別添1

厚生労働科学研究研究費補助金


萌芽的先端医療技術推進研究事業


# がん特異的増殖機能を有するウイルス製剤と高感度GFP蛍光検出装置を用いた体外超早期がん診断および体内微小リンパ節転移診断システムに関する研究


(H17-ナノ-014)


平成17年度　総括研究報告書



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平成18（2006）年　4月

別添2

# 目　　　次

別添3

厚生労働科学研究費補助金（萌芽的先端医療技術推進推進研究事業）
（総括・分担）研究報告書

# がん特異的増殖機能を有するウイルス製剤と高感度GFP蛍光検出装置を用いた体外超早期がん診断および体内微小リンパ節転移診断システムに関する研究

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【研究要旨】近年増加を続けるがん患者の生存率や治療成績の向上には、早期発見、適格な悪性度の予知、適切な治療方針の決定が重要な因子となる。末梢血中の浮遊がん細胞の検出は原発巣が認識されないがん患者の早期発見に有効であり、また悪性度評価に基づく予後の予知にも有用であると報告されている。さらに微小リンパ節転移の検出は、患者のQOLを重視した必要最小限の低侵襲外科手術の確立に役立つ。本研究では、テロメラーゼ活性（hTERT遺伝子発現）依存性にがん細胞で選択的に増殖し、オワンクラゲ由来の蛍光遺伝子GFP (Green Fluorescence Protein)を発現する改変アデノウイルス製剤 Telomelysin-GFP (OBP-401)を標識薬剤とし、がん患者末梢血中に流れる浮遊がん細胞を高率に再現性をもって、かつ定量的に検出する体外的超早期がん診断システムを開発することを目的とする。まず、末梢血中の浮遊がん細胞の検出のための研究では、健常人の末梢血に各種ヒト培養がん細胞(肺癌、乳癌、大腸癌、etc)を加え、赤血球の溶血の後に一定量のOBP-401ウイルス製剤を添加したところ、50-70％のがん細胞の蛍光を検出することが可能であった。またパイロット・スタディーとして、文書でインフォームド・コンセントを得た後に進行胃癌・大腸癌患者の末梢血を採取し、溶血、OBP-401添加、24時間後の蛍光顕微鏡での鏡検を行ったところ、進行度に応じて末梢血中にGFP蛍光陽性の細胞を検出することができた。次に、マウスモデルにおける微小リンパ節転移の検出の研究では、ヒト大腸がん細胞とヌードマウスを用いた同所性直腸がんモデルにおいて、OBP-401ウイルス製剤を直腸腫瘍に直接投与し、5日後に開腹、キセノン光で蛍光を励起して高感度3CCDカメラにて観察した。最終的に組織学的に確認したところ、大動脈周囲のGFP陽性リンパ節では高頻度に微小転移が検出された。感度は、sensitivity 92.3%、specificity 86.6%であり、1 mm以下の微小転移巣を蛍光 spot として同定することが可能であった。このリンパ節転移診断システムは患者に優しい低侵襲手術の確立に寄与し、過剰な手術を避けることによる医療費の節減にも有効と思われる。

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## A. 研究目的

日本人の主要な死因が感染症から成人病、いわゆる生活習慣病へと移行する中で、特に癌は1981年以来日本人の死亡原因の第1位となっており、今後の本邦の癌罹患数は2015年には男性55万4000人、女性33万6000人となると推測されている。癌患者の生存率や治療成績の向上には、早期発見、適格な悪性度の予知、適切な治療方針の決定が重要な因子となる。近年、末梢血中の浮遊癌細胞の検出は原発巣が認識されない癌患者の早期発見に有効であり (Racila et al, PNAS 95:4589, 1998)、また悪性度評価に基づく予後の予知にも有用であったと報告されている (Cristofanilli et al, N Engl J Med, 351:781, 2004)。さらに、微小リンパ節転移の検出は、患者のQOLを重視した必要最小限の低侵襲外科手術の確立に役立つ (Umeoka et al, Cancer Res, 64:6259, 2004)。

本研究では、テロメラーゼ活性依存性に癌細胞で選択的に増殖してオワンクラゲ由来の蛍光遺伝子GFP (Green Fluorescence Protein)を発現する改変アデノウイルス製剤 Telomelysin-GFP (OBP-401)を標識薬剤とし、末梢血中に流れる浮遊癌細胞を高率に再現性をもって、かつ定量的に検出する体外的超早期癌診断システムを開発することを目的とする。また、外科手術前にOBP-401を腫瘍内に投与してリンパ流に乗せ、ペンプローブ型の高感度GFP蛍光検出装置を用いることで、微小リンパ節転移を手術中にリアルタイムに検出して切除範囲を同定する外科手術ナビゲーション・システムとしての有効性も検討する。肺癌や胃癌などの進行固形癌手術の際に、OBP-401はリンパ節内の微小転移巣で増殖しGFP蛍光を発するため、原発巣周辺の組織にプローブを接触させることで転移リンパ節を検出することができ、リンパ節廓清範囲決定の有効な指標となると期待される。OBP-401ウイルスの生物製剤としての安全性確認の後に、sensitivityおよびspecificityを検証するためのトランスレーショナル・リサーチを計画し、検出装置とともにその実用化を目指す。

B. 研究方法

1）OBP-401（Telomelysin-GFP）の構造

　OBP-401は幼児の「かぜ」症状の原因となるアデノウイルス5型を基本骨格とし、テロメラーゼ構成成分であるhTERT（human telomerase reverse transcriptase）遺伝子のプロモーターの下流にウイルス増殖に必須のE1AおよびE1B遺伝子がIRES配列で連結して組み込まれている。また、ウイルスゲノムのE3領域に、サイトメガロウイルス（CMV）プロモーターとオワンクラゲ由来のGFP（Green Fluorescent Protein）蛍光発現遺伝子が挿入されている（図1）。OBP-401は癌細胞で選択的に増殖し、GFP蛍光を発するとともに、最終的には細胞死を誘導する。一方、テロメラーゼ活性を持たない正常細胞では、その増殖は抑制され、GFPもみられず、細胞死も生じることはない。

2）血中浮遊癌細胞の検出感度、再現性の検討

　健常人から提供された末梢血5mlに、各種ヒト培養癌細胞を段階的に希釈して浮遊させ、溶血で赤血球を除いた後に、遠心にて細胞成分を分離、一定量のOBP-401ウイルスと24時間混合培養する。回収した細胞をPBSに浮遊させ、スライドガラス上で蛍光顕微鏡下に陽性細胞を確認、カウントする（図2）。

3）マウス同所性直腸癌モデルにおける微小転移リンパ節検出の検討

　ヒト大腸癌細胞株をヌードマウスの直腸に移植して約4-6週間置くと、傍大動脈リンパ節に高率に転移が発生する。この大腸癌リンパ節転移モデルを用いて、OBP-401を直腸腫瘍内に投与し、開腹して転移リンパ節のGFP蛍光陽性率を観察する。さらに、摘出リンパ節を組織学的に検討して、実際の転移陽性リンパ節個数を同定する。複数のマウスにて同実験を繰り返し、sensitivityおよびspecificityを確認する。

（倫理面への配慮）

　本研究は「ヒトのクローンに関する研究等」に該当するおそれはないと判断される。ヒト臨床検体を用いる際には、「臨床研究倫理指針」に基づいてプロトコールを作成し施行する。また、本研究に用いられるGMP規格のOBP-401は米国のベンチャー企業にて製造・調整され、米国食品医薬品庁（FDA；Food and Drug Administration）の推奨するアデノウイルス製剤に関する安全性チェックを受けた後に遺伝子・細胞治療センターで保管される。微小転移リンパ節検出システムの臨床研究実施計画書は「遺伝子治療臨床研究に関する指針」に基づいて作成され、またインフォームドコンセントの説明と同意文書は新GCPに乗っ取って作成される。学内の遺伝子治療臨床研究審査委員会での審議の後、厚生労働省科学技術部会に提出する予定である。さらに、「遺伝子組換え生物等の規制による生物の多様性の確保に関する法律」に基づき、「第二種使用規程承認申請」を行う予定である。
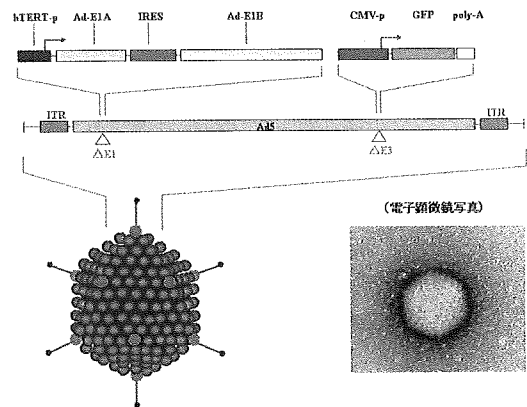
C. 研究結果

1）OBP-401（Telomelysin-GFP）の構造



（電子顕微鏡写真）

図1　OBP-401の構造と概観

2）血中浮遊癌細胞の検出感度、再現性の検討

　末梢血中の浮遊がん細胞の検出：健常人の末梢血5mlをチューブに準備し、各種ヒト培養がん細胞（肺癌、乳癌、大腸癌、etc）を0、10、100、1000、10000個になるように調整、赤血球を溶血した後に一定量（10e4 PFU/250 μl）のOBP-401ウイルス製剤を加えた。24時間37℃の恒温槽で振盪し、蛍光顕微鏡下に観察したところ、10-100個のがん細胞を加えたもので50-70％のがん細胞の蛍光を検出することが可能であった（表1、図3）。これらの結果より、上皮系のがん細胞でもチューブ内に浮遊した状態で24時間以上維持することが可能であり、その細胞内で感染したOBP-401も増殖できることが明らかとなった。



