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厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

マイクロRNAを指標にして癌を標的破壊する純和製抗癌ウイルス製剤の開発とその臨床応用に関する研究

平成２３年度　総括・分担研究報告書

研究代表者　中村　貴史

平成２３（２０１１）年　５月

別紙１

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別紙３

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総括研究報告書

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研究代表者　中村　貴史　東京大学医科学研究所　特任准教授

研究要旨

現在世界中において、生きたウイルスを利用して癌を治療する癌ウイルス療法に関する前臨床研究、及び臨床試験が積極的に行われている。これは、ウイルスが本来持っている癌細胞に感染後、癌組織内で増殖しながら死滅させるという性質を利用する方法である。本研究では、純国産ワクシニアウイルスワクチン株の安全性に注目し、遺伝子組換え技術により改良を加え、“純和製抗癌ウイルス製剤”として活用する。これまでの研究では、正常組織と比べ肺癌、膵臓癌、乳癌、及び悪性リンパ腫などで発現が低下しているlet7aの標的配列をウイルス伝播増殖に重要であるウイルス膜蛋白B5R遺伝子の3'非翻訳領域に挿入することで、癌細胞ではB5Rを発現させる（＝ウイルスは増殖する）が、正常細胞ではB5Rを発現させない（＝ウイルスは増殖しない）ようワクチン株を改良した。本研究では、腫瘍特異性をさらに向上させるため、このmiRNA制御に加え、ウイルスTK遺伝子を欠失させた多因子制御ワクシニアウイルス（MDVV）を作成し、担癌マウスモデルにおけるMDVVの全身投与によって副作用なく転移した癌を標的破壊できることを実証した。

研究分担者氏名・所属研究機関名及び所属研究機関における職名

東條有伸・東京大学医科学研究所・教授

A. 研究目的

現在世界中において、生きたウイルスを利用して癌を治療する癌ウイルス療法に関する前臨床研究、及び臨床試験が積極的に行われている。これは、ウイルスが本来持っている癌細胞に感染後、癌組織内で増殖しながら死滅させるという性質を利用する方法である。本研究の目的は、純国産ワクシニアウイルスワクチン株の安全性に注目し、遺伝子組換え技術により改良を加え、“純和製抗癌ウイルス製剤”として活用することにある。本研究では、現行の治療法に極めて高い抵抗性を示す難治性悪性腫瘍に対する純和製抗癌ウイルスによる革新的な治療法の確立を目指す。さらに、臨床応用に向けウイルス製剤の GMP 製造や品質管理に関する基盤技術を構築することによって、本研究の成果をシームレスに臨床応用へと直結させることを目指す。

B. 研究方法

本研究では、純国産ワクシニアウイルスワクチン株を基に、３種類の遺伝子組換えワクシニアウイルス（無制御ワクシニアウイルス、miRNA 制御ワクシニアウイルス、多因子制御ワクシニアウイルス）を作製した（図１）。無制御ウイルスでは、感染細胞内において B5R が恒常的に発現する。一方 miRNA 制御ウイルスでは、ウイルスの B5R

遺伝子の3′非翻訳領域へ、正常組織と比べ肺癌、膵臓癌、卵巣癌や造血器腫瘍などで発現が低下している let7a の標的配列が挿入されている。これより let7a によって B5R の発現が制御される。又、両者のウイルスとも、ホタルルシフェラーゼ遺伝子の発現ユニットが HA 遺伝子に挿入され、これによって HA 遺伝子は機能しない。尚、HA 遺伝子の欠失はウイルス複製能に影響を及ぼさない。多因子制御ワクシニアウイルス（MDVV）では、この let7a による B5R の発現制御に加え、ホタルルシフェラーゼ遺伝子の発現ユニットが HA ではなく TK 遺伝子に挿入され、これによって TK 遺伝子が機能しない。

免疫不全 SCID マウスの腹腔内にウミシイタケルシフェラーゼ発現ヒト膵臓癌細胞 BxPC-3（5×10^6個）を投与し、その 7 日後に各ワクシニアウイルス（10^6 pfu）を腹腔内に投与した。その後、(腫瘍発育抑制効果と生存期間延長効果)、安全性（体重減少や皮膚などの一般状態)、及び腫瘍特異的増殖性を評価した（図２)。尚、この担癌モデルマウスにおいて、マウス正常組織と BxPC-3 腫瘍の let7a 発現レベルを TaqMan miRNA assay 法によって比較解析した。

又、BxPC-3 はウミシイタケルシフェラーゼを発現しているので、セレンテラジン投与によってマウス体内の腫瘍分布を非侵襲的にモニターできる。一方、各ワクシニアウイルスはホタルルシフェラーゼを発現しているので、ルシフェリン投与によってマウス体内のウイルス分布を非侵襲的にモニターできる。

（倫理面への配慮）

本研究を実施するにあたり、DNA 組換え実験は、東京大学医科学研究所遺伝子組換え生物等安全委員会で承認されている。その中の自立的な増殖力、感染力を保持した組換えワクシニアウイルスの作成・使用に関しては、文部科学省の第二種使用等拡散防止措置指針に従い、大臣確認実験承認を得ている。又、全ての動物実験は、東京大学医科学研究所動物実験委員会の承認を得ており、その実施にあたっては、同研究所動物実験指針を遵守し動物愛護上の配慮を十分に行っている。

Ｃ．研究結果

ヒト膵臓癌細胞 BxPC-3 の腹膜播種マウスモデルにおいて、その let7a の発現は、マウス正常組織に比べ BxPC-3 腫瘍で低下していることが明らかになった（図３)。BxPC-3 腹膜播種マウスモデル（各群 10 匹）において、多因子制御ウイルス MDVV は、生理食塩水を投与したコントロールや他のウイルスと比べ、生存を延長させ強力な抗癌効果を示した（図４)。無制御ウイルスは、全身の正常組織へ伝播増殖し、最終的にはそれに伴うウイルス毒性（急激な体重減少）によってマウスは死亡した。又 miRNA 制御ウイルスは、無制御ウイルスと比べると全身の正常組織への伝播増殖性は顕著に低下していたが、最終的にはウイルス毒性を示した。それに対し MDVV は、再発した腫瘍によって死亡するマウスがいたものの、全身の正常組織へ伝播増殖することはなかった。

Ｄ．考察

この結果は、生体内の腫瘍分布とウイルス分布のイメージングによって、その詳細が明らかとなった。MDVV 治療群では治療 11 日後の腹腔内の腫瘍がほぼ消失していたが、コントロール群では治療効果がなく腫瘍が増大していた（図５)。又、マウス体内のウイルス分布の解析より、投与３日後には腹腔内腫瘍における MDVV の増殖が確認され、その増殖による腫瘍の破壊に伴って 10 日後にはウイルス増殖も消失していた（図６)。一方、無制御ウイルス、miRNA 制御ウイルスは、MDVV と同等の抗癌効果を示したが(図５)、投与 10 日後には全身の正常組織へも伝播増殖し、その毒性でマウスは死亡した（図６)。

Ｅ．結論

以上より、MDVV は極めて高い腫瘍特異的増殖能を有し、その腹膜播種マウスモデルにおいても副作用なく強力な抗癌効果を示した。これは、miRNA によるウイルス伝播増殖能の制御に加え、ウイルス TK 遺伝子が機能を失うと、正常細胞におけるウイルスの複製能は低下するが、癌細胞にはこの遺

