Development of optimized AAV3 serotype vectors: mechanism of high-efficiency transduction of human liver cancer cells

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Our recent studies have revealed that among the 10 different commonly used adeno-associated virus (AAV) serotypes, AAV3 vectors transduce human liver cancer cells extremely efficiently because these cells express high levels of human hepatocyte growth factor receptor (hHGFR), and AAV3 utilizes hHGFR as a cellular co-receptor for viral entry. In this report, we provide further evidence that both extracellular as well as intracellular kinase domains of hHGFR are involved in AAV3 vector entry and AAV3-mediated transgene expression. We also document that AAV3 vectors are targeted for degradation by the host cell proteasome machinery, and that site-directed mutagenesis of surface-exposed tyrosine (Y) to phenylalanine (F) residues on AAV3 capsids significantly improves the transduction efficiency of Y701F, Y705F and Y731F mutant AAV3 vectors. The transduction efficiency of the Y705+Y731F double-mutant vector is significantly higher than each of the single mutants in liver cancer cells in vitro. In immunodeficient mouse xenograft models, direct intratumoral injection of AAV3 vectors also led to high-efficiency transduction of human liver tumor cells in vivo. We also document here that the optimized tyrosine-mutant AAV3 vectors lead to increased transduction efficiency following both intratumoral and tail-vein injections in vivo. The optimized tyrosine-mutant AAV3 serotype vectors containing proapoptotic genes should prove useful for the potential gene therapy of human liver cancers.

Keywords: AAV vectors; tyrosine mutants; human hepatocyte growth factor receptor; human liver cancer

INTRODUCTION
Adeno-associated virus 2 (AAV2), a non-pathogenic human parvovirus, contains a single-stranded DNA genome and possesses a wide tissue tropism that transcends the species barrier. Recombinant AAV2 vectors have gained attention as a promising vector system for the potential gene therapy of a variety of human diseases, and are currently in use in a number of gene therapy clinical trials. More recently, several additional AAV serotypes have been isolated, and have been shown to transduce specific cell types efficiently. Although various steps in the life cycle of AAV2 are reasonably well understood, less is known about the other serotypes.

Of the 10 commonly used AAV serotypes, AAV3 has been reported to transduce cells and tissues poorly. However, recent studies from our laboratory revealed that AAV3 vectors transduce established human hepatoblastoma (HB) and human hepatocellular carcinoma (HCC) cell lines, as well as primary human hepatocytes, extremely efficiently. Subsequently, we documented that AAV3 infection was strongly inhibited by hepatocyte growth factor (HGF), HGF receptor (HGFR)-specific siRNA, and anti-HGFR antibody, which suggested that AAV3 utilizes HGFR as a cellular receptor/co-receptor for viral entry. The precise underlying molecular mechanisms of HGFR-mediated viral entry as well as additional steps in the life cycle of AAV3 remain unexplored.

Others and we have previously reported that the ubiquitin–proteasome pathway has a crucial role in intracellular trafficking of AAV vectors. We have also reported that intact AAV2 capsids can be phosphorylated at tyrosine residues by epidermal growth factor receptor protein tyrosine kinase (EGFR–PTK), and that tyrosine phosphorylation of AAV capsids negatively affects viral intracellular trafficking and transgene expression. These observations led to the suggestion that tyrosine phosphorylation is a signal for ubiquitination of AAV capsids, followed by proteasome-mediated degradation.

This led to the hypothesis that mutations of the surface-exposed tyrosine residues (Y) to phenylalanine (F) might allow the vectors to...
evade phosphorylation, ubiquitination and proteasome-mediated degradation. Indeed, mutations of the surface-exposed tyrosine residues in AAV2 vectors led to high-efficiency transduction at lower doses both in HeLa cells in vitro and murine hepatocytes in vivo.\textsuperscript{34} Therapeutic levels of expression of human factor IX have been obtained in several different strains of mice using the single and multiple tyrosine-mutant AAV2 vectors.\textsuperscript{34,35} Additional studies have corroborated that similar tyrosine–phenylalanine (Y–F) mutations in AAV serotypes 6, 8 and 9 also lead to augmented transgene expression.\textsuperscript{36–38} Six of seven surface-exposed tyrosine residues in AAV2 are also conserved in AAV3, but their involvement in AAV3-mediated transduction has not been evaluated.

In this study, we report that: (i) AAV3 vector-mediated transduction is dramatically increased in T47D cells, a human breast cancer cell line that expresses undetectable levels of the endogenous human HGFR (hHGFR),\textsuperscript{39} following stable transfection and overexpression of hHGFR; (ii) the tyrosine kinase activity associated with hHGFR negatively affects the transduction efficiency of AAV3 vectors; (iii) the use of proteasome inhibitors significantly improves AAV3 vector-mediated transduction; (iv) site-directed mutagenesis of two surface-exposed tyrosine residues on the AAV3 capsid leads to improved transduction efficiency; (v) a specific combination of two tyrosine mutations further improves the extent of transgene expression; and (vi) AAV3 vectors efficiently transduce human HB and HCC tumors in a murine xenograft model in vivo, following both intratumoral or systemic administration. These optimized AAV3 vectors may be useful for the potential gene therapy of liver cancer in humans.

RESULTS

Human HGFR is required for AAV3 infectivity

We recently provided preliminary evidence that AAV3 utilizes hHGFR as a cellular co-receptor.\textsuperscript{50} To unequivocally corroborate this contention, we used a human breast cancer cell line T47D, which expresses undetectable levels of endogenous hHGFR,\textsuperscript{39} as well as T47D cells stably transfected with a hHGFR expression plasmids (T47D+hHGFR).\textsuperscript{39} The expression of hHGFR protein in the established cell line T47D+hHGFR was confirmed by western blot analysis (data shown below in Figure 2c). Equivalent numbers of T47D and T47D+hHGFR cells were transduced with various multiplicities of infection of self-complementary (sc) AAV3–chicken β-actin promoter (CBAP)–enhanced green fluorescence protein (EGFP) vectors under identical conditions, and transgene expression was determined at 72 h after transduction. These results, shown in Figure 1a, document that the transduction efficiency of AAV3 vectors is approximately 8- to 13-fold higher in cells that express hHGFR than those that do not. AAV3 vector-mediated transduction of T47D+hHGFR cells could be completely blocked in the presence of 5 μg ml\textsuperscript{–1} of hHGFR (Figure 1b). Taken together, these data provide conclusive evidence that cell surface expression of hHGFR is required for successful transduction by AAV3 vectors.

Inhibition of HGFR PTK activity enhances the transduction efficiency of AAV3 vectors

We next wished to examine whether in addition to the extracellular domain, the intracellular domain of HGFR, which contains PTK activity, is also involved in AAV3 infection. Binding of its ligand HGF results in dimerization of the receptor and intermolecular transphosphorylation of multiple tyrosine residues in the intracellular domain.\textsuperscript{40} T47D+hHGFR cells were treated for 2 h with increasing concentrations of a specific HGFR kinase inhibitor, BMS-777607 (BMS),\textsuperscript{41,42} Cells were subsequently infected with scAAV3 vectors at 2000 vector genomes (vgs) per cell. These results are shown in Figure 2a. It is evident that BMS-777607 treatment led to approximately twofold increase in AAV3 transduction efficiency. Although the \( P \)-value was higher when BMS-777607 was used at the highest concentration of 10 μM, compared with the lower concentration of 1 μM, this change was most likely due to drug toxicity. In previous studies, it has been reported that BMS-777607 treatment had no significant effect on cell growth at doses \( \leq 1 \) μM. However, doses of 10 μM did result in significant reduction in cell proliferation, which suggests that this concentration is toxic to cells.\textsuperscript{42} In the next experiment, to rule out any possible non-specific nature of this drug, the parental T47D cells were included as a control. Both cell types were treated with 1 μM BMS-777607 for 2 h and then infected with scAAV3 vectors at 10 000 vgs per cell. The results, shown in Figure 2b, indicated that although BMS-777607 treatment significantly enhances AAV3 infectivity in T47D+hHGFR cells, it has no effect in T47D cells that lack the expression of hHGFR.

We also wished to examine whether inhibition of the HGFR kinase led to alterations in the phosphorylation status of specific cellular proteins involved in the downstream signaling pathway. Total and phosphorylation levels of the HGFR protein in both T47D and T47D+hHGFR lysates were determined following a 2-h drug-incubation period. Activation of signaling pathways downstream from HGFR

![Figure 1](image_url)
kinase, ERK1/2 and Akt, were analyzed using phosphorylation-specific antibodies. These results, shown in Figure 2c, confirmed that although little expression of hHGFR occurs in T47D cells, the level of expression is significantly higher in T47D+hHGFR cells for both total HGFR and phosphorylated HGFR, which is consistent with previously published reports.39 Treatment of T47D+hHGFR cells with BMS-777607 completely blocked the phosphorylation of HGFR, but not total HGFR. In addition, BMS-777607 treatment had no effect on the expression of phosphorylated Akt and ERK1/2. These results suggest that the enhancement of AAV3 vector infectivity by the BMS-777607 treatment is due to inhibition of HGFR kinase.

To date, only AAV2 has been reported to use hHGFR as a co-receptor.43 The roles of hHGFR and hHGFR kinase inhibitor on other AAV serotypes are not known. To rule out any non-specific enhancement of transduction by BMS-777607, other serotypes of AAV, which are not dependent on HGFR, as well as AAV2 vectors were compared for transduction efficiency following treatment of cells with BMS-777607. These results, shown in Figure 3, indicate that although AAV2 and AAV3 vectors can efficiently transduce T47D+hHGFR cells, other serotypes (AAV4–AAV9) can only transduce these cells at a very low efficiency. This result suggests that hHGFR is not involved in the life cycle of these AAV serotypes. Treatment of cells with BMS-777607 significantly increased the transduction efficiency of both the AAV2 and AAV3 vectors, but not the other AAV serotypes, which suggests that the effect of the BMS-777607 treatment is AAV serotype-specific.

Proteasome inhibitors increase the transduction efficiency of AAV3 vectors

Previous studies by others and us have shown that proteasome inhibitors, such as MG132, can significantly enhance the transduction efficiency of AAV2 vectors by facilitating intracellular trafficking.31,44 To evaluate whether MG132 can also improve AAV3 trafficking in target cells, Huh7, a well-established human HCC cell line,45 and Hep293TT, a recently established human HB cell line,46 were either mock treated or treated with increasing concentrations of MG132. Following a 2-h treatment, cells were infected with scAAV3–CBαp–EGFP vectors. HeLa cells, treated with 5 μM MG132 and transduced with scAAV2 vectors, were included as a positive control. Transgene expression was determined by fluorescence microscopy at 72 h after transduction. These data are shown in Figures 4a and b. As can be seen, pretreatment with MG132 significantly increased the transduction efficiency of scAAV2 vectors in HeLa cells, which is consistent with our previously published studies.31 Interestingly, a dose-dependent increase in the transduction efficiency of scAAV3 vectors in both Huh7 and Hep293TT cells occurred following MG132 treatment, suggesting that AAV3 vectors also undergo ubiquitination, followed by proteasome-mediated degradation.

Previous studies from our laboratory have also shown that inhibition of EGFR–PTK signaling by tyrphostin 23 (Tyr23), a specific inhibitor of EGFR–PTK,47 modulates the ubiquitin/proteasome pathway, which in turn facilitates intracellular trafficking and transgene expression mediated by AAV2 vectors.31 Hep293TT cells were mock treated or treated with Tyr23 for 2 h and transduced with scAAV3 vectors. HeLa cells, pretreated with Tyr23 and transduced with scAAV2 vectors, were included as an appropriate control. Transgene expression was determined at 72 h after transduction. These results, shown in Figures 4c and d, indicate that Tyr23 treatment led to a significant increase in the transduction efficiency of both scAAV2 and scAAV3 vectors. The increased transgene expression was independent.
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378 cells under identical conditions. As can be seen in Figure 5a, the extent compared with its wild-type (WT) AA V3 counterpart using Huh7 (F501Y). The transduction efficiency of the mutant vector was tested by generating a mutant AAV3 vector in which the reduction of the transduction efficiency of AAV3 vectors. This hypothesis reasoned that mutation of F501 back to a tyrosine residue would reduce the transduction efficiency of AAV3 vectors. These results further corroborate the involvement of the host cell ubiquitin/proteasome machinery in the life cycle of AAV3 vectors as well.

Site-directed mutagenesis of surface-exposed tyrosine residues significantly improves the transduction efficiency of scAAV3 vectors

Recent studies from our laboratory have shown that there are seven surface-exposed tyrosine residues (Y252, Y272, Y444, Y500, Y700, Y704 and Y730) on AAV2 capsids that are phosphorylated by EGFR–PTK and negatively affect the transduction efficiency of AAV2 vectors. Alignment of amino-acid sequences from AAV2 and AAV3 capsids indicated that six of these tyrosine residues (Y252, Y272, Y444, Y701, Y705, and Y731) are conserved in AAV3 capsid (Table 1). One tyrosine residue, Y500 in AAV2, is present as F501 in AAV3. As we and others have shown that Y–F mutations in several AAV serotypes enhance transgene expression by circumventing ubiquitination and proteasome-mediated degradation, we reasoned that mutation of F501 back to a tyrosine residue would reduce the transduction efficiency of AAV3 vectors. This hypothesis was tested by generating a mutant AAV3 vector in which the phenylalanine residue was substituted with a tyrosine residue (F501Y). The transduction efficiency of the mutant vector was compared with its wild-type (WT) AAV3 counterpart using Huh7 cells under identical conditions. As can be seen in Figure 5a, the extent of the transgene expression mediated by the F501Y mutant vector was reduced by ~50% compared with the WT AAV3 vector.

To further test our hypothesis that tyrosine mutations on AAV3 capsids would lead to decreased EGFR–PTK-mediated phosphorylation, followed by reduced ubiquitination and impaired proteasome-mediated degradation resulting in increased transgