analyzed by RT-PCR to determine SeV NP mRNA as described under "Experimental Procedures." *Lane 1*, HeLa cells carrying SeVdp(*KO/Hyg/EGFP/Luc2CP*) and treated with siL527 as described in *D*; *lanes 2–7*, naïve HeLa cells containing SeVdp(+) cells at the ratio indicated.

harboring SeVdp(*KO/Hyg/EGFP/Luc2CP*) had been treated with the siRNA for 8 days and were further cultured for 4 weeks in the absence of the siRNA, all the cells became susceptible to hygromycin B, indicating that the SeVdp genome was no longer present in the cells (Fig. 4*D*). We further confirmed the complete erasure of the SeVdp genome by sensitive RT-PCR analysis; we could not detect any SeV genome under conditions capable of detecting a single SeV(+) cell among 10^5 naïve cells (Fig. 4*E*). From these data, we conclude that siRNA against the L gene is an effective tool for erasing the SeVdp genome thoroughly from the cells.

Generation of Mouse iPS Cells with SeVdp Vectors Installed with Reprogramming Genes—We then constructed an SeVdp vector installed with four reprogramming genes (SeVdp(*c-Myc/Klf4/Oct4/Sox2*)) (Fig. 5*A*) and examined its potential to reprogram mouse fibroblasts. First, we compared the efficiency of reprogramming by an infection of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) with that produced by the coinfection of ecotropic retrovirus vectors installed with the same genes separately (RvMX4), which is a current standard approach for iPS generation (24) (Fig. 5*C*). For assessing the expression of Nanog, a well known marker of fully reprogrammed iPS cells, we used embryonic fibroblasts derived from a Nanog-GFP knock-in mouse (MEF/Nanog-GFP) (24) and monitored the expression of GFP. We found that SeVdp(*c-Myc/Klf4/Oct4/Sox2*) and pMX4 reprogrammed 0.83 and 0.01% of MEF cells to Nanog (+) iPS cell-like colonies, respectively, on day 14 after vector infection (Fig. 5*C*). As the efficiency of emergence of Nanog-GFP (+) colonies from MEF with retrovirus vectors was consistent with that reported previously (24), we concluded that the SeVdp vector could reprogram MEF about 100 times more efficiently than standard procedures using retrovirus vectors. We also prepared SeVdp vectors installed with *Klf4/Oct4/Sox2* genes (SeVdp(*Klf4/Oct4/Sox2*)) and with a *c-Myc* gene (SeVdp(*Zeo/hKO/c-Myc*)) (Fig. 5*B*) separately. We found that the coinfection with these two SeVdp vectors reprogrammed MEF/Nanog-EGFP much less efficiently than did the single infection with SeVdp(*c-Myc/Klf4/Oct4/Sox2*) (Fig. 5*D*). These results clearly proved our assumption that installing all the necessary genes on a single vector was critical for maximizing the potential of the vector to reprogram cells.

After treating these Nanog (+) cells with siRNA L527, we obtained SeV antigen-free iPS cells with typical characteristics (Fig. 5). First, they expressed ES/iPS cell markers detectable by fluorescence microscopy (*Nanog*, *Sox2*, and *SSEA-1*) (Fig. 5*E*) as well as by RT-PCR (*Nanog*, *Sox2*, *Oct4*, *c-Myc*, *ECAT1*, and *FGF4*) (Fig. 5*F*). The primer sets used in this RT-PCR assay detected the expression of mouse genes but not that of human genes installed on the SeVdp vector. Some of the endogenous iPS marker genes (Nanog, Oct4, and Sox2) were detectable as early as on day 5 after gene transfer (Fig. 5*F*, *lane 3*), suggesting rapid cell reprogramming by the SeVdp vector. Second, the promoter regions of the *Oct4* and *Nanog* genes were epigenetically remodeled similar to ES cells (Fig. 5*G*). Third, telomerase activity was increased by 50–200-fold to the same level as in ES cells (Fig. 5*H*). Fourth, they differentiated into derivatives of all three germ layers in teratomas (Fig.



5*I*). Finally, we can generate chimera animals by injecting the SeVdp-iPS cells into embryos (Fig. 5*J*).