伝子の機能を補う酵素が豊富に存在するためウイルスの複製能は低下せず、結果的に腫瘍特異性が向上したためである。

F．健康危険情報
なし

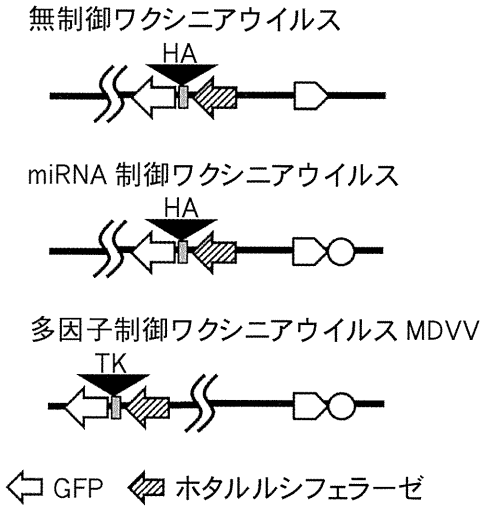

図1　遺伝子組換え MDVV の構築

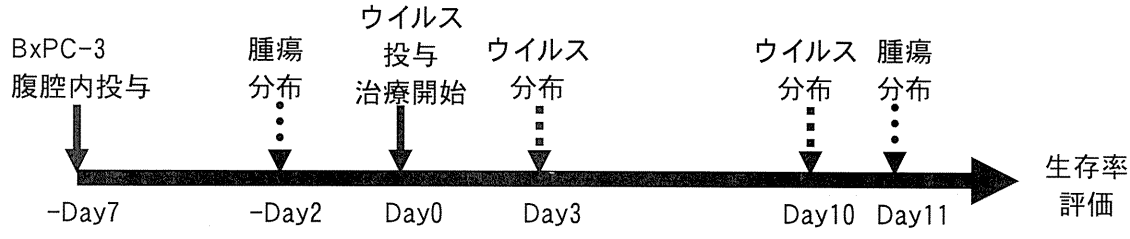

図2　実験スケジュール

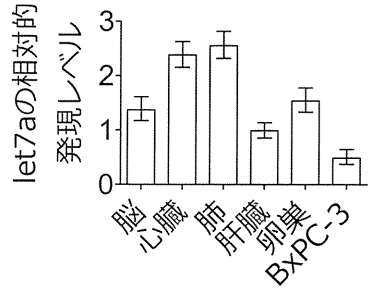

図3　定量 RT-PCR による let7a の発現解析

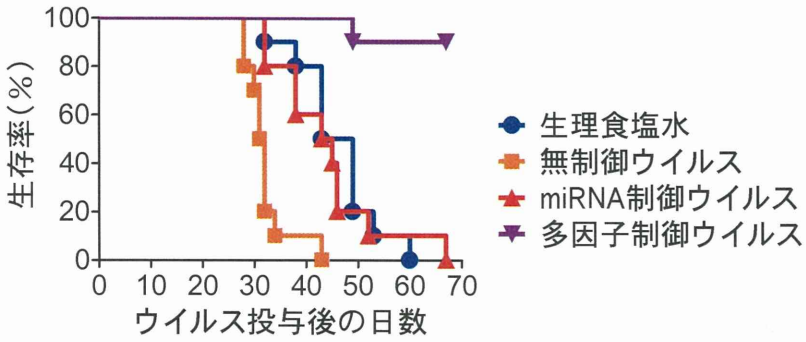

図4　MDVV の腫瘍特異的増殖による強力な抗癌効果と高い安全性

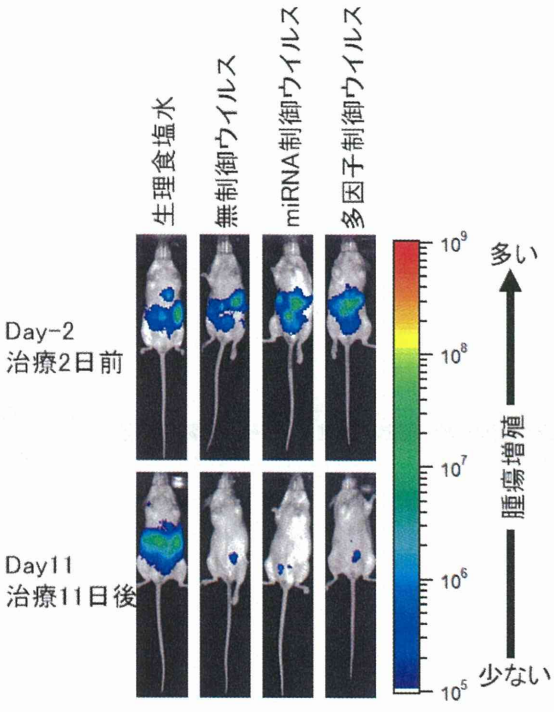

図5　腫瘍分布のイメージング

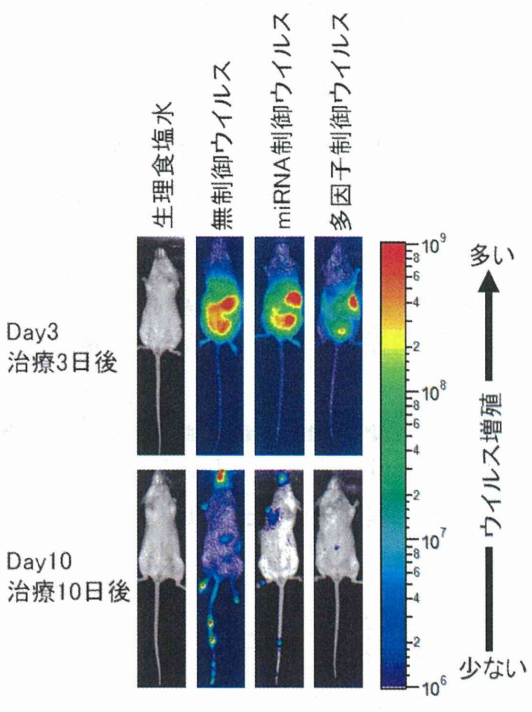

図6　ウイルス分布のイメージング

G．研究発表

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5. 東條有伸、『造血幹細胞移植医療の最先端と課題』 第 100 回日本病理学会総会（横浜）ワークショップ 13 2011 年 4 月 30 日

H. 知的財産権の出願・登録状況

1. 特許取得
発明の名称：マイクロＲＮＡ制御組換えワクシニアウイルス及びその使用
発　明　者：中村貴史、他4人
出　願　人：東京大学、北海道大学、国立感染症研究所長
出　願　日：2011年3月15日
出願番号：PCT/JP2011/056693

2. 実用新案登録
なし

3. その他
なし

別紙４

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

成人Ｔ細胞白血病(ATL)に対する純和製抗癌ウイルスの抗腫瘍効果と安全性の評価に関する研究

研究分担者　東條　有伸　東京大学医科学研究所　教授

研究要旨

HTLV-1感染に起因して発症するATLやHAMに対する有効な治療法の確立が望まれている。感染した癌細胞内で増殖しながら死滅させるというウイルス本来の性質（腫瘍溶解性）を利用する癌ウイルス療法は、従来の化学療法や放射線療法と比較して、様々なメカニズムによって腫瘍を攻撃できる利点がある。本研究では、純国産ワクシニアウイルスワクチン株の安全性に注目し、遺伝子組換え技術により改良を加え、“純和製抗癌ウイルス製剤”として活用する。ATL細胞株を用いた担癌マウスモデルにおいて、miRNAの制御に加え、ウイルスTK遺伝子を欠失させた多因子制御ワクシニアウイルス（MDVV）は、腫瘍特異的増殖による強力な抗癌効果と高い安全性を示した。特にこのモデルでは血中を介して効率よく腫瘍組織に到達できることが実証できたので、実際の症例で多臓器へ浸潤しているATL細胞を標的破壊する治療法として期待できる。

A. 研究目的

現在世界中において、生きたウイルスを利用して癌を治療する癌ウイルス療法に関する前臨床研究、及び臨床試験が積極的に行われている。これは、ウイルスが本来持っている癌細胞に感染後、癌組織内で増殖しながら死滅させるという性質を利用する方法である。本研究の目的は、純国産ワクシニアウイルスワクチン株の安全性に注目し、遺伝子組換え技術により改良を加え、“純和製抗癌ウイルス製剤”として活用することにある。本研究では、現行の治療法に極めて高い抵抗性を示す成人Ｔ細胞白血病(ATL)に対する純和製抗癌ウイルスによる革新的な治療法の確立を目指す。

B. 研究方法

本研究では、純国産ワクシニアウイルスワクチン株を基に、多因子制御ワクシニアウイルス（MDVV)を作製した。ウイルスゲノムのB5R遺伝子の3′非翻訳領域へ、正常組織と比べ肺癌、膵臓癌、卵巣癌や造血器腫瘍などで発現が低下しているlet7aの標的配列を挿入した。これよりlet7aによってB5Rの発現が制御される。又、このlet7aによるB5Rの発現制御に加え、ホタルルシフェラーゼ遺伝子の発現ユニットをTK遺伝子に挿入した。これによってTK遺伝子が機能しない。

NK細胞枯渇処理NOD/SCIDマウスにウミシイタケルシフェラーゼ発現ATL細胞株MT-2を腹腔内投与し、11日後にホタルルシフェラーゼ発現MDVVを腹腔内投与した。その後、抗腫瘍効果、及び腫瘍特異的増殖性を評価した。

一方、TL-Om1細胞をNOGマウスの皮下に接種し、腫瘍が約100mm^{3}になった時点で、尾静脈よりMDVVを投与した。その３日後に体内でのウイルス分布をモニターし、その腫瘍特異的増殖性を評価した。

又、これらの担癌モデルマウスにおいて、マウス正常組織と ATL 腫瘍の let7a 発現レベルを TaqMan miRNA assay 法によって比較解析した。

（倫理面への配慮）

本研究を実施するにあたり、DNA 組換え実験は、東京大学医科学研究所遺伝子組換え生物等安全委員会で承認されている。その中の自立的な増殖力、感染力を保持した組換えワクシニアウイルスの作成・使用に関しては、文部科学省の第二種使用等拡散防止措置指針に従い、大臣確認実験承認を得ている。又、全ての動物実験は、東京大学医科学研究所動物実験委員会の承認を得ており、その実施にあたっては、同研究所動物実験指針を遵守し動物愛護上の配慮を十分に行っている。

C．研究結果

ATL 担癌モデルマウスにおいて、その let7a の発現は、マウス正常組織に比べ ATL 腫瘍で低下していることが明らかになった（図１）。

NK 細胞枯渇処理 NOD/SCID マウスにウミシイタケルシフェラーゼ発現 ATL 細胞株 MT-2 を腹腔内投与し、10 日後にマウス体内の腫瘍をセレンテラジン投与によってモニターした。腹腔内における腫瘍の増殖が確認できたので、11 日後にホタルルシフェラーゼ発現 MDVV ウイルスを腹腔内投与した。14 日後にルシフェリンをマウスに投与後、マウス体内のウイルス分布を非侵襲的にモニターした。その結果、腹腔内腫瘍でのウイルス増殖が確認された。そして、20 日後に腫瘍はほぼ消失し、それに伴いウイルス増殖も減少することが 21 日後に観察された。一方、腹腔内に腫瘍がないマウスにウイルスを投与しても、その正常組織でのウイルス増殖は確認できなかった（図２）。

TL-Oml 皮下腫瘍 NOG マウスにおいて、MDVV は血中を介して効率よく腫瘍組織に到達することが可能であった（図３）。

D．考察

上記の担癌モデルマウスにおいて、血中を介して効率よく腫瘍組織に到達できることが実証できたので、実際の症例で多臓器へ浸潤している ATL 細胞を標的破壊する治療法として期待できる。今後は、このモデルにおいて抗腫瘍効果（腫瘍発育抑制効果と生存期間延長効果）、及び安全性（体重減少や皮膚などの一般状態）を評価する予定である。

E．結論

以上より、ATL 腫瘍細胞株を使った研究では、let7a の発現が正常細胞と比べ癌細胞で低下しており、その ATL 細胞株の担癌マウスモデルにおいて、MDVV は腫瘍特異的増殖による強力な抗癌効果を示した。さらに MDVV は、血中を介して効率よく腫瘍組織に到達することが可能であった。

F．健康危険情報

なし

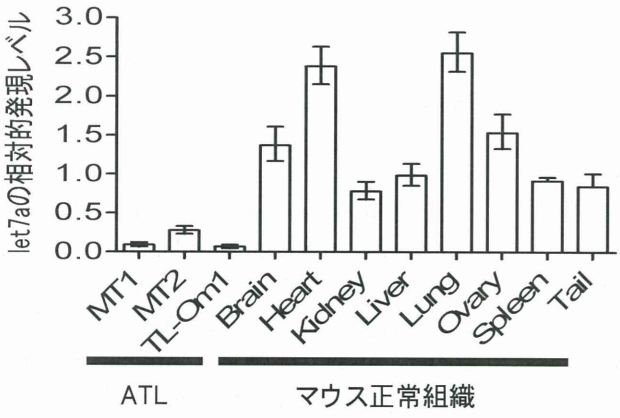

図1　定量 RT-PCR による let7a の発現解析

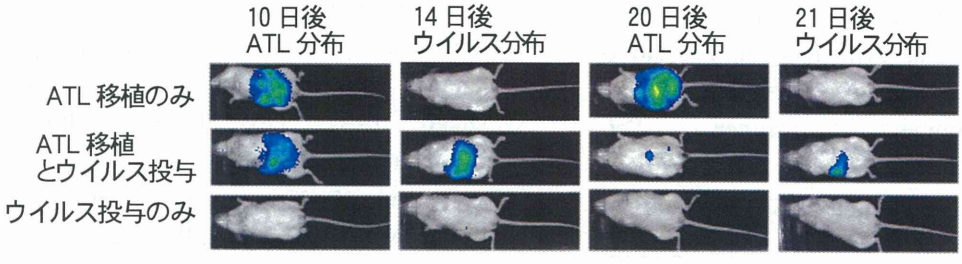

図2　MDVV の腫瘍特異的増殖による強力な抗癌効果

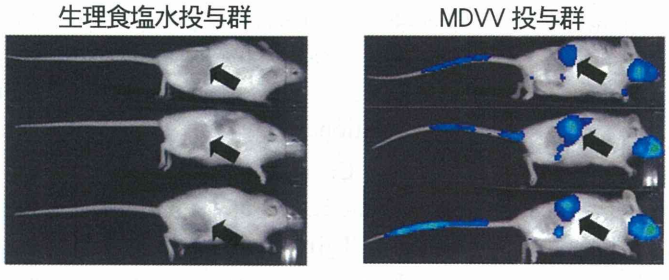

図3　血中を介した MDVV の腫瘍標的化

⬅ は皮下腫瘍組織を示す

G．研究発表

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2. 東條有伸、『造血幹細胞移植医療の最先端と課題』 第 100 回日本病理学会総会（横浜）ワークショップ 13 2011 年 4 月 30 日

H．知的財産権の出願・登録状況

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

別紙５

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hikichi M, Kidokoro M, Haraguchi T, Iba H, Shida H, Tahara H, Nakamura T.	MicroRNA regulation of glycoprotein B5R in oncolytic vaccinia virus reduces viral pathogenicity without impairing its antitumor efficacy.	Molecular Therapy	19	1107-1115	2011

MicroRNA Regulation of Glycoprotein B5R in Oncolytic Vaccinia Virus Reduces Viral Pathogenicity Without Impairing Its Antitumor Efficacy

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Vaccinia virus, once widely used for smallpox vaccine, has recently been engineered and used as an oncolytic virus for cancer virotherapy. Their replication has been restricted to tumors by disrupting viral genes and complementing them with products that are found specifically in tumor cells. Here, we show that microRNA (miRNA) regulation also enables tumor-specific viral replication by altering the expression of a targeted viral gene. Since the deletion of viral glycoprotein B5R not only decreases viral pathogenicity but also impairs the oncolytic activity of vaccinia virus, we used miRNA-based gene regulation to suppress B5R expression through let-7a, a miRNA that is downregulated in many tumors. The expression of B5R and the replication of miRNA-regulated vaccinia virus (MRVV) with target sequences complementary to let-7a in the 3′-untranslated region (UTR) of the *B5R* gene depended on the endogenous expression level of let-7a in the infected cells. Intratumoral administration of MRVV in mice with human cancer xenografts that expressed low levels of let-7a resulted in tumor-specific viral replication and significant tumor regression without side effects, which were observed in the control virus. These results demonstrate that miRNA-based gene regulation is a potentially novel and versatile platform for engineering vaccinia viruses for cancer virotherapy.

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INTRODUCTION

Oncolytic viruses are promising therapeutic agents for cancer and are currently under preclinical and clinical investigation.[1] For example, vaccinia virus is a potential oncolytic virus because it has broad tropism in mammalian cells, a fast replication cycle, and no risk of integration into the host genome.[2] The replication cycle of vaccinia viruses only requires about 8 hours and results in cell lysis and release of progeny viruses. Furthermore, there is no risk of the viral genome integrating into the host genome because vaccinia viruses complete their entire life cycle in the cytoplasm, unlike most other DNA viruses. However, since viral toxicity is a potentially serious problem, the virus has been engineered to reduce its pathogenicity while retaining its oncolytic properties.[3,4]

The attenuated, replicating vaccinia virus strain LC16m8 is an attractive backbone for engineering a novel oncolytic agent because the strain has an extremely low neurovirulence profile.[5,6] In addition, LC16m8 has been safely administered to >100,000 infants and adults for smallpox vaccination and induced levels of immunity similar to those of the original Lister strain without serious side effects.[5,7,8] LC16m8 was isolated from LC16mO, which is a clone that was isolated from Lister strain through LC16, by repeated passages in primary rabbit kidney cells and selection for their temperature sensitivities.[5,6] As a result of attenuation, LC16m8 has a single nucleotide deletion in the open reading frame of the B5R gene.[9,10] B5R is a 42 kDa glycoprotein that is involved in virus morphogenesis, trafficking, and dissemination.[11–19] Previously, the *B5R* gene was deleted from LC16m8 to develop a more genetically stable strain, LC16m8Δ.[20] In this study, we first compared the oncolytic potential of B5R-negative LC16m8Δ with B5R-positive LC16mO in mouse xenograft tumor model to determine the contribution of B5R to the pathogenicity and oncolytic potential of vaccinia virus.

Two strategies have been proposed to reduce the pathogenicity of vaccinia virus in normal cells and selectively target its oncolytic effects to tumor cells. In one strategy, insertional inactivation of vaccinia virus genes encoding thymidine kinase and/or epidermal growth factor-like vaccinia growth factor inhibits pathogenic viral replication in normal cells, while retaining its therapeutic replication in tumor cells that constitutively express thymidine kinase at high levels and have strong activation of the epidermal growth factor receptor pathway.[3,4] Thus, the success of this approach requires complementation of the disrupted viral genes by-products that are specifically found in tumor cells. Another strategy, transcriptional

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targeting, uses tissue-specific promoters to restrict the replication of oncolytic viruses that have been developed from DNA viruses, such as adenovirus and herpes simplex virus, to malignant tissues.[21,22] However, this approach is not applicable to vaccinia viruses due to its cytoplasmic life cycle. As a result, an alternative strategy is needed to reduce the pathogenicity of vaccinia virus without impairing its oncolytic activity.

In this study, we used microRNA (miRNA)-based regulation of B5R to specifically target the oncolytic effects of vaccinia virus to tumor cells. Although this strategy has been applied to the development of oncolytic viruses from other DNA[23,24] and RNA[25,26] viruses, this is first application of the strategy to vaccinia virus. miRNAs are small noncoding RNAs (~22 nucleotides) that repress gene expression by binding to complementary sequences in the 3′-untranslated region (UTR) of messenger RNAs.[27,28] These post-transcriptional regulators play important roles in the control of tissue specification, tumorigenesis, and tumor progression. Since many miRNAs are differentially expressed in different tissues[29] and tumors,[30] they can be used to selectively promote viral replication in tumor cells expressing low levels of miRNA while inhibiting viral replication in normal cells that express higher levels of miRNA. An example of such a miRNA is let-7a, which belongs to the let-7 family of miRNAs that has lower expression in many kinds of cancer cells than in normal cells.[31–36] We successfully developed a miRNA-regulated vaccinia virus (MRVV) with let-7a miRNA complementary target sequences in the 3′UTR of *B5R*. This MRVV selectively replicates and induces oncolysis in tumor cells without toxicity in normal cells.

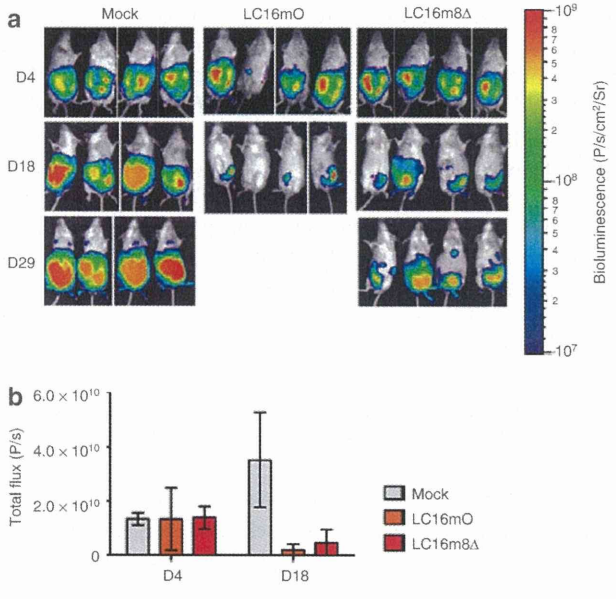

Figure 1 Comparison of the oncolytic effects of LC16m8Δ and LC16mO in mice bearing intraperitoneal xenografts. (**a**) BxPC-3 cells stably expressing luciferase (5×10^6 cells) were intraperitoneally injected into female severe combined immunodeficiency mice on day 0. Seven days after tumor implantation, the mice were intraperitoneally injected with a single dose of LC16mO or LC16m8Δ (1×10^7 plaque-forming unit/mouse). (**b**) *In vivo* tumor growth was monitored noninvasively by bioluminescence imaging after intraperitoneal administration of D-luciferin on days 4, 18, and 29. Quantification of the bioluminescence signals (photons/s) in the imaging data from days 4 and 18 in **a**. The data are presented as mean ± SD ($n = 4$).