プロトコール

末梢血採血（5ml）

溶血（溶血剤と混和、室温で5分間静置）

遠心後、PBS 10mlに懸濁

遠心後、RPMI 0.75mlに懸濁（1.5mlエッペンドルフチューブ）

OBP-401 1×10⁴PFU/250mlを添加

ローターインキュベーターで振盪培養（37℃、24時間）

遠心後、上清を除去し、固定液30mlに懸濁

5mlをスライドガラスに滴下、蛍光細胞数をカウント

チューブカウント3回の平均と30ml全体の細胞数を算出
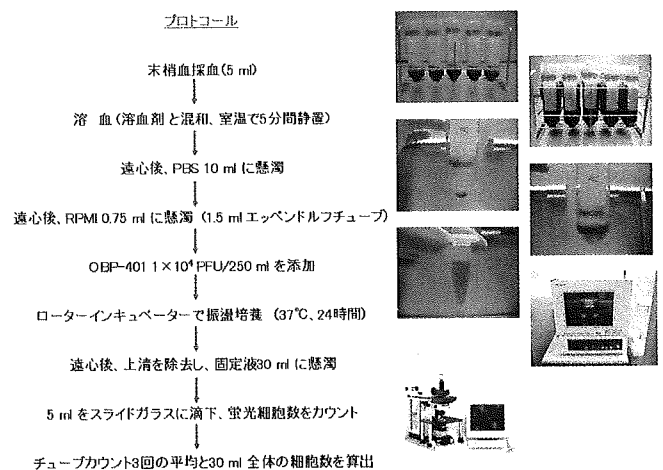
図2　浮遊癌細胞検出のための血液処理方法

　またパイロット・スタディーとして、文書でインフォームド・コンセントを得た後に進行胃癌・大腸癌患者の末梢血5mlを採取し、溶血、OBP-401添加、24時間後の蛍光顕微鏡での鏡検を行ったところ、進行度に応じて末梢血中にGFP蛍光陽性の細胞を検出することができた。健常人末梢血にOBP-401を添加しても、

2

GFP 陽性細胞は認められず、バックグランドの疑陽性は極めて低いことが確認された。進行癌患者の末梢血中の GFP 陽性細胞は 100 個以下であり、蛍光顕微鏡下の鏡検でも十分に定量可能であると考えられる。

表1　末梢血中の H1299 肺癌細胞の検出効率

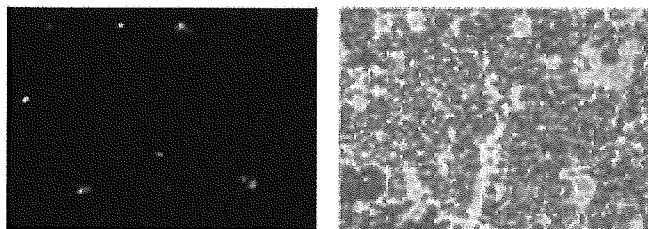| Tube | | A | B | C | D | E |
|---|---|---|---|---|---|---|
| 癌細胞数 (個) | | 10000 | 1000 | 100 | 10 | 0 |
| GFP 陽性細胞数 (個) | Exp. 1 | >4774 | 1236 | 70 | 8 | 0 |
| | Exp. 2 | N/A | 752 | 72 | 6 | 0 |



図3　末梢血中の GFP 陽性 H1299 肺癌細胞

## 3) マウス同所性直腸癌モデルにおける微小転移リンパ節検出の検討

マウスモデルにおける微小リンパ節転移の検出：ヒト大腸がん細胞をヌードマウスの直腸粘膜下に移植すると、同所性直腸がんが形成され、約 5-6 週間後に大動脈周囲に高頻度にリンパ節転移が認められる（図4）。
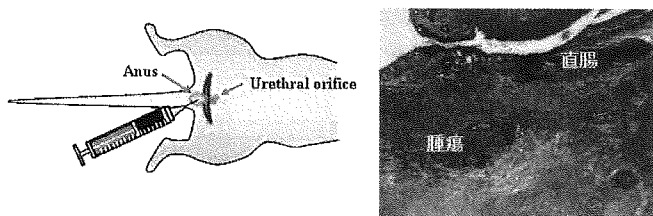


図4　リンパ節転移を来たす同所性ヒト直腸癌モデル

このモデルにおいて、OBP-401 ウイルス製剤を直腸腫瘍に直接投与し、5 日後に開腹し、キセノン光で蛍光を励起して高感度 3CCD カメラにて観察した。最終的に組織学的に確認したところ、GFP 陽性リンパ節では高頻度に微小転移が検出された。感度は、sensitivity 92.3%、specificity 86.6%であり、1 mm 以下の微小転移巣を蛍光 spot として同定することが可能であった（表2）。また、ペンプローブ型の高感度 GFP 蛍光検出装置の試作を計画しており、プローブの先端形状や操作性などについての検討項目も上がってきている。具体的には、励起光は独立して比較的広い範囲の術野に照射し、プローブの先端には高感度カメラのみを搭載することで軽量化を図る。また、モニターも術野外に大型のものを設置することで、プローブの操作性を優先する。将来的には、ゴーグル型のウェアラブルのものも開発することは可能である。
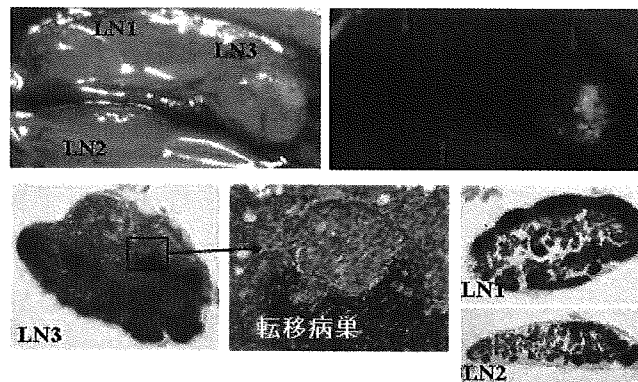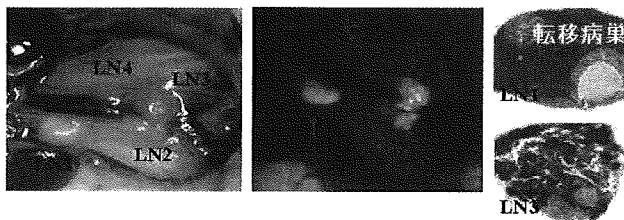


図5　OBP-401 による転移リンパ節特異的 GFP 発現

表2　転移リンパ節における GFP 蛍光と組織学的解析

| マウス No. | 転移 | GFP 蛍光 | | 合計 (%) |
|---|---|---|---|---|
| | | 陽性 | 陰性 | |
| ＃1 | 陽性 | 1 | 0 | 1 (33.3) |
| | 陰性 | 0 | 2 | 2 (66.6) |
| ＃2 | 陽性 | 3 | 0 | 3 (75.0) |
| | 陰性 | 0 | 1 | 1 (25.0) |
| ＃3 | 陽性 | 1 | 0 | 1 (33.3) |
| | 陰性 | 0 | 2 | 2 (66.6) |
| ＃4 | 陽性 | 0 | 0 | 0 (0) |
| | 陰性 | 0 | 4 | 4 (100) |
| ＃5 | 陽性 | 1 | 1 | 2 (66.6) |
| | 陰性 | 0 | 1 | 1 (33.3) |
| ＃6 | 陽性 | 3 | 0 | 3 (60.0) |
| | 陰性 | 1 | 1 | 2 (40.0) |
| ＃7 | 陽性 | 3 | 0 | 3 (50.0) |
| | 陰性 | 1 | 2 | 3 (50.0) |

## D. 考察

ウイルスは本来ヒトの細胞に感染して、その構造蛋白質を産生することで複製・増殖する。その増殖機能に選択性を付加することにより、ウイルスをがん細胞を標識する診断用製剤として用いることが可能となる。「かぜ」症状の原因となるアデノウイルス5型を基本骨格とするOBP-401は、ウイルス増殖に必須のE1遺伝子をテロメラーゼ構成分子であるhTERT (human telomerase reverse transcriptase) 遺伝子のプロモーターで制御することで、がん細胞のみで増殖するように改変されたウイルス製剤である。さらに、E3領域にGFP遺伝子が組込まれており、がん細胞のみで緑色蛍

光を発する。末梢血中の浮遊がん細胞の検出には、サイトケラチンなどを標的としたPCR法が多く行われているが、がん細胞特異性や感度の問題で一般医療とはなっていないのが現状である。また、蛍光色素で標識した抗体を用いた方法も報告されているが、増幅機能がないため、その検出感度は満足できるものではない。

われわれが標的とするテロメラーゼは80-90%の癌で極めて高い活性の上昇がみられ、正常組織で活性が検出されるリンパ球や小腸上皮での発現レベルは低いため、OBP-401のがん選択性はかなり厳格かつ広範である。また、OBP-401はがん細胞で複製・増殖するため、経時的なGFP蛍光強度の増強が期待できることが画期的であり、ウイルスを細胞標識に用いる点が独創的であると言える。さらに、GFPは導入遺伝子発現などの目的で多くの基礎研究で使用されているため、蛍光顕微鏡やFACSなどの検出技術の開発が進んでいる。緑色蛍光を選択的に感知するRGB処理などの工夫は必要であるが、OBP-401のGFP蛍光を高感度に検出する装置の開発は技術的には可能と思われる。