DISCUSSION

Discovery of the methods for achieving cell reprogramming with ectopic expression of defined factors has had a great impact on modern bioscience. However, to establish this technology for practical applications, we still have to overcome several difficult hurdles, such as the dramatic improvement of reprogramming efficiency and reproducible generation of fully differentiation-competent iPS cells without allowing the expression of residual exogenous genes on the chromosomes. Among these issues, chromosomal integration of reprogramming genes is the most critical factor affecting the characteristics of iPS cells (9). Incomplete suppression of exogenous reprogramming genes might affect the pluripotency of iPS cells. Furthermore, even if the integrated gene cassette is suppressed by epigenetic modification, differentiation of mouse iPS cells has frequently reactivated the integrated genes and induced cancers in iPS cell-derived offspring, as some of the reprogramming genes have intrinsic tumorigenic activity (24). Therefore, methodologies for generating fully pluripotent human iPS cells carrying no remnant of exogenous genes have been investigated but with limited success (9). As far as we know, the SeVdp vector described here is the only system that fulfills all the requirements to accomplish this goal with high efficiency and without laborious procedures.

Efficient gene delivery and expression are the primary factors affecting iPS cell generation, and this SeVdp vector has a great advantage over other delivery systems. As a typical recombinant viral vector, it can deliver genes much more efficiently than a nonviral system. In addition, its exceptionally broad host range should be quite beneficial; these viruses can infect almost all cell types from avian to human (42). Among others, the efficiency of the SeVdp vector to induce stable gene expression in human HSCs (~80%) is remarkably higher than that of retroviral vectors under multiple infection cycles with the aid of recombinant fibronectin fragments (~30%) (43). These characteristics have emerged partly because the SeVdp recognizes ubiquitous sialic acid as the primary receptor. Furthermore, the SeVdp has its own RNA-dependent RNA polymerase and requires only ubiquitous cytosolic proteins for transcription/replication (44). Thus, the SeVdp vector induces active transcription just after the nucleocapsid is delivered into the cytoplasm.

The enablement of stable gene expression without chromosomal integration is the most remarkable characteristic of these SeVdp vectors. DNA-based vectors (including retro/lentiviral vectors) can accomplish stable gene expression either by chromosomal integration or by episomal replication, depending on the cellular replication machinery. However, these characteristics make it difficult to remove the stabilized DNA from the cells; this depends either on a complex excision process using DNA recombinase or on passive elimination in the absence of selection. On the other hand, recombinant RNA viral vectors (except for retro/lentiviral vectors) cannot achieve stable gene expression, partly because they trigger cellular defense systems and induce apoptotic death in the host cells. The SeVdp vector is the only RNA-based platform inert enough to allow stable gene expression in sensitive HSCs, thanks to its unique gene mutations/alterations for escaping the host defense system. Furthermore, as the stability of SeVdp genomic RNA depends on the activity of viral RNA polymerase, interference of the polymerase with siRNA can be used to eliminate the genome from infected cells, as shown here.

Coinfection of conventional F-defective SeV vectors installed with *Oct4*, *Sox2*, *Klf4*, and *c-Myc* separately has been reported to generate iPS cells (45, 46). Although these reports demonstrated the potential of SeV vectors in cell reprogramming, our data have clearly demonstrated that this SeVdp vector provides a superior alternative to this approach. Among other factors, the cytopathic nature of the conventional SeV vectors based on wild-type SeV, including simple F-defective SeV vectors (30, 47), limits their utility in cell re-

FIGURE 5. **Reprogramming of MEFs with SeVdp vectors installed with reprogramming genes.** *A*, the genome structure of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. *B*, shown is genome structure of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*), coexisting in a single cell, is shown. *C* and *D*, shown is the efficiency to reprogram MEF/Nanog-GFP. MEF/Nanog-GFP cells (1.25×10^5) were infected with SeVdp vectors, and retroviral vectors were installed with *c-Myc/Klf4/Oct4/Sox2* as described under "Experimental Procedures." Then 1.0×10^3 of infected cells were seeded onto the feeder cells in 6-well plates and cultured for 14 days (*C*) or for the indicated days (*D*). The number of iPS colonies expressing GFP was determined under fluorescent microscopy. Reprogramming efficiency was indicated as the ratio of the number of EGFP-positive colonies to that of MEF/Nanog-GFP seeded in the well (*C*) or to that of infected MEF/Nanog-GFP seeded in the well (*D*). *C*, shown is a comparison of reprogramming efficiency with the SeVdp(*c-Myc/Klf4/Oct4/Sox2*) vector and with retroviral vectors. *SeVdp(M/K/O/S)*, SeVdp(*c-Myc/Klf4/Oct4/Sox2*); *RvMX4*, coinfection of ecotropic retroviral vectors installed with *c-Myc*, *Klf4*, *Oct4*, and *Sox2* separately. *D*, shown is a comparison of reprogramming efficiency by a single infection of SeVdp(*c-Myc/Klf4/Oct4/Sox2*) (*SeVdp(M/K/O/S)*) and by coinfections of SeVdp(*Klf4/Oct4/Sox2*) and SeVdp(*Zeo/hKO/c-Myc*) (*SeVdp(K/O/S)* + *SeVdp(M)*). *E*, characterization of mouse iPS cells generated with SeVdp(*c-Myc/Klf4/Oct4/Sox2*) is shown. The ES-like colonies emerging from MEF/Nanog-GFP cell lines were fixed, incubated with specific primary antibodies against SeV NP antigen (*left*), Sox2 (*middle*), and SSEA-1 (*right*), then stained with secondary antibodies conjugated with Alexa 555. The cells were then counterstained with DAPI and examined by fluorescence microscopy as described under "Experimental Procedures." *Nanog* (*left*), expression of GFP driven by the Nanog promoter. *F*, gene expression analysis with semiquantitative RT-PCR is shown. Aliquots (2 μg) of total RNA prepared from the cells indicated were analyzed as described under "Experimental Procedures." *Lane 1*, MEF; *lane 2*, MEF infected with control vector (SeVdp(*Bsr/ΔF/KO*)); *lane 3*, MEF infected with SeVdp(*M/K/O/S*) on day 5 infection; *lane 4*, SeVdp-iPS cell clone #2-1; *lane 5*, SeVdp-iPS cell clone #9-1; *lane 6*, SeVdp-iPS cell clone #13; *lane 7*, SeVdp-iPS cell clone #16; *lane 8*, SeVdp-iPS cell clone #21; *lane 9*, mouse iPS cell generated with retrovirus vectors (RvMX4); *lane 10*, mouse ES cell (clone D3). *ECAT1*, ES cell-associated transcript 1; *FGF4*, fibroblast growth factor 4. *G*, methylation analysis of Oct4 and Nanog promoters is shown. Methylation profile of CpG in genomic DNA was analyzed by bisulfite sequence analysis as described under "Experimental Procedures." *Open circles*, unmethylated cytosine; *closed circles*, methylated cytosine. The ratio of methylated cytosine is indicated as a percentage of total cytosine residues analyzed. *H*, a telomerase assay is shown. Telomerase activity in total cell extract prepared from 1×10^3 cells was analyzed as described under "Experimental Procedures" and is indicated as the amount of $(dT\text{-}TAGGG)_n$ synthesized. *I*, histology of teratomas derived from SeVdp-iPS cells is shown. Teratoma formation was studied at 6 weeks after the subcutaneous injection of 1×10^6 SeVdp-iPS cells from clone #13 into SCID mice. *a–c*, low magnification; *scale bar* = 100 μm. *d–h*, high magnification observation; *scale bar* = 20 μm. *d*, cartilage; *e*, epidermis; *f*, hair follicle; *g*, sweat gland; *h*, intestinal gland. *J*, adult chimeras derived from SeVdp-iPS cells (clone #13) are shown. *Dark hair* indicates donor contribution.

programming. As revealed in our previous work (12) and in the present article, the cytopathogenicity of SeV depends on multiple factors including escaping from cytokine induction and acute membrane dysfunction, and it is not possible to evade this by simple deletion of all the structural genes (*M*, *F*, and *HN*) (27). Furthermore, the use of a single-gene defective SeV vector raises safety and regulatory concerns, as the potential of the SeV vector to self-replicate was diminished but was not completely abolished by a single gene defect, as shown in Table 1. This issue was neither investigated nor addressed in previous reports describing single gene defective vectors (30, 47) but has been resolved here for the first time.

Cell reprogramming depends on the simultaneous delivery of multiple genes, on their balanced expression, and on their prompt suppression/removal. These factors affect both the efficiency of iPS cell generation and the quality of the iPS cells (36, 39). Nevertheless, this issue has not been investigated in detail, partly because most of the current viral vectors can accommodate only one or a few extra genes. The capacity of SeVdp vectors to install four reprogramming genes on a single vector was critical both for expressing these genes at a prefixed balance (Fig. 3) and for highly efficient reprogramming (Fig. 5); coinfection of two independent SeVdp vectors failed to accomplish either of these goals (Figs. 3 and 5). This latter phenomenon might be caused by homologous viral interference, which has been observed after coinfections of two independent paramyxoviruses including SeV (48). Otherwise, this phenomenon could reflect that the SeVdp genome is a multicopy replicon with about 40,000 copies per cell (12). In general, it is difficult to manage to produce two (or more) independent multicopy replicons under equal balance when they share the same replication machinery. Active and rapid erasure of SeVdp vectors with specific siRNAs (Fig. 4) is also advantageous to the generation of homogeneous and vector-free iPS cells compared with the passive and unmanageable vector removal that depends on the sequential passages of the cells (45, 46).

Synthetic modified mRNA encoding reprogramming factors has been reported to generate iPS cells highly efficiently (49). This nonviral approach and our SeVdp vector have their own advantages and disadvantages, but the escape from the cellular antiviral defense system is a critical characteristic common to these advanced reprogramming systems. The former has the advantage that the combination of exogenous reprogramming genes and the balance or duration of their expression can be adjusted flexibly. The nonviral approach has another advantage, as the use of recombinant viruses is regulated strictly in general. On the other hand, it is highly dependent on the gene delivery system, as it requires repetitive transfection for 16 days. Therefore, it might not be applicable to cells that are difficult to transfect, such as primary peripheral blood cells. In contrast, adjustment of reprogramming genes and of their expression is not so easy using SeVdp vectors and needs the construction of different vectors for tuning these features. However, once established, the SeVdp vector can always induce steady expression of the installed genes at a fixed balance, and this feature allows highly reproducible and uniform cell reprogramming. Furthermore, prolonged gene expression by a single infection procedure and the wider host range available are advantageous for practical applications, such as reprogramming of blood cells. Combining the results obtained with these two approaches depending on specific research purposes will enable us to create a more advanced system for cell reprogramming.

The field of cell reprogramming is expanding very rapidly, and the tools for safer and efficient reprogramming have become increasingly important both for basic research and for development of medical applications. We are presently investigating the utility of the SeVdp vectors in the genomic reprogramming of human cells.

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Freeze-drying of proteins with glass-forming oligosaccharide-derived sugar alcohols

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ABSTRACT

Physical properties and protein-stabilizing effects of sugar alcohols in frozen aqueous solutions and freeze-dried solids were studied. Various frozen sugar alcohol solutions showed a glass transition of the maximally freeze-concentrated phase at temperatures (T_g's) that depended largely on the solute molecular weights. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure freeze-dried solids. Microscopic observation of frozen maltitol and lactitol solutions under vacuum (FDM) indicated onset of physical collapse at temperatures (T_c) several degrees higher than their T_g's. Freeze-drying of pentitols (e.g., xylitol) and hexitols (e.g., sorbitol, mannitol) resulted in collapsed or crystallized solids. The glass-forming sugar alcohols prevented activity loss of a model protein (LDH: lactate dehydrogenase) during freeze-drying and subsequent storage at 50 °C. They also protected bovine serum albumin (BSA) from lyophilization-induced secondary structure perturbation. The glass-forming sugar alcohols showed lower susceptibility to Maillard reaction with co-lyophilized L-lysine compared to reducing and non-reducing disaccharides during storage at elevated temperature. Application of the oligosaccharide-derived sugar alcohols as alternative stabilizers in lyophilized protein formulations was discussed.

1. Introduction

Freeze-drying is a popular method to ensure long-term stability of therapeutic proteins that are not stable enough in the aqueous solutions during distribution and long-term storage (Manning et al., 1989; Nail et al., 2002). Removal of surrounding water molecules by lyophilization significantly reduces gradual chemical and physical degradation of proteins, whereas the dehydration often induces structural perturbation that leads to misfolding and/or aggregation in the re-hydrated solutions (Arakawa et al., 2001). Protecting the protein conformation in freeze-dried formulations through appropriate process control and ingredient optimization is essential to ensure the pharmacological effects, as well as to reduce the risk of product immunogenicity (Hermeling et al., 2004).