RESULTS

Glycoprotein B5R is associated with viral pathogenicity and oncolytic activity

The *in vivo* oncolytic potentials of B5R-negative LC16m8Δ and B5R-positive LC16mO viruses in xenograft mouse model are compared in **Figure 1**. Four days after tumor implantation (day 4), the tumor growth in all of the implanted mice was similar. On day 18, tumor growth was significantly inhibited ($P < 0.001$) in both the LC16mO- and the LC16m8Δ-treated groups compared with the control group (**Figure 1a,b**). However, there was no significant difference in tumor volume reduction between the LC16mO- and LC16m8Δ-treated groups on this day (**Figure 1b**). In addition, all of the LC16mO-treated mice died or were sacrificed on days 21–28 because they exhibited symptoms of severe viral toxicity, such as weight loss and pock lesions on their tail, paws, face, and other areas of the body surface (data not shown). Although LC16m8Δ-treated mice did not show these symptoms, tumor regrowth was observed after day 29 (**Figure 1a**).

A schematic representation of the restoration of *B5R* in recombinant vaccinia virus LC16m8Δ-B5R is shown in **Figure 2a**. LC16m8Δ lysed A549, BxPC-3, Caco-2, and HeLa cells more efficiently than HEp-2, MDA-MB-231, PANC-1, and SK-N-AS cells. Although the cytolytic activity of LC16m8Δ was much lower than that of LC16mO in all tumors, B5R expression fully restored its oncolytic activity (**Figure 2b**).

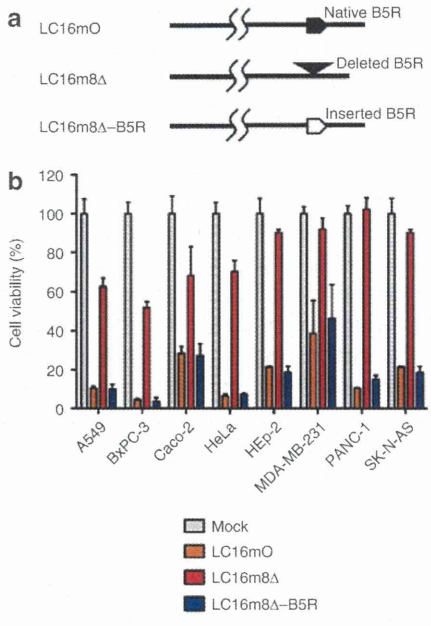

Figure 2 Relationship between B5R expression and oncolytic activity. (**a**) Schematic representation of the recombinant vaccinia virus LC16m8Δ-B5R. (**b**) Human cell lines were infected with B5R-positive or B5R-negative viruses at a multiplicity of infection of 0.5. The cell viabilities were determined 120 hours postinfection and are expressed as percentages of the cell survival of mock-infected cultures. The data are presented as mean + SD ($n = 3$).

B5R expression and the replication of miRNA-regulated vaccinia virus are dependent on endogenous let-7a

A schematic representation of MRVV with B5R-EGFP (BG) fusion protein (MRVV/BG) is shown in **Figure 3a**. The expression level of mature let-7a miRNA in normal human lung fibroblasts (NHLF) cells was three to four times higher than that in human lung and pancreatic carcinoma cell lines A549, BxPC-3, and PANC-1. On the other hand, there was less than a twofold difference in let-7a expression between NHLF and cervical carcinoma cell line HeLa (**Figure 3b**). These expression levels of let-7a correlate with functional activities of let-7a as measured by luciferase reporter assay. As shown in **Figure 3c**, the presence of four copies of let-7a target sequences in the 3′UTR of firefly luciferase (*FLuc*) mRNA in HeLa cells resulted in >96% of suppression of *FLuc* expression compared with the presence of four copies of the disrupted target sequences. In contrast, the suppression of *FLuc* expression in A549 and BxPC-3 cells was much weaker than that in HeLa cells. Thus, HeLa cells have five to seven times more let-7a activity than A549 and BxPC-3 cells (**Figure 3c**).

During infection of HeLa and NHLF cells, LC16m8Δ-B5Rgfp$_{let7a}$ did not induce a cytopathic effect (CPE) with B5R-enhanced green fluorescent protein (EGFP) expression, whereas LC16mO and the control viruses LC16m8Δ-B5Rgfp (lacking miRNA target sequences) and LC16m8Δ-B5Rgfp$_{let7a\text{-}mut}$ (containing the disrupted miRNA target sequences) resulted in a massive CPE after B5R-EGFP expression (**Figure 3d**). Simultaneously, the replication of LC16m8Δ-B5Rgfp$_{let7a}$ replication in HeLa and NHLF cells, which was equivalent to that of LC16m8Δ, was reduced by two log orders compared with that of LC16mO, LC16m8Δ-B5Rgfp, and LC16m8Δ-B5Rgfp$_{let7a\text{-}mut}$

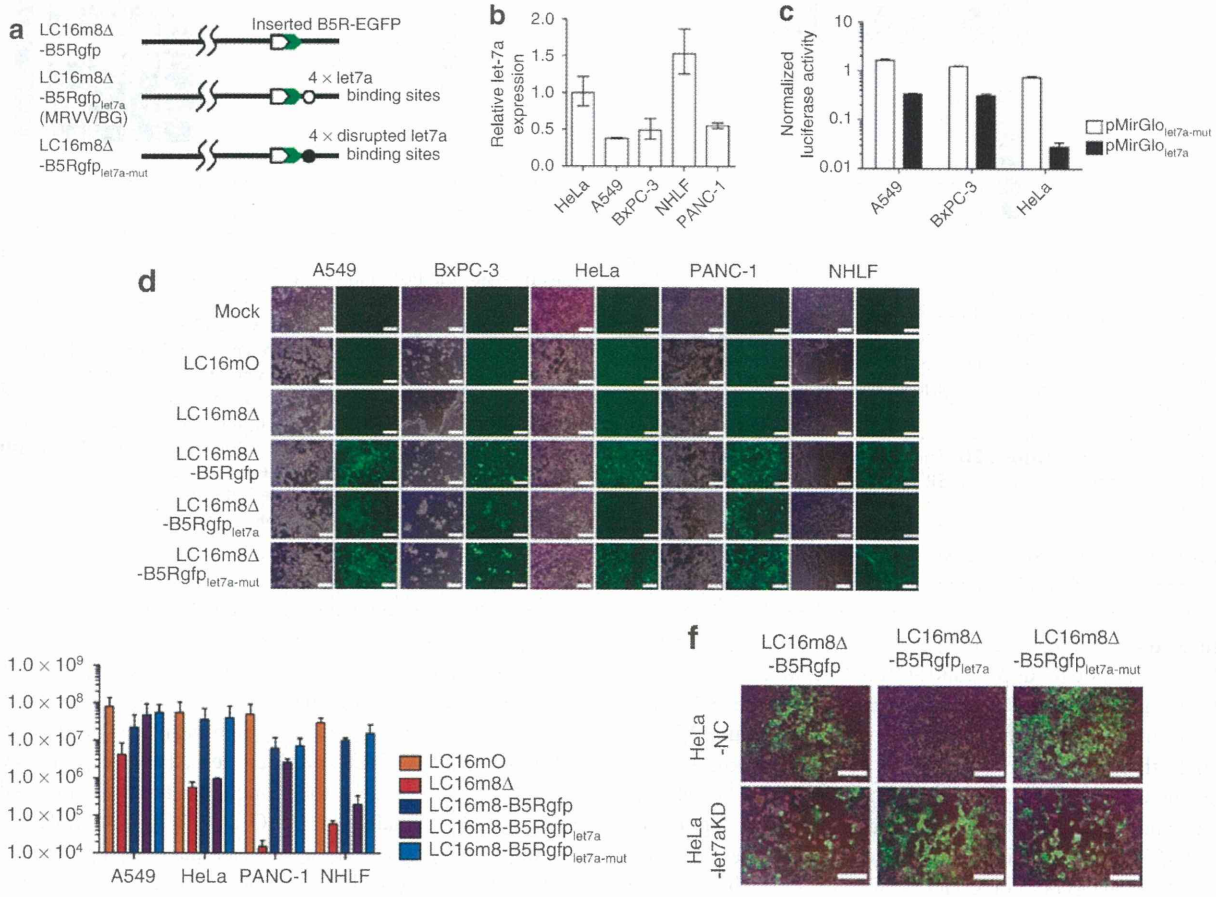

Figure 3 Construction and characterization of recombinant microRNA (miRNA)-regulated vaccinia virus (MRVV). (**a**) Schematic representation of the recombinant vaccinia virus genome showing the modified B5R protein fused with enhanced green fluorescent protein at its C-terminus (MRVV/BG). Four copies of let-7a miRNA complementary or disrupted target sequences, flanked by *NheI*/*AgeI* restriction sites, were incorporated into the 3′-untranslated region of the *B5R* gene. (**b**) Relative expression of mature let-7a miRNA in the indicated cell lines by real-time PCR analysis. The data are the let-7a level normalized with the U6 small nuclear RNA level relative to that in HeLa cells and are represented by the mean ± SD (n = 3). (**c**) The cell lines expressing different levels of let-7a were transfected with pMirGlo$_{let7a\text{-}mut}$ or pMirGlo$_{let7a}$ plasmid containing two expression units encoding firefly luciferase (*FLuc*) used as the primary reporter to monitor mRNA regulation and Renilla luciferase (*RLuc*) acting as a transfection control. Dual luciferase assay was performed 24 hours post-transfection. The *FLuc* activity is normalized to the *RLuc* activity. The data are presented as mean + SD (n = 3). (**d**) The cell lines expressing different levels of let-7a were infected with the MRVV/BG at an multiplicity of infection (MOI) of 0.1 and photographed using phase-contrast or fluorescence microscopy of the same field 3 days later. Bar = 200 µm. (**e**) One-step growth of the MRVV/BG was determined by titration of the viruses that were collected from the infected cells shown in **(d)**. The data are presented as mean + SD (n = 3). (**f**) HeLa-let7aKD cells (let-7a miRNA knockdown) or HeLa-NC cells (negative control) were infected with the MRVV/BG at an MOI of 0.1 and photographed 3 days later. The combined phase-contrast and fluorescence images are shown. Bar = 200 µm.

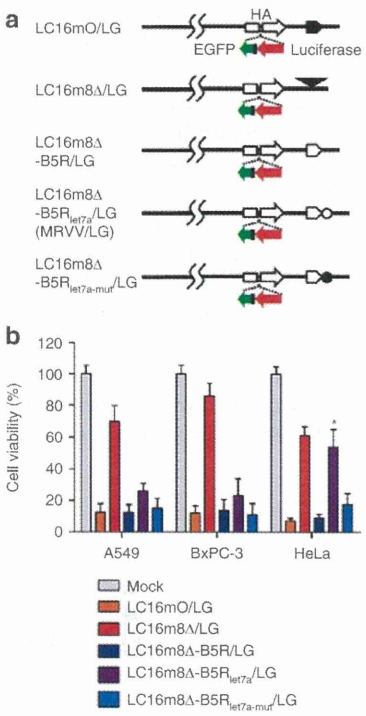

Figure 4 Effect of transgene insertion into microRNA (miRNA)-regulated vaccinia virus (MRVV) on its oncolytic activity *in vitro*. (**a**) Schematic representation of the recombinant vaccinia virus genome with the expression cassette that encodes both luciferase and enhanced green fluorescent protein reporters miRNA-regulated vaccinia virus (MRVV) luciferase (L) and enhanced green fluorescent protein (G) reporters (MRVV/LG). The symbols are the same as those used in **Figures 2a** and **3a**. (**b**) The cell viability after infection with MRVV/LG was determined as described in **Figure 2b**. The data are presented as mean + SD ($n = 3$). *$P < 0.001$ for LC16m8Δ-B5R$_{let7a}$/LG versus LC16m8Δ-B5R$_{let7a-mut}$/LG in HeLa cells.

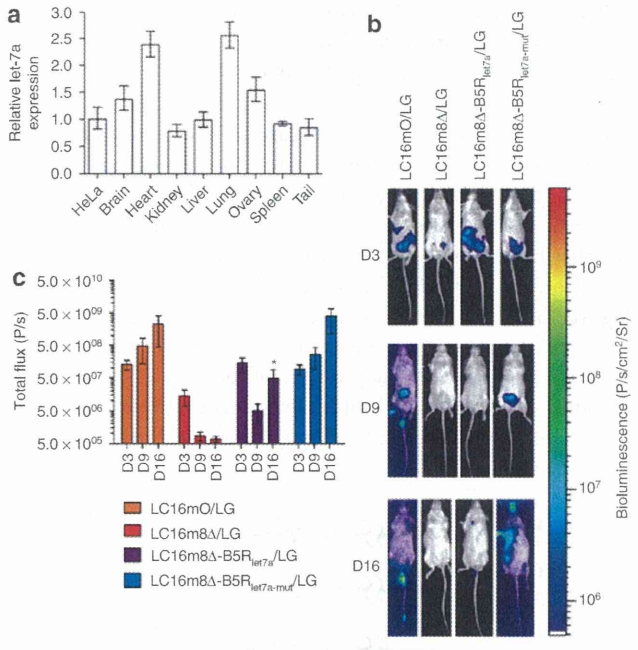

Figure 5 Biodistribution and replication of microRNA (miRNA)-regulated vaccinia virus (MRVV) luciferase (L) and enhanced green fluorescent protein (G) reporters (MRVV/LG) *in vivo*. (**a**) Relative expression of mature let-7a miRNA in the indicated mouse normal tissues by real-time PCR analysis. The data are the let-7a level normalized with the U6 small nuclear RNA level relative to that in HeLa cells and are represented by the mean ± SD ($n = 3$). (**b**) Representative images of the biodistribution of MRVV/LG in severe combined immunodeficiency mice that were intraperitoneally injected with 1×10^7 plaque-forming unit of MRVV/LG ($n = 3$). The biodistributions were visualized by intraperitoneal injection of D-luciferin at 3, 9, and 16 days after viral administration. The vaccinia virus designations are the same as those used in **Figure 4a**. (**c**) Quantitation of the bioluminescence signals in photons/s, calculated from the imaging data in **(b)**. The data are presented as mean ± SD ($n = 3$). *$P < 0.001$ for LC16m8Δ-B5R$_{let7a}$/LG versus LC16m8Δ-B5R$_{let7a-mut}$/LG on day 16.

(**Figure 3e**). In contrast, there were no differences in the CPE and replication in A549, BxPC-3, and PANC-1 cells among LC16m8Δ-B5Rgfp, LC16m8Δ-B5Rgfp$_{let7a}$, and LC16m8Δ-B5Rgfp$_{let7a-mut}$ (**Figure 3d,e**). Furthermore, the CPE and replication of LC16m8Δ-B5Rgfp, LC16m8Δ-B5Rgfp$_{let7a}$, and LC16m8Δ-B5Rgfp$_{let7a-mut}$ in A549, BxPC-3, and PANC-1 cells were comparable with those of LC16mO but were much greater than those of LC16m8Δ (**Figure 3d,e**). In addition, the presence of miRNA-based gene regulation was confirmed by using HeLa-let7aKD cells where TuD RNA largely suppresses endogenous let-7a activity (**Supplementary Figure S1**). LC16m8Δ-B5Rgfp$_{let7a}$ induced a CPE after B5R-EGFP expression in HeLa-let7aKD cells but not in HeLa-NC cells, although LC16m8Δ-B5Rgfp and LC16m8Δ-B5Rgfp$_{let7a-mut}$ showed a massive CPE after B5R-EGFP expression in both cell types (**Figure 3f**). Collectively, these results clearly demonstrate that B5R expression and the replication of LC16m8Δ-B5Rgfp$_{let7a}$ were regulated by endogenous let-7a.