1年目のフィージビリティー・スタディーにより、健常人末梢血にOBP-401を添加しても、GFP陽性細胞は認められず、バックグランドの疑陽性は極めて低いことが確認された。一方、進行癌患者の末梢血を用いたパイロット・スタディーでは、GFP陽性細胞は100個以下であり、蛍光顕微鏡下の鏡検でも十分に定量可能であることが明らかとなった。したがって、中央集中的に特殊な検出機器を用いて測定するよりは、より簡便な検出キットを開発し、いろいろなレベルの医療機関で施行できるようなシステムを確立する方が実用的であると思われる。18年度以降は、臨床研究として各種進行癌患者の末梢血における浮遊がん細胞数を測定し、がん種による差や進行度との相関などについて検討する。また、OBP-401製剤の選択的ながん細胞標識機能の感度や検出限界を検討するとともに、従来のPCR法との比較検討などを行い、体外診断システムとしての有効性を検証する。さらに、浮遊がん細胞が検出された患者において、化学療法施行後に経時的にGFP陽性細胞数の変化を観察し、治療効果判定に有用か否かを検証する。従来の腫瘍マーカーは血中からのクリアランスのために一定の半減期が存在し、リアルタイムに治療効果を反映しない可能性がある。一方、本技術は、OBP-401ウイルス製剤の増幅というステップがあるために、生きたがん細胞のみを検出しており、よりリアルタイムに病状を捉えていると言える。本技術は、ウイルスそのものをがん細胞の標識薬剤として用いる点で独創的であり、細胞分画とウイルスを混ぜて蛍光顕微鏡下に鏡検するのみで陽性細胞を確認できることから、簡便性にも優れている。

手術の縮小による低侵襲化を目指す場合にほしい情報の一つに転移リンパ節の有無があり、それを知る方法としてセンチネルリンパ節(Sentinel Node; SN)が注目されている。しかし、SNはリンパの流れる方向を確認するのみであり、胃がん手術などの際に約10%にみられるskip転移が問題となってくる。外科手術前にOBP-401を腫瘍内に投与してリンパ流に乗せ、ペンプローブ型の高感度GFP蛍光検出装置を用いることで、微小リンパ節転移を手術中にリアルタイムに検出して切除範囲を同定する外科手術ナビゲーション・システムとして応用できる。肺がんや胃がんなどの進行固形がん手術の際に、OBP-401はリンパ節内の微小転移巣で増殖しGFP蛍光を発するため、原発巣周辺の組織にプローブを接触させることで転移リンパ節を検出することができ、リンパ節廓清範囲決定の有効な指標となると期待される。OBP-401ウイルスの生物製剤としての安全性確認の後に、sensitivityおよびspecificityを検証するためのトランスレーショナル・リサーチを計画し、検出装置とともにその実用化を目指す。18年度以降は、転移サイズとGFP蛍光強度の相関や経時的な蛍光強度の変化、蛍光減弱の経過などを検討するとともに、ペンプローブ型の高感度GFP蛍光検出装置を試作し、外科手術ナビゲーション・システムとしての応用の可能性を探る。具体的には、ヌードマウスとヒト大腸がん細胞を用いた同所性直腸がんモデルにおいて、ペンプローブ型検出器の有用性を検討する。現在の高感度3CCDカメラとペンプローブ型検出器のGFP蛍光の検出限界を比較検討し、さらに正常組織の後方に存在する場合、どの程度の深部までの転移リンパ節が確認できるかを検証する。このリンパ節転移診断システムは患者に優しい低侵襲手術の確立に寄与し、過剰な手術を避けることによる医療費の節減にも有効と思われる。

E. 結論

テロメラーゼ活性依存性に癌細胞で選択的に増殖して蛍光遺伝子 GFP を発現する改変アデノウイルス製剤 OBP-401 は、標識薬剤として末梢血中の浮遊癌細胞を高率に検出する体外的超早期癌診断システムおよび微小リンパ節転移検出外科手術ナビゲーション・システムに応用可能である。

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H. 知的財産権の出願・登録状況

1. 特許出願

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別添4

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| 藤原俊義<br>田中紀章 | p53遺伝子発現アデノウイルスベクターを用いた肺癌遺伝子治療. | **Surgery Frontier** | 12 | 53-56 | 2005 |
| 藤原俊義<br>田中紀章 | 悪性腫瘍に対するウイルス製剤：Telomelysinの開発と臨床応用の可能性 | **Medical Technology** | 33 | 961-966 | 2005 |

# Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD')

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Replication-competent oncolytic viruses are being developed for human cancer therapy. We previously reported that an attenuated adenovirus (OBP-301, 'Telomelysin'), in which the hTERT promoter element drives expression of E1A and E1B genes linked with an IRES, could replicate in cancer cells, and causes selective lysis of cancer cells. We further constructed OBP-405 ('Telomelysin-RGD') that contains an RGD motif in the HI loop of the fiber knob. We examined whether OBP-405 could be effective in overcoming the limitations of OBP-301, specifically their inefficient infection into cells lacking the primary receptor, the coxsackievirus and adenovirus receptor (CAR). By flow cytometric analysis, H1299 (lung) and SW620 (colorectal) tumor cells showed high levels of CAR expression, whereas LN444 (glioblastoma), LNZ308 (glioblastoma), and H1299-R5 (lung) tumor cells were negative for CAR expression. A quantitative real-time PCR analysis demonstrated that fiber-modified OBP-405 infected more efficiently than OBP-301, although the intracellular replication rate of both viruses was consistent. The comparative antitumor effect of fiber-modified OBP-405 and unmodified OBP-301 for human cancer cells was evaluated in vitro by XTT assay as well as in vivo by using athymic mice carrying xenografts. OBP-405 had a profound oncolytic effect on human cancer cell lines compared to OBP-301, in particular on cells with low CAR expression. Intratumoral injection of $10^7$ plaque-forming units of OBP-405 into CAR-negative H1299-R5 lung tumor xenografts in nu/nu mice resulted in a significant inhibition of tumor growth and long-term survival in all treated mice. Moreover, selective replication of OBP-405 in the distant, uninjected H1299-R5 tumors was demonstrated. Our results suggest that fiber-modified replication-competent adenovirus OBP-405 exhibits a broad target range by increasing infection efficiency, an outcome that has important implications for the treatment of human cancers.

## Introduction

The optimal treatment for human cancer requires an improvement of therapeutic ratio to increase cytotoxic efficacy on the tumor cells and decrease that on the normal cells. This may not be an easy task because most of normal cells surrounding tumors are sensitive to the cytotoxic treatment. Thus, to establish reliable therapeutic strategies for human cancer, it is important to seek the genetic and epigenetic targets present only in cancer cells. The emerging fields of functional genomics and functional proteomics provide an expanding repertoire of clinically applicable targeted therapeutics (Kohn et al., 2004). Telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes (Greider and Blackburn, 1985; Collins and Mitchell, 2002), and contains the enzymatic subunit human telomerase reverse transcriptase (hTERT) (Nakamura et al., 1997). The hTERT proximal promoter can be used as a molecular switch for the selective expression of target genes in tumor cells (Koga et al., 2000; Komata et al., 2001; Gu et al., 2000, 2002), since almost all advanced human cancer cells express telomerase and most normal cells do not (Kim et al., 1994; Shay and Wright, 1996). Genetically modified adenoviruses have emerged as a new biological anticancer agent (McCormick, 2001; Fang and Roth, 2003). We previously constructed an adenovirus vector (OBP-301, 'Telomelysin'), in which the hTERT promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site (IRES), and showed that OBP-301 induced selective

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E1A and E1B expression in human cancer cells, but not in normal cells (Kawashima et al., 2004). Therefore, OBP-301 can replicate and lyse only cancer cells, but not normal cells. In addition, OBP-301 will infect neighboring cancer cells, and induce oncolysis throughout the whole tumor mass in vivo.

Infection efficiency of the presently available adenoviral agent, which is derived from human adenovirus serotype 5, varies widely depending on the expression of Coxsackie-adenovirus receptor (CAR) (Wickham et al., 1993). The initial step of adenovirus infection involves at least two sequential steps. The first step is the attachment of the virus to the cell surface through binding of the knob domain of the fiber to CAR (Bergelson et al., 1997). Following attachment, the viral internalization into the cells occurs by the interaction of RGD (Arg-Gly-Asp) motifs of penton base with integrin receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$, expressed on most cell types (Wickham et al., 1993). Therefore, the interaction of the fiber knob with CAR on the cell is the key mediator by which adenoviral agents enter the cells. Modification of fiber protein is an attractive strategy for overcoming the limitations imposed by the CAR dependence of adenovirus infection (Wickham et al., 1997; Dmitriev et al., 1998; Krasnykh et al., 1998; Mizuguchi et al., 2001).

We modified the fiber of OBP-301 to contain RGD peptide, which binds with high affinity to integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) on the cell surface, on the HI loop of the fiber protein. The resultant adenovirus, termed OBP-405 or 'Telomelysin-RGD', mediated not only CAR-dependent virus entry but also CAR-independent, RGD-integrin ($\alpha v\beta 3$ and $\alpha v\beta 5$)-dependent virus entry. We explored whether OBP-405 containing RGD peptide on the fiber knob had more oncolytic efficacy on several types of human cancer cells (CAR-positive or -negative), as compared with OBP-301 containing wild-type fiber in vitro and in vivo.

## Results

### Expression of CAR and integrins in human cancer and normal cells

To investigate the antitumor effect of the fiber-modified OBP-405, we used several human cell lines. We first examined the expression levels of CAR and $\alpha v$ integrin family, $\alpha v\beta 3$ and $\alpha v\beta 5$, on each cell surface by flow cytometric analysis (Figure 1). Apparent amounts of CAR expression were detected on H1299 and SW620 cells, whereas LN444, LNZ308, and H1299-R5 cells expressed extremely low levels of CAR. The normal human lung fibroblast (NHLF) cell also exhibited detectable CAR expression. In contrast to CAR, $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins were readily expressed in all cell lines.

### Increased infection efficiency and selective replication of OBP-405

To assess whether incorporation of an RGD motif into the HI loop of the fiber knob domain would enhance its

infectivity, CAR-positive parental H1299, CAR-negative LN444, and NHLF (normal cell) were infected with OBP-301 or OBP-405 at an multiplicity of infection (MOI) of 1. Quantitative real-time PCR analysis with DNA extracted 2 h after infection demonstrated that the amount of E1A DNA after OBP-405 infection was higher than that after OBP-301 infection in two human cancer cell lines, whereas infectivity of both viruses was almost similar in NHLF (Figure 2a). These results suggest that RGD-modified OBP-405 showed increased infectivity to neoplastic cells regardless of the levels of CAR expression, although the infectivity enhancement was greater in CAR-negative cancer cells.

We next examined the replication ability of OBP-301 and OBP-405 in different cell lines by measuring the relative amounts of E1A DNA. H1299, LN444, and NHLF cells were harvested at the indicated time points over 72 h after infection with OBP-301 or OBP-405, and subjected to quantitative real-time PCR analysis. The ratios were normalized by dividing the value of cells obtained 2 h after viral infection. As shown in Figure 2b, both OBP-301 and OBP-405 replicated 5–6 logs by 72 h after infection; their replication, however, were attenuated up to 3 logs in normal NHLF cells.

### Enhanced viral spread of OBP-405 in human cancer cells

To examine whether increased infectivity of OBP-405 could facilitate viral spread, H1299, LN444, and NHLF cells cultured in chamber slides were infected either with OBP-301 or OBP-405 at an MOI of 1, and then immunohistochemically stained for viral hexon at 24 and 48 h of postinfection. Viral hexon was detectable in H1299 cells infected with OBP-301 and OBP-405 in a time-dependent manner, although the amount of positive cells after OBP-405 infection was higher than that after OBP-301 infection (Figure 3a). In contrast, viral hexon was only present in CAR-negative LN444 cells infected with OBP-405, but not in OBP-301-infected LN444 cells (Figure 3b), suggesting the selective replication of OBP-405 in CAR-negative human cancer cells. NHLF human normal cells exhibited no hexon-positive cells after OBP-301 or OBP-405 infection (Figure 3c).

### In vitro cytopathic efficacy of OBP-405 in CAR-negative human cancer cells

To test whether the increased infectivity and replication of OBP-405 translated to improved oncolysis, we compared the cytopathic effect of OBP-405 with that of OBP-301 on various human cell lines in vitro (Figure 4). Both OBP-301 and OBP-405 killed CAR-positive H1299 and SW620 human cancer cells in a dose-dependent manner; OBP-405 at an MOI of 0.1, however, killed these cells as efficiently as OBP-301 at an MOI of 1, suggesting that OBP-405 showed approximately 10-fold more profound tumor cell killing compared with OBP-301. In contrast, only OBP-405 was lytic in CAR-negative LN444, LNZ308, and H1299-R5 cells, likely due to the higher infectivity of OBP-405
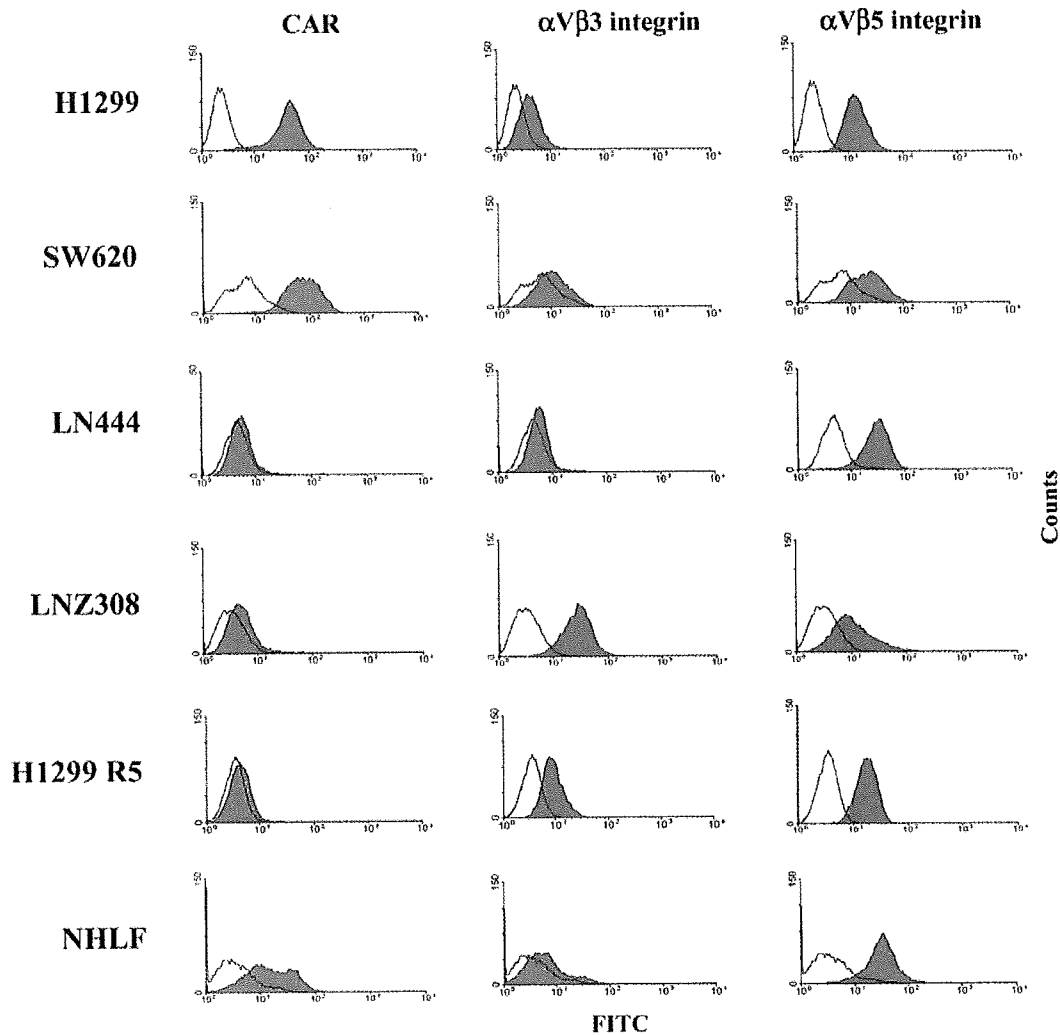
**Figure 1** Flow cytometric analysis of CAR and integrin ($\alpha v \beta 3$ and $\alpha v \beta 5$) expression in human cancer and normal cell lines. Cells were incubated with anti-CAR (RmcB), anti-$\alpha v \beta 3$ integrin (LM609), and anti-$\alpha v \beta 5$ integrin (P1F6) monoclonal antibodies, followed by detection with FITC-labeled goat anti-mouse IgG secondary antibody. An isotype-matched normal mouse IgG1 conjugated to FITC was used as a control in all experiments (solid line)

in these cell lines. Most of these cells were dead within 5 days after OBP-405 infection, whereas OBP-301-infected cells were still intact at 5 days postinfection (Figure 4b). Neither OBP-301 nor OBP-405 exhibited cytopathic effect on NHLF cells.

*Enhanced oncolysis of CAR-negative tumor xenografts by OBP-405*

We next examined whether OBP-405 cause enhanced oncolysis and spread *in vivo*. Subcutaneous H1299-R5 tumor xenografts with a diameter of 5–6 mm received three daily courses of intratumoral injection of $1 \times 10^7$ plaque-forming units (PFU) of OBP-301, OBP-405 or replication-deficient control adenovirus (dl312), or PBS (mock). As shown in Figure 5, administration of OBP-405 resulted in significant growth suppression compared to mock- or dl312-treated tumors 34 days after virus

injection ($P < 0.01$). In addition, one of the six mice treated with OBP-405 showed the complete eradication of the established H1299-R5 tumor. We previously reported that intratumral injection of OBP-301 significantly inhibited the growth of H1299 tumor xenografts; a modest, insignificant growth inhibition, however, occurred with administration of OBP-301 on H1299-R5 tumors. Treatment with replication-deficient dl312 had no apparent effect on the growth of H1299-R5 tumors.

*Targeting replication of OBP-405 in tumor tissues in vivo*

To evaluate selective replication of OBP-301 and OBP-405 *in vivo*, we examined mouse tissues including implanted tumors for the presence of viral DNA and protein by quantitative real-time PCR and immunohistochemistry, respectively, following intratumoral viral
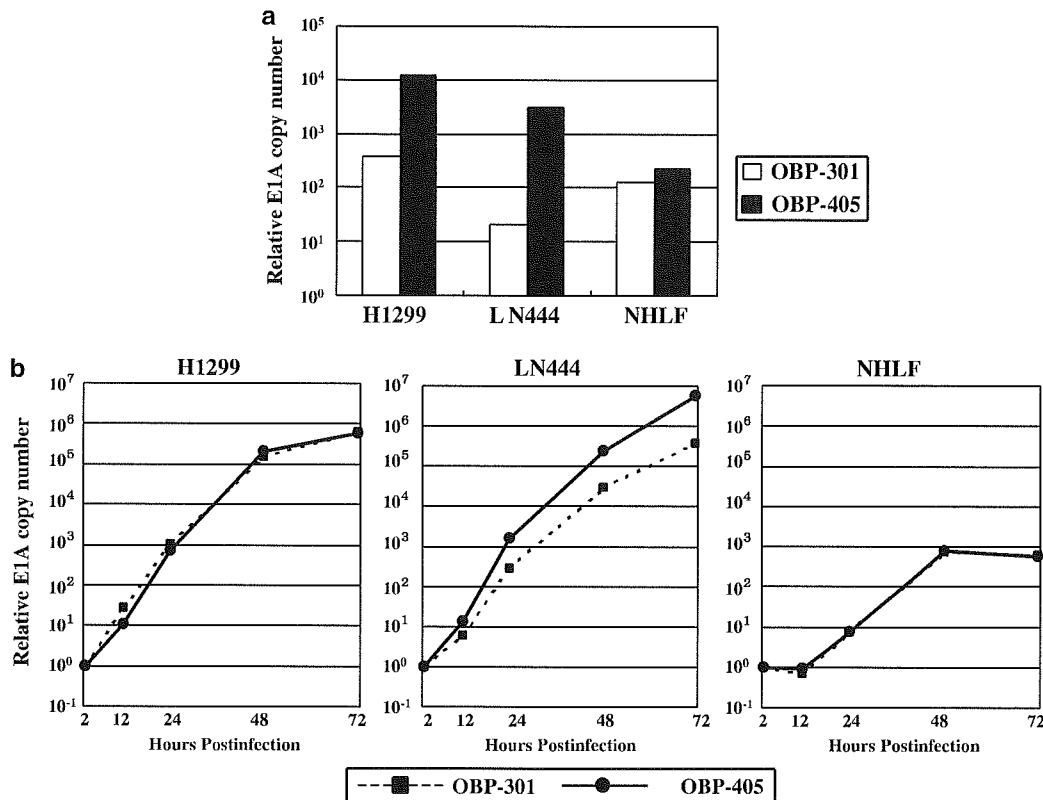
**Figure 2** (a) Comparative analysis of the infection efficiency with OBP-301 and OBP-405 in H1299 (CAR-positive lung cancer), LN444 (CAR-negative glioblastoma), and NHLF (normal fibroblast) cells. Cells were infected with either OBP-301 or OBP-405 at an MOI of 1 for 2 h, and the viral infection rate was evaluated by measuring the E1A copy number using the real-time quantitative PCR method. (b) Assessment of viral DNA replication in H1299, LN444, and NHLF cells. Cells were infected with either OBP-301 or OBP-405 at an MOI of 1 for 2 h. Following the removal of virus inocula, cells were further incubated for the indicated periods of time, and then subjected to the real-time quantitative PCR assay. The amounts of viral E1A copy number are defined as the fold increase for each sample relative to that at 2 h (2 h equals 1)

injection. Mice with established subcutaneous H1299-R5 tumors received three daily courses of intratumoral injection of $1 \times 10^7$ PFU of OBP-301 or OBP-405, or PBS (mock), and killed 7 days after treatment. E1A DNA was not detected in any normal tissues examined (liver, kidney, pancreas, and spleen), however, it was apparently detected in tumors (Figure 6a). Tumors from mice treated with OBP-405 contained 100-fold more E1A DNA than tumors from OBP-301-treated mice (Figure 6a). Immunohistochemical staining of adenoviral hexon protein revealed that OBP-405 mediated viral spread throughout the tumor tissues that was less evident in OBP-301-treated animals (Figure 6b). In other normal organs, adenoviral hexon protein was absent (data not shown).

To directly address whether OBP-405 is not toxic, we measured levels of liver enzymes as an indicator of hepatocellular damages 7 days after intratumoral injection of $1 \times 10^7$ PFU of viruses. As shown in Table 1, no significant elevation of liver enzymes was observed in mice intratumorally injected with OBP-301 or OBP-405. In addition, histopathological analysis of liver sections demonstrated that there were no apoptotic hapatocytes or other histological signs of hapatocellular

damages in mice treated with either OBP-301 or OBP-405 (data not shown).

*Viral spread of OBP-405 in distant tumor tissues after intratumoral injection*

We finally tested whether intratumoral injection of OBP-405 could mediate a therapeutic benefit on distant, uninjected H1299-R5 tumors in a dual tumor model. H1299-R5 tumors were established in the flanks at both left and right sides of nu/nu mice and viral replication in the left tumors was assessed after intratumoral inoculation of $1 \times 10^7$ PFU of either OBP-301 or OBP-405 into tumors in the right flank. Quantitative real-time PCR analysis on postinfection day 14 demonstrated that OBP-405 caused approximately 100-fold more efficient replication than OBP-301 in H1299-R5 tumors injected with viruses, whereas only OBP-405 replicated on distant, uninjected H1299-R5 tumors (Figure 7a).

At four weeks after viral injection, OBP-301 also replicated in uninjected H1299-R5 tumors; OBP-405, however, resulted in an approximately 100-fold more replication at uninjected sites (Figure 7b). In contrast,
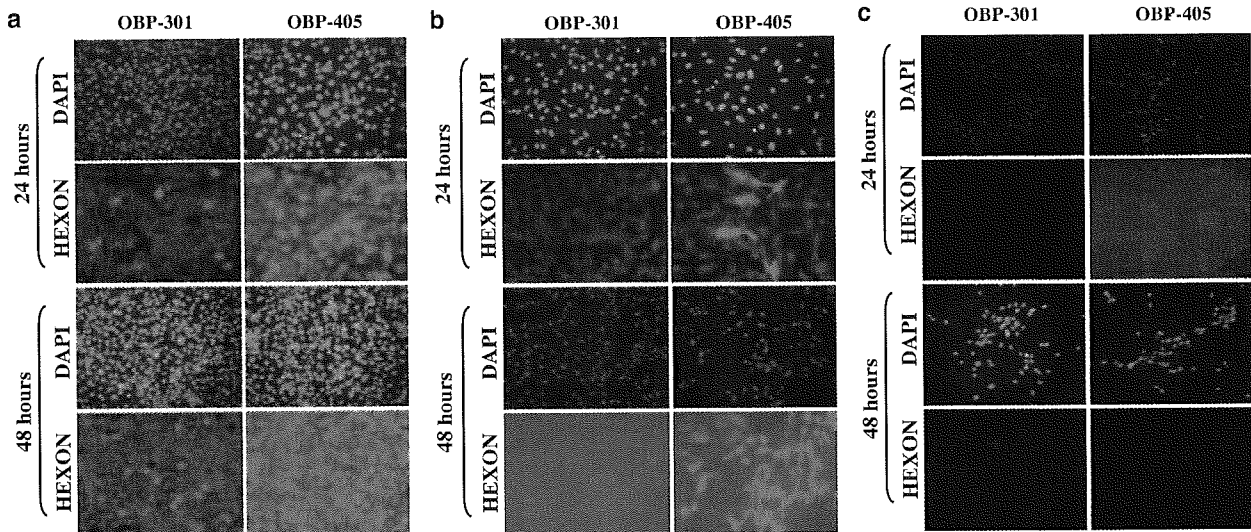
**Figure 3** *In vitro* virus spread in CAR-positive H1299 (a), CAR-negative LN444 (b), and NHLF (c) cells. Cells cultured in chamber slides were infected with OBP-301 or OBP-405 at an MOI of 1. Cells stained with FITC-labeled goat anti-hexon antibody to monitor the replication of viruses 24 and 48 h after infection are shown. Cell nuclei were counterstained with DAPI. Virus replication was assessed with fluorescence microscopy, and the blue and green fluorescence correspond to cell nuclei and adenovirus hexon, respectively. Original magnification, ×200

H1299-R5 tumor treated with OBP-405 had completely disappeared, and the level of E1A copy number of OBP-301 was almost consistent with that at 14 day postinfection. These results suggest that OBP-405 could more efficiently replicate in both injected and uninjected tumors, when CAR-negative H1299-R tumors were treated. Notably, no E1A DNA could be detected in the blood of mice treated with OBP-301 or OBP-405, indicating that viral replication in tumors dose not correlate with the level of viruses in the blood circulation (Figure 7a and b).

Moreover, we histologically confirmed a profound replication of OBP-405 in untreated H1299-R5 tumors. As shown in Figure 7c, immunohistochemical analysis for the detection of adenoviral hexon demonstrated that the percentage of positive-staining cells was apparently higher in uninjected tumors of OBP-405-treated mice than those of OBP-301-treated mice. Hematoxylin/eosin analysis revealed apparent tumor cell death at the central portions of the tumors; morphological changes, however, that are associated with the apoptotic phenotype such as nuclear fragmentation and chromatin condensation were not evident (data not shown), suggesting that oncolysis by viral replication might be nonapoptotic cell death.

## Discussion

Viral replication generally results in tissue destruction. Oncolytic viruses have been developed as an anticancer agent, because controlled replication in the tumors causes selective killing of tumor cells and minimizes the undesired effects on normal cells (Kirn et al., 2001). Amplified viruses can infect adjacent tumor cells as well as reach distant metastatic tumors with the blood circulation. Therefore, oncolytic viruses can amplify the administered dose as a result of *in vivo* viral replication. This might be one of the potential advantages of oncolytic viruses compared with conventional cancer therapies. We previously reported that hTERT promoter-specific replication-competent adenovirus OBP-301 could replicate and eventually lyse the telomerase-expressing tumors cells, leading to the viral spread to adjacent cells (Kawashima et al., 2004). OBP-301 could infect both normal and tumor cells, but the virus would only replicate in those cells that have robust telomerase activity. OBP-301 induced oncolysis in a variety of human cancer cell lines; tumors that lost CAR expression, however, might be refractory to infection with OBP-301, because subgroup C adenoviruses, including serotypes 2 and 5, rely on CAR as the primary binding

**Figure 4** Oncolytic effect of OBP-301 and OBP-405 *in vitro* on human cancer and normal cell lines. (a) CAR-positive (H1299 and SW620) and CAR-negative (LN444, LNZ308, and H1299-R5) cell lines and normal cells (NHLF) were stained with Coomassie brilliant blue 5 day after infection with OBP-301 or OBP-405. Blue areas indicate viable cells; white areas show loss of cells through cell lysis. (b) Cells were infected with OBP-301 or OBP-405 at the indicated MOI values, and surviving cells were quantitated over 5 days by XTT assay. Statistical analysis was performed using Student's *t*-test for differences among groups. Statistical significance (*) was defined as $P < 0.01$
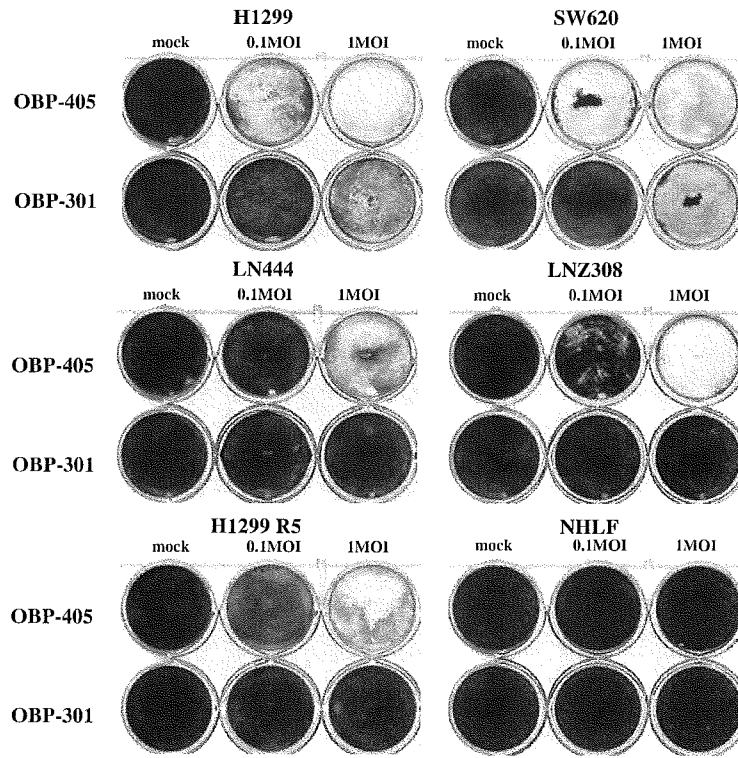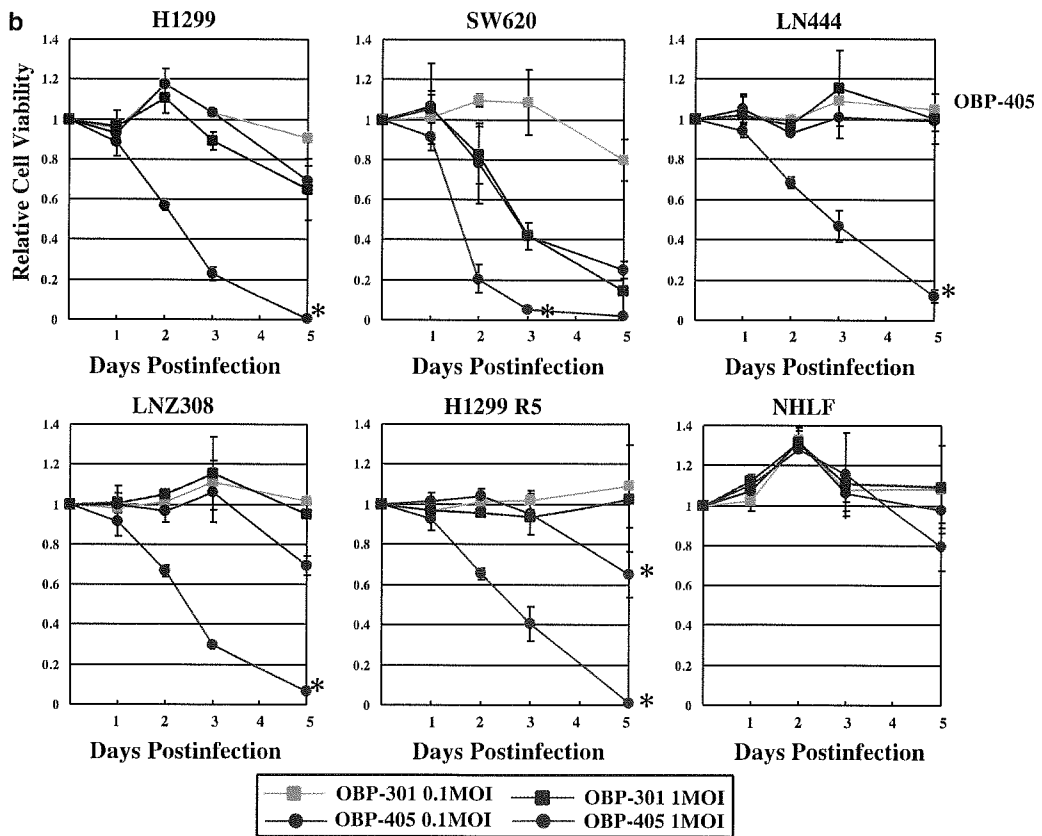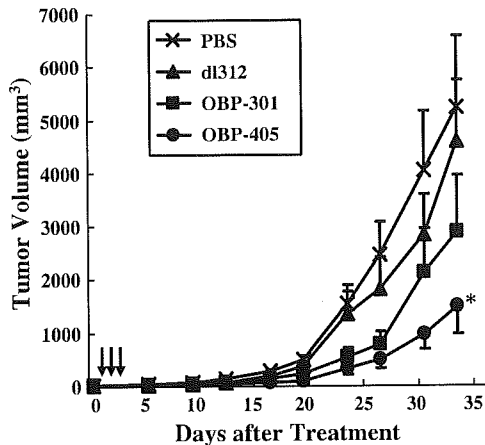
**Figure 5** Antitumor effects of intratumorally injected OBP-301 or OBP-405 against established flank H1299-R5 xenograft tumors in *nu/nu* mice. PBS and replication-deficient dl312 were used as a control. Six mice were used for each group. The tumor growth was expressed by the tumor mean volume ±s.e. Statistical significance (*) was defined as $P<0.01$ (Student's *t*-test)

site on the target cells (Bergelson *et al.*, 1997). In fact, CAR deficiency in primary tumors has been reported (Miller *et al.*, 1998; Li *et al.*, 1999). Here, we demonstrate that the modification of the adenovirus fiber knob by the addition of an RGD-containing peptide in the HI loop increased its infectious efficiency and enabled the virus to kill CAR-negative tumor cells. The fiber-modified new oncolytic adenovirus OBP-405 was more effective to inhibit the growth of CAR-negative tumors *in vivo*, comprised of OBP-301.

A wide spectrum of CAR levels exists among many types of human cancer lines (Figure 1), although the regulation as well as the function of this transmembrane protein are poorly understood. If expression of CAR on target cells could be increased, this could potentially yield improved efficacy of adenovirus-based therapies. We previously established H1299-R5 human lung cancer cell line refractory to adenovirus infection by five-time repeated infections (Tango *et al.*, 2004). The observation that CAR expression markedly diminished as the cells are repeatedly infected with adenovirus suggests that the levels of CAR expression could be altered. Indeed, It has been reported that the chemotherapeutic agents are effective in increasing CAR expression (Hemminki *et al.*, 2003); in our preliminary experiments, however, CAR expression could not be modified in H1299-R5 cells by any chemotherapeutic agents tested, including the EGF receptor-tyrosine kinase inhibitor ZD1839 (Gefitinib, 'Iressa') (data not shown). Therefore, it seems to be difficult to consistently upregulate CAR expression in various types of human cancer cells.

A variety of strategies have been devised to increase adenovirus infection to cells with low or absent CAR. For our study, we have chosen to alter the tropism of oncolytic virus by the modification of the fiber. Making the fiber-modified oncolytic adenovirus, we supposed that the virus could infect not only by CAR-dependent

entry but also by CAR-independent, RGD-integrin ($\alpha v\beta3$ and $\alpha v\beta5$)-dependent entry. As expected, OBP-405 was taken up efficiently by both CAR-positive and CAR-negative human cancer cells; the infectivity of OBP-405 was 10- and 1000-fold higher in CAR-positive and CAR-negative human cancer cell lines, respectively, than that of OBP-301 (Figure 2a). In contrast, the replication yields of OBP-301 and OBP-405 were persistent in both cell lines (Figure 2b), indicating that the tropism modification is not anticipated to alter fundamental aspects of the viral replication cycle. The increased initial virus entry into the cells results in earlier detection (data not shown) and augmented yields of OBP-405 compared with unmodified OBP-301 (Figure 3). Enhancing the infection efficiency of OBP-405 translated into increased oncolytic effects (Figure 4). OBP-301 showed complete oncolysis at as low as 1 MOI in H299 and SW620 cells, suggesting that OBP-301 is sufficient to treat CAR-positive human cancer cells; OBP-301, however, could not kill CAR-negative cell lines at all. Notably, OBP-405 did effectively kill LN444, LNZ308, and H1299-R5 cells at an MOI of 1, indicating that the infection enhancement of OBP-405 contributed to its efficacy on CAR-negative cancer cells. Another important finding is that OBP-405 elicited no increased infectivity as well as cytopathic effect to normal cells despite of CAR expression (Figures 3 and 4).

We also demonstrated the superior oncolytic effect of OBP-405 in the subcutaneous xenograft model of CAR-negative H1299-R5 cells. Intratumoral injection of OBP-405 for three consecutive days resulted in the significant inhibition of H1299-R5 tumor growth (Figure 5) and selective spread of viruses throughout the tumor tissues (Figure 6b). Although the RGD fiber knob modification of selectively replicating adenoviruses, such as AdΔ24 containing the Rb-binding mutation in E1A (Lamfers *et al.*, 2002) and the cyclooxygenase-2 (Cox-2) promoter-based adenovirus (Davydova *et al.*, 2004), has been previously reported to reduce tumor size *in vivo*, the major advantage of OBP-405 is the broad applicability for many types of human cancers because of the telomerase-specific hTERT promoter. In fact, many studies have reported that telomerase is present in nearly all immortal cell lines and ~90% of human tumors but seldom in normal somatic cells (Kim *et al.*, 1994; Shay and Wright, 1996). In addition to the antitumor effect, when the tropism of the virus is modified, it has to be addressed whether a pattern of biodistribution could be affected. We observed that OBP-405 showed a tumor-restricted pattern of biodistribution in mice after intratumoral administration (Figure 6a) and no hepatotoxicity despite of high levels of CAR and $\alpha v$ integrin expression in the liver (Tomko *et al.*, 1997; Fechner *et al.*, 2000) (Table 1). Viral replication and spread of OBP-405 could be detected at least for 4 weeks (Figure 7b), whereas OBP-405 was negative in any normal specimen throughout the period (data not shown). A limitation of our biodistribution data is that the hTERT promoter is not expected to function in mice as it does in humans. Indeed, some studies have reported that mouse and rat tumors do not support efficient replication of human
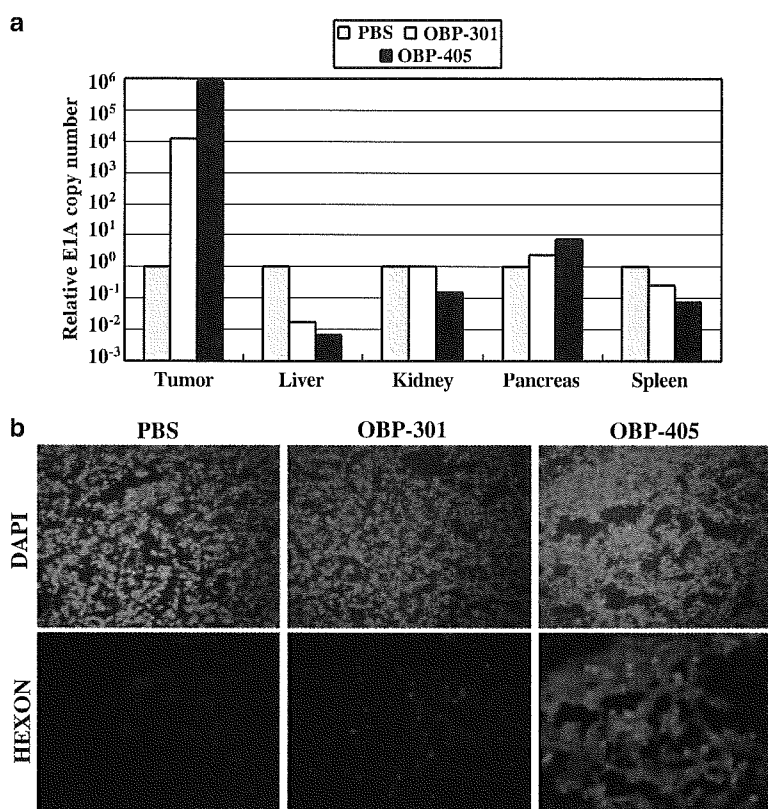
**a**



**b**



Figure 6 Spread and replication of OBP-301 or OBP-405 following intratumoral administration in *nu/nu* mice transplanted with H1299-R5 tumor cells. **(a)** DNA was extracted from subcutaneous tumor and various tissues in *nu/nu* mice at 7 day postinfection. Viral DNA was detected by quantitative PCR amplification of the adenoviral E1A sequence. The amounts of viral E1A copy number are defined as the fold increase for each sample relative to that with PBS (PBS equals 1). The results are representative of three separate experiments. **(b)** Sections of H1299-R5 tumors were immunofluorescently stained with anti-hexon antibody 7 days after virus injection, followed by counterstaining with DAPI. Magnification, × 200

Table 1 Assessment of hepatotoxicity in *nu/nu* mice intratumorally injected with OBP-301 or OBP-405

| | T-Bil (mg/dl) | AST (IU/l) | ALT (IU/l) | LDH (IU/l) | GGT (IU/l) | ALP (IU/l) |
|---|---|---|---|---|---|---|
| PBS | 0.4 | 87 | 31 | 258 | <10 | 475 |
| OBP-301 | 0.3 | 118 | 40 | 388 | <10 | 382 |
| OBP-405 | 0.4 | 69 | 27 | 190 | <10 | 492 |

Blood was obtained from H1299-R5 tumor-bearing mice 7 days after intratumoral injection of PBS or $1 \times 10^7$ PFU of OBP-301 or OBP-405, and the levels of liver enzymes were analysed

adenoviruses (Ginsberg *et al.*, 1991; Prince *et al.*, 1993). However, as preliminary data, we confirmed that OBP-405 could infect and efficiently lyse murine adenocarcinoma cell line Colon-26 (data not shown). Therefore, OBP-405 is considered to be specific and safe within its therapeutic window.

To treat distant, metastatic tumors, an infusion of chemotherapeutic drugs by intravenous administration will need to distribute a sufficient quantity of agents to the tumor sites; oncolytic viruses, however, could replicate in the tumor, cause oncolysis, and then release

virus particles that will reach to the distant metastatic lesions. Therefore, intratumoral administration that causes the release of newly formed virus from infected tumor cells might be theoretically suitable for oncolytic virus rather than systemic administration. OBP-405 cleared rapidly from the body after intravenous administration (data not shown). This is one of the reasons why we also used intratumoral injection of OBP-405 for the toxicity analysis. In fact, a phase I clinical study demonstrated PSA-specific oncolytic virus shedding in the blood after intraprostatic delivery (DeWeese *et al.*,
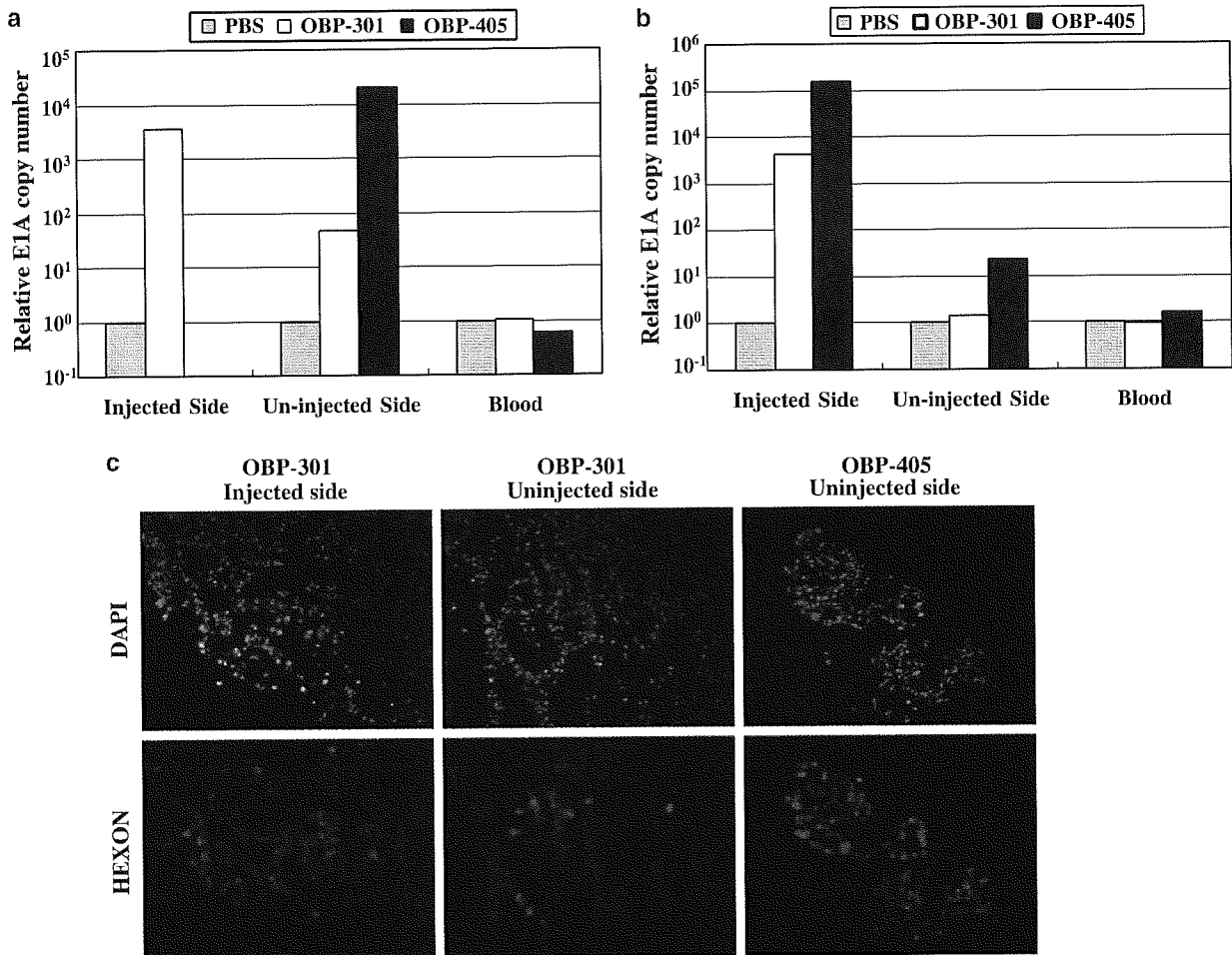
**Figure 7** Replication of OBP-301 and OBP-405 in the tumor distant from the site of virus inoculation. Bilateral H1299-R5 tumors were implanted in nu/nu mice, and the right-sided tumors received intratumoral injection of PBS or $1 \times 10^7$ PFU of OBP-301 or OBP-405. The results are representative of three separate experiments. (a, b) Quantitative real-time PCR amplification of the E1A gene with DNA isolated from the right (injected) and left (uninjected) H1299-R5 tumors at 2 weeks (a) and 1 month (b) post-treatment. (c) Sections of H1299-R5 tumors were immunofluorescently stained with anti-hexon antibody 1 month after virus injection, followed by counterstaining with DAPI. Magnification, × 200