Some non-reducing saccharides (e.g., sucrose, trehalose) are popular stabilizers that protect proteins from the chemical and physical degradations in aqueous solutions, during freeze-drying, and in subsequent storage (Arakawa and Timasheff, 1982; Carpenter and Crowe, 1989; Franks, 1992; Wang, 2000). They protect protein conformation in the solids thermodynamically through direct interactions (e.g., hydrogen bonds) that substitute surrounding water molecules and reduce protein chemical degradation kinetically by embedding the protein in a glass-state lower molecular mobility environment. High molecular mobility of the glass-state disaccharide solids, however, induces slow but not negligible chemical degradation of embedded proteins over pharmaceutically relevant timescales. Unexpected exposure of the solids to humid or high-temperature environments often induces physical changes of the dried cakes (e.g., shrinkage) (Breen et al., 2001; Tian et al., 2007).

Application of other excipients that stabilize proteins by themselves and/or in combination with disaccharides would provide further choices to improve the formulation quality (Wang, 2000; Costantino, 2004). Some excipients (e.g., polymers, sodium phosphates) raise the glass transition temperature (T_g) of co-lyophilized disaccharide-based solids, which limited molecular mobility should confer robustness against undesirable storage conditions (Ohtake et al., 2004). Some amino acids and their salts (e.g., L-arginine citrate) form glass-state amorphous solids that protect proteins from inactivation during freeze-drying (Tian et al., 2007; Izutsu et al., 2009). In addition to the structural stabilization, varied physicochemical properties of amino acids would provide some unique effects (e.g., reducing the protein aggregation in aque-

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ous solutions) that are preferable for pharmaceutical formulations (Arakawa et al., 2007).

The purpose of this study was to systematically examine the physical properties and protein-stabilizing effects of oligosaccharide-derived sugar alcohols for their application in freeze-dried formulations. It has been established that various sugar alcohols (e.g., sorbitol, xylitol, lactitol) protect proteins from heat-induced denaturation in aqueous solutions through a thermodynamic mechanism (preferential exclusion) identical to that of saccharides and other polyols (e.g., glycerol) (Arakawa and Timasheff, 1982; Gekko, 1982). Some pentitols and hexitols (e.g., xylitol, sorbitol) protect biological macromolecules (e.g., proteins) and microorganisms from inactivation and/or viability loss during freeze-thawing and during freeze-drying (Tamoto et al., 1961; Carpenter and Crowe, 1988). Varied physical properties (i.e., crystallinity, molecular mobility) have been considered as key factors that determine effects of sugar alcohols to stabilize proteins in frozen solutions and freeze-dried solids (Griebenow and Klibanov, 1995; Carrasquillo et al., 2000; Liao et al., 2002). For example, high propensity to crystallize in the frozen solution (e.g., mannitol) or to collapse during primary drying (e.g., sorbitol, xylitol) makes them inappropriate for main stabilizer in freeze-drying. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) should have greater opportunities to structurally and kinetically stabilize proteins during freeze-drying and subsequent storage. Maltitol and lactitol are popular excipients for oral (tablet) formulations, and are also widely used in food industries as glass-forming additives upon cooling of edible hot-melt compositions (Slade et al., 2006). Information on the physical properties (e.g., thermal transition temperatures) and protein-stabilizing effects (e.g., enzyme activity, protein secondary structure) should be relevant in the application of sugar alcohols to the freeze-dried formulations.

2. Materials and methods

2.1. Materials

All chemicals employed in this study were of analytical grades and were obtained from the following commercial sources: L-lactic dehydrogenase (LDH, rabbit muscle), bovine serum albumin (BSA, essentially fatty acid free), glucose, trehalose dihydrate, sorbitol, and sucrose (Sigma Chemical, St. Louis, MO); maltitol, maltotriitol and maltotetraitol (Hayashibara Biochemical Laboratories, Okayama, Japan); maltose, lactose, mannitol, xylitol, lactitol monohydrate, and other chemicals (Wako Pure Chemical, Osaka, Japan); methanol dehydrate (Kanto Kagaku, Tokyo, Japan). The protein solutions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (1500 g × 5 min) and filtered (0.45 μm PVDF filters, Millipore, Bedford, MA) to remove insoluble aggregates before the freeze-drying study.

2.2. Freeze-drying

A freeze-drier (FreeZone-6; Labconco, Kansas City, MO) was used for lyophilization. Aliquots (0.3 ml) of aqueous solutions in flat-bottom glass vials (13 mm diameter, SVF-3; Nichiden-Rika Glass, Kobe, Japan) were placed on the shelf of the lyophilizer. The shelf was cooled to −40 °C at 0.5 °C/min, and then maintained at this temperature for 2 h before the primary drying process. The frozen solutions were dried under a vacuum (4.0 Pa) while maintaining the shelf temperature at −40 °C for 15 h, −30 °C for 6 h, and 35 °C for 6 h. The shelf was heated at 0.2 °C/min between the thermal steps. The vials were closed with rubber stoppers under a vacuum.

2.3. Thermal analysis

Thermal analysis of frozen solutions and dried solids was performed by using a differential scanning calorimeter (Q-10; TA Instruments, New Castle, DE) and software (Universal Analysis 2000; TA Instruments). Aliquots of aqueous solutions (10 μl) in hermetic aluminum cells were cooled from room temperature to −70 °C at 10 °C/min, and then scanned by heating at 5 °C/min. Freeze-dried solids (1–2 mg) in hermetic aluminum cells were subjected to the thermal analysis from −20 °C at 5 °C/min under a nitrogen gas flow. Cooled-melt saccharide and sugar alcohol solids obtained by a brief period of heating (1 min at 160 °C for maltose monohydrate, xylitol, sorbitol, maltitol, and lactitol monohydrate; at 180 °C for glucose; at 200 °C for sucrose, mannitol, and maltotriitol; and at 220 °C for lactose and trehalose monohydrate) and subsequent rapid cooling (−50 °C) in hermetic aluminum cells were scanned at 5 °C/min to obtain the glass transition temperatures. The glass transition temperatures were determined as the maximum inflection point of the discontinuities in the heat flow curves.

2.4. Freeze-drying microscopy (FDM)

We observed the behavior of frozen aqueous excipient solutions under a vacuum using a freeze-drying microscope system (Lyostat2; Biopharma Technology, Winchester, UK) with an optical microscope (BX51; Olympus, Tokyo). Aqueous solutions (2 μl) sandwiched between cover slips (70 μm apart) were frozen at −40 °C and then maintained at that temperature for 5 min. Each sample was heated under a vacuum (12.9 Pa) at 5 °C/min to a temperature approximately 5 °C below its T_g' as obtained by thermal analysis, and then scanned at an angle speed of 1 °C/min after reaching T_g'. The collapse onset temperature (T_c) of the frozen solution was determined from the first appearance of translucent dots behind the ice sublimation interface ($n = 3$).

2.5. Powder X-ray diffraction (XRD) and residual water measurements

The powder X-ray diffraction patterns were measured at room temperature by using a Rint-Altima diffractometer (Rigaku, Tokyo, Japan) with Cu Kα radiation at 40 kV/40 mA. The samples were scanned in the area of $5° < 2\theta < 35°$ at an angle speed of 5°/min. The lyophilized solids were suspended in dehydrated methanol to obtain residual water by a volumetric Karl-Fischer titrator (AQV-6; Hiranuma Sangyo, Ibaraki, Japan). Residual water contents were shown as ratios (%) to the estimated solid weights in the vials.

2.6. Freeze-drying and activity measurement of LDH

Aqueous solutions (0.5 ml) containing LDH (0.05 mg/ml), excipients (100 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0) were lyophilized in the flat-bottom glass vials. Some freeze-dried solids plugged with rubber stoppers were stored at 50 °C for 7 days in a temperature chamber (Model SH-221, Espec, Osaka, Japan). Pyruvate and NADH were used as substrates to obtain LDH activity from the absorbance reduction at 340 nm (25 °C). Residual enzyme activity was shown as the ratio (%) to that of the solution before freezing ($n = 6$) (Izutsu et al., 1994).

2.7. Fourier-transform infrared (FT-IR) analysis of freeze-dried BSA

A Fourier-transform infrared spectrophotometer (MB-104; Bomen, Quebec, Canada) with a dry gas generator (Balston, Haverhill, MA) and software (PROTA; BioTools, Jupiter, FL and GRAMS/32; Galactic Ind., Salem, NH) was used to obtain mid-infrared spectra of