Transgene insertion into microRNA-regulated vaccinia virus does not affect let-7a miRNA-regulated oncolytic activity

A schematic representation of MRVV with luciferase (L) and EGFP (G) reporters (MRVV/LG) is shown in **Figure 4a**. Although LC16m8Δ-B5R$_{let7a}$/LG lysed A549 and BxPC-3 cells with low levels of let-7a more efficiently than LC16m8Δ/LG, there was no significant difference in the oncolytic activities of LC16m8Δ-B5R$_{let7a}$/LG and LC16m8Δ/LG against HeLa cells with higher levels of let-7a (**Figure 4b**). In addition, the oncolytic activity of LC16m8Δ-B5R$_{let7a}$/LG was significantly lower than that of LC16m8Δ-B5R$_{let7a-mut}$/LG in HeLa cells; however, there was no significant difference in their oncolytic activities in A549 and BxPC-3 cells. Finally, LC16mO/LG, LC16m8Δ-B5R/LG, and LC16m8Δ-B5R$_{let7a-mut}$/LG showed a similar oncolytic effect against A549, BxPC-3, and HeLa cells. These results indicated that transgene insertion into the vaccinia virus genome did not affect let-7a miRNA-regulated oncolytic activity.

miRNA-based regulation of vaccinia virus inhibits viral replication in normal tissues

It has been reported that let-7a is highly conserved in humans and mice and is ubiquitous and abundant in normal tissues.[27,37,38] Similarly, we confirmed ubiquitous let-7a RNA accumulation in all mouse tissues tested (**Figure 5a**). Real-time PCR analysis showed that the brain, heart, lung, and ovary have higher expression level of let-7a than HeLa cells do, while the kidney, liver, spleen, and

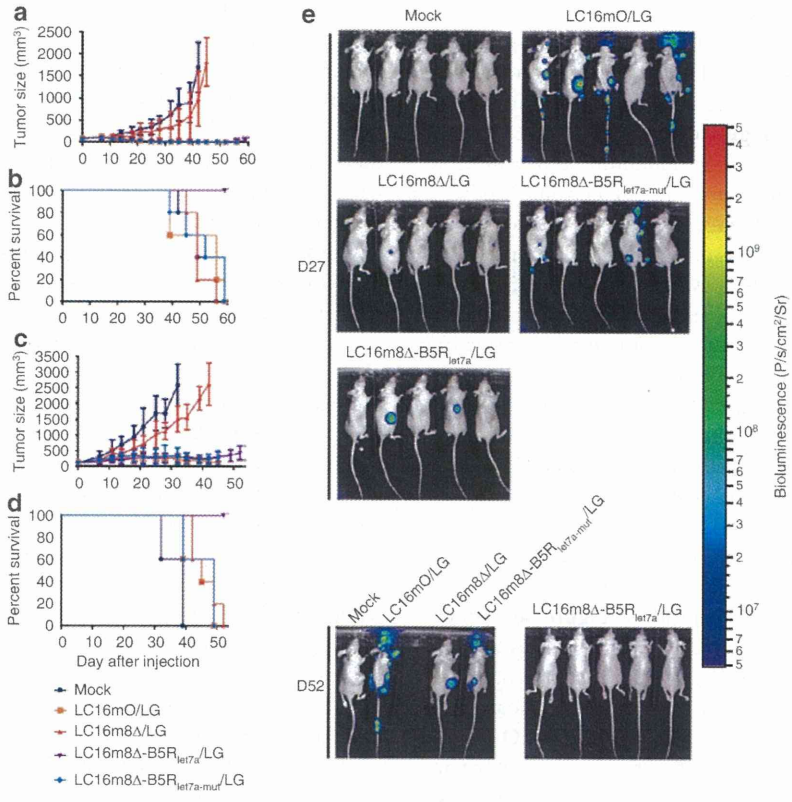

Figure 6 MicroRNA (miRNA)-regulated vaccinia virus (MRVV) luciferase (L) and enhanced green fluorescent protein (G) reporters (MRVV/LG) reduces viral pathogenicity while maintaining oncolytic activity *in vivo*. (**a**,**c**) Nude mice bearing established subcutaneous (**a**) BxPC-3 tumors or (**c**) A549 tumors were treated with intratumoral injections of MRVV/LG [1×10^7 plaque-forming unit (pfu)/injection, 3×10^7 pfu/mouse] on days 0, 3, and 6. The vaccinia virus designations are the same as those used in **Figure 4a**. The data are presented as mean ± SD ($n = 5$). (**b**,**d**) Survival curves of the mice that are shown in **a** and **c**. (**e**) *In vivo* biodistribution of MRVV/LG, determined by noninvasive imaging after intraperitoneal injection of D-luciferin into the mice that are shown in **(a, b)** on days 27 and 52.

tail have let-7a expression comparable to HeLa cells. Therefore, *in vivo* let-7a regulation was evaluated by a single intraperitoneal injection of each virus and noninvasive bioluminescence imaging in severe combined immunodeficiency (SCID) mice. Three days after injection of LC16mO/LG, LC16m8Δ/LG, LC16m8Δ-B5R$_{let7a}$/LG, or LC16m8Δ-B5R$_{let7a-mut}$/LG into SCID mice (day 3), the biodistribution of these viruses was concentrated in the abdomen. On days 9 and 16, the LC16mO/LG and LC16m8Δ-B5R$_{let7a-mut}$/LG viruses spread to several areas of their body, including the tail, paws, and face, where pock lesions were observed; however, the LC16m8Δ/LG and LC16m8Δ-B5R$_{let7a}$/LG viruses did not spread much (**Figure 5b**). In addition, there were significant differences in transgene expression levels and replication between LC16m8Δ-B5R$_{let7a}$/LG (or LC16m8Δ/LG) and LC16m8Δ-B5R$_{let7a-mut}$/LG (or LC16mO/LG) on day 16 but not on days 3 and 9 (**Figure 5c**). To clear the relationship between B5R expression and the replication of MRVV in normal tissues, LC16m8Δ-B5Rgfp, LC16m8Δ-B5Rgfp$_{let7a}$, or LC16m8Δ-B5Rgfp$_{let7a-mut}$ was intraperitoneally injected into SCID mice, and the virus-associated B5R-EGFP expression was examined 15 days after injection. As expected, LC16m8Δ-B5Rgfp and LC16m8Δ-B5Rgfp$_{let7a-mut}$ caused pock lesions on the tail, where B5R-EGFP expression was detected. In contrast, no pock lesion and B5R-EGFP expression were observed on the tail of LC16m8Δ-B5Rgfp$_{let7a}$-injected mice (**Supplementary Figure S2**). Taken together, these results demonstrate that let-7a miRNA-based regulation inhibits vaccinia virus replication in normal cells by downregulating B5R in cells infected with MRVV.

miRNA-regulated vaccinia virus reduces viral pathogenicity while maintaining oncolytic activity after tumor-specific replication in mouse tumor models

LC16m8Δ-B5R$_{let7a}$/LG induced a significantly stronger antitumor effect than LC16m8Δ/LG in nude mice with subcutaneous BxPC-3 tumors ($P < 0.001$ on days 39–45; **Figure 6a**) or A549 tumors ($P < 0.001$ on days 25–42; **Figure 6c**) without the severe viral toxicity associated with LC16mO/LG and LC16m8Δ-B5R$_{let7a-mut}$/LG. Furthermore, in the BxPC-3 model, four out of five LC16m8Δ-B5R$_{let7a}$/LG-treated mice showed complete tumor regression without any symptoms of toxicity at the end of the experiment. Similarly, in the A549 model, the LC16m8Δ-B5R$_{let7a}$/LG-treated mice showed reduced tumor growth but not complete tumor regression. Although the degree of tumor regression in the LC16mO/LG- and LC16m8Δ-B5R$_{let7a-mut}$/LG-treated mice was similar to that of the LC16m8Δ-B5R$_{let7a}$/LG-treated mice in both tumor models, all of the mice treated with the former viruses died or were sacrificed on days 39–59 due to severe viral toxicity, such as pock lesions and weight loss. Finally, all of the mock- or

LC16m8Δ-treated mice were sacrificed by the end of the experiment due to their great tumor burden. Thus, infection with the LC16m8Δ-B5R$_{let7a}$/LG virus resulted in a significantly longer survival than infection with any of the other viruses in both mouse xenograft models ($P < 0.005$; **Figure 6b,d**).

These results were confirmed by bioluminescence imaging, which showed tumor-specific replication of LC16m8Δ-B5R$_{let7a}$/LG in BxPC-3 xenografts (**Figure 6e**) and A549 xenografts (**Supplementary Figure S3**). Three and 10 days after intratumoral injection of LC16mO/LG, LC16m8Δ/LG, LC16m8Δ-B5R$_{let7a}$/LG, or LC16m8Δ-B5R$_{let7a\text{-}mut}$/LG (days 3 and 10), the biodistribution of these viruses was concentrated in the tumor in nude mice bearing BxPC-3 (data not shown) or A549 xenografts (**Supplementary Figure S3**). On day 20, the LC16mO/LG and LC16m8Δ-B5R$_{let7a\text{-}mut}$/LG viruses spread to several areas of their body, including the tail, paws, and face, where pock lesions were observed; however, the LC16m8Δ/LG and LC16m8Δ-B5R$_{let7a}$/LG viruses did not spread (**Supplementary Figure S3**). On day 27, there was no viral replication in three of the tumor-free mice treated with LC16m8Δ-B5R$_{let7a}$/LG, whereas two of the mice with reduced tumor growth still showed tumor-specific viral replication (**Figure 6e**). On day 52, no viral replication was observed in normal tissues after the tumor had regressed completely in all five mice. In contrast, on day 27, viruses had already spread from tumor to normal tissues even in the tumor-free mice treated with LC16mO/LG and LC16m8Δ-B5R$_{let7a\text{-}mut}$/LG. By day 52, all of the surviving mice had widespread viruses in their tail, paws, ears, and face (**Figure 6e**). Finally, LC16m8Δ/LG replication was tumor-specific; however, it was much slower than that of the other viruses, which resulted in weaker oncolytic activity (**Figure 6e** and **Supplementary Figure S3**).

DISCUSSION

The viral glycoprotein B5R plays important roles in packaging intracellular matured virions with membranes derived from the trans-Golgi network or early endosomes to form intracellular enveloped virions.[12,16,17] Intracellular enveloped virions are transported along microtubules to the cell periphery[15,18] where they adhere to the cell surface as a cell-associated enveloped virions. B5R, along with the A36R and A33R proteins, is also involved in the Src kinase-dependent process of forming actin-containing microvilli and releasing cell-associated enveloped virions from the cell surface to form extracellular enveloped virions (EEVs).[13,14] Since EEVs are critical for cell-to-cell and long-range virus spreading, B5R dysfunction markedly reduces the formation of EEVs and results in small viral plaques *in vitro* and highly attenuated viruses *in vivo*.[10,11,17,19,20]

In addition, this study demonstrated that the deletion of B5R weakens its oncolytic activity, as shown by the reduced antitumor efficacy of B5R-negative LC16m8Δ in mouse xenograft tumor models (**Figures 1a** and **6a,c**). These results were confirmed by replication of LC16m8Δ *in vivo*, which was not only spatially restricted within the injected tumor but also slower replicating than B5R-positive viruses (**Figure 6e** and **Supplementary Figure S3**). The EEV is also surrounded by a host cell-derived envelope that contains several host complement control proteins and a few exposed viral proteins.[39–41] Among these proteins, B5R is the only target for EEV-neutralizing antibodies.[39] Nevertheless, high EEV-producing strains of vaccinia virus spread between tumors more efficiently than low EEV-producing strains, even in the presence of EEV-neutralizing antibodies, in a syngeneic mouse tumor model.[42] Collectively, these results suggest that strategies that regulate the expression of B5R, such as miRNA, are promising approaches for engineering safe and effective vaccinia viruses for cancer virotherapy.

Recently, tumor-targeting approaches using miRNA have been used to develop oncolytic viruses based on adenovirus,[23] coxsackievirus A21,[26] herpes simplex virus 1,[24] and vesicular stomatitis virus.[25] The tumor-specific replication of these engineered viruses has decreased their pathogenic effects in normal tissues. For example, the insertion of miRNA target sequences for muscle-specific miRNA into coxsackievirus A21 decreased myositis without compromising antitumor activity.[26] Similarly, insertion of miRNA target sequences for hepatocyte-specific miRNA into adenovirus reduced hepatotoxicity.[23] Since vaccinia virus exhibits broad host cell tropism, we used tumor-suppressed miRNA rather than tissue-specific miRNA to develop the MRVV. In addition, we selected let-7a miRNA because the let-7 family of miRNAs is highly conserved and abundantly expressed in many types of normal cells.[27,38] However, the expression of let-7a is downregulated in tumor cells isolated from patients with breast,[31] hepatocellular,[32] lung,[33,34] melanoma,[35] and pancreatic[36] carcinomas.

For example, let-7a is reportedly expressed in ~50% of tumor cell lines and tumor tissues from patients with lung or pancreatic cancer at <20% of the expression in normal cells and tissues adjacent to the tumors.[33,36] Similarly, the expression of let-7a in the human A549 lung, BxPC-3 pancreatic, and HeLa cervical carcinoma cell lines used in this study was reduced by ~25, 30, and 65% of the expression in NHLF, respectively (**Figure 3b**). Remarkably, the HeLa cells have five to seven times more let-7a activity than the A549 and BxPC-3 cells despite an approximately twofold difference of let-7a expression between these cells (**Figure 3c**). Considering that it has been proposed that target suppression depends on a threshold miRNA concentration,[43] the concentration of let-7a in HeLa cells may be sufficient to reach the threshold expression level necessary for strong suppression. On the other hand, perfectly complementary target sites for let-7a of MRVV may be subject to regulation by all the other members of the let-7 family that are expressed in HeLa cells as described previously.[43] Anyhow, B5R expression and the replication of MRVV/BG were almost completely inhibited by let-7a miRNA-based regulation in not only the NHLF but also HeLa cells (**Figure 3d–f**). Furthermore, B5R-EGFP expression and the replication of MRVV/BG were also abrogated on the mouse tail that has let-7a expression comparable to HeLa cells (**Figure 5a** and **Supplementary Figure S2**). Our findings are consistent with previous reports concerning let-7a miRNA-based regulation of vesicular stomatitis viral[25] and polioviral[37] replication in HeLa cells and mouse models. In contrast, the expression of let-7a in the A549, BxPC-3, and PANC-1 cells was low enough to induce efficient replication of MRVV/BG in these tumor cells and xenografts, although the residual let-7a activity slightly repressed the B5R expression of MRVV/BG *in vitro* (**Figure 3d**) and *in vivo* (**Supplementary Figure S4**).