2001). These findings indicate that the intratumorally administered virus that reached the circulation could potentially replicate and lyse metastatic tumors. We observed evidence of OBP-405 replication in the distant, uninjected H1299-R5 tumors after its intratumoral administration into the contralateral tumors by quantification of virus DNA (Figure 7). Moreover, OBP-405 continued to replicate in the distant tumors even after the injected tumors disappeared, although the presence of OBP-405 in the blood circulation could not be detected over time. One possible explanation for this result is that the amount of OBP-405 in the circulation might be quite small due to its short half-life (approximately 2 min) (Huard et al., 1995; Wood et al., 1999), but sufficient to initiate replication once it reached the distant tumors.

In conclusion, we demonstrate that the fiber-modified telomerase-specific replication-selective adenovirus OBP-405 permits CAR-independent cell entry and

effective destruction of tumors lacking the primary CAR. The feasibility of original OBP-301 (Telomelysin) for human cancer therapy will be confirmed in clinical trials in the near future; some CAR-negative tumors, however, may be refractory to OBP-301. Under such circumstances, OBP-405 is a powerful way of overcoming low infectivity and increasing antitumor activity. Our data may be consequential for the development of virotherapy for human cancers.

## Materials and methods

### Cells and culture conditions

The H1299 and H1299-R5 human non-small-cell lung cancer cell lines and the SW620 human colon cancer cell line were cultured in RPMI 1640 medium supplemented with 10% FCS. H1299-R5 is a subline of H1299 that are refractory to adenovirus infection due to the decreased CAR expression

(Tango et al., 2004). The human glioma cell lines LN444 and LNZ308 (kindly provided by Dr N Ishi, Hokkaido University, Hokkaido, Japan), and the transformed embryonic kidney cell line 293 were cultured in DMEM containing high glucose (4.5 g/l) and supplemented with 10% FCS. The normal human lung fibroblast cell line NHLF was purchased from TaKaRa Biomedicals (Kyoto, Japan) and cultured in the medium recommended by the manufacturer.

### Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 ('Telomelysin') was previously constructed and characterized (Kawashima et al., 2004; Umeoka et al., 2004). OBP-405 ('Telomelysin-RGD') that has mutant fiber containing the RGD peptide, CDCRGDCFC, in the HI loop of the fiber knob was created using the method developed by Mizuguchi et al. (2001). OBP-301 and OBP-405 viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titer and infectious titer was accomplished spectrophotometrically by the method of Maizel et al. (1968) and by the method of Kanegae et al. (1994), respectively.

### Flow cytometry

The cells ($2 \times 10^5$ cells) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology, NY, USA), anti-human integrin $\alpha v \beta 3$ (LM609; Chemicon International, Temecula, CA, USA), or anti-human integrin $\alpha v \beta 5$ (P1F6; Chemicon International, Temecula, CA, USA). Then, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco, USA) and analysed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA).

### Quantitative real-time PCR assay

DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA), and quantitative real-time PCR assay for the E1A gene was performed using a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of specific primers used for E1A were as follows: sense: 5'-CCT GTG TCT AGA GAA TGC AA-3' and antisense: 5'-ACA GCT CAA GTC CAA AGG TT-3'). PCR amplification began with a 600-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 8 s. Data analysis was performed using LightCycler Software (Roche Molecular Biochemicals). The ratios normalized by dividing the value of untreated cells were presented for each sample.

### Viral spread assay

H1299, LN444, and NHLF were cultured in two-well chamber slides and infected with OBP-301 or OBP-405 at an MOI of 1. The slides were fixed with 4% paraformaldehyde 24 or 48 h after infection, blocked, incubated with FITC-labeled goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc., Temecula, CA, USA), and counterstained with DAPI (1 μg/ml; Molecular Probes, Eugene, OR, USA). The slides were photographed under the fluorescence microscopy and then analysed using the software (Viewfinder; Pixera, CA, USA).

### Cell killing assay

Cells were plated at 100 000 cells/well on 12-well plates and infected either with OBP-301 or OBP-405 at an MOI of 0, 0.1, or 1 for 2 h. The medium with 10% FBS was then added following the removal of viruses. Coomassie brilliant blue staining was performed on day 5.

### Cell viability assay

An XTT assay was performed to measure cell viability. Cells were plated on 96-well plates at $5 \times 10^3$/well, 24 h before infection and infected either with OBP-301 or OBP-405 at an MOI of 0, 0.1, or 1. Cell viability was determined at the indicated times by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the manufacturer's protocol.

### In vivo human tumor model

Human lung cancer H1299-R5 cells ($1 \times 10^7$ cells/mouse) were subcutaneously injected into the flank of 5–6-week-old female BALB/c nu/nu mice and permitted to grow to approximately 5–6 mm in diameter. At that time, the mice were randomly assigned into four groups, and a 100 μl solution containing $1 \times 10^7$ PFU of dl312, OBP-301, or OBP-405, or PBS was injected into the tumor on days 1, 2, and 3. Tumors were measured for perpendicular diameters every 3 or 4 days, and tumor volume was calculated using the following formula: tumor volume $(mm^3) = a \times b^2 \times 0.5$, where $a$ is the longest diameter, $b$ is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry.

### In vivo toxicity study

Mice bearing H299-R5 tumors received intratumoral injection of $1 \times 10^7$ PFU of OBP-301 or OBP-405, or PBS. At 1 week after treatment, blood samples were obtained and the serum levels of total bilirubin (T-Bil), aspartate amino transferase (AST), alanine amino transferase (ALT), lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were determined by automated colorimetric assays to assess the hepatotoxicity.

### In vivo viral replication

OBP-301 or OBP-405 at $1 \times 10^7$ PFU/100 μl, or PBS were intratumorally injected into H1299-R5-bearing mice. After 1 week, the tumors and organs were harvested and DNA was extracted from each tissue. To compare the viral replication in the tumor and other normal organs, quantitative real-time PCR for the E1A gene was performed with a LightCycler instrument. The tumors and organs were immediately embedded in Tissue Tek (Sakura, Tokyo, Japan), cut into 5 μm-thick sections, and assessed by immunofluorescence detection of the adenoviral hexon protein using a goat anti-hexon polyclonal antibody (Chemicon, Temecula, CA, USA). To assess the viral replication on distant, uninjected tumors, H1299-R5 cells ($1 \times 10^7$ cells/mouse) were injected subcutaneously into bilateral flanks of mice. At 2 weeks or 1 month after intratumoral inoculation of OBP-301 or OBP-405 at $1 \times 10^7$ PFU/100 μl into tumors in the right flank, the bilateral tumors and blood were collected from mice and DNA was extracted. Quantitative real-time PCR as well as immunofluorescence staining for the hexon protein were performed.

*Statistical analysis*

Determinations of significant differences among groups were assessed by calculating the value of Student's *t* using the original data analysis.

## Abbreviations

hTERT, human telomerase reverse transcriptase; IRES, internal ribosome entry site; CAR, Coxsackie-adenovirus receptor; NHLF, normal human lung fibroblasts; MOI, multiplicity of infection; PFU, plaque-forming units.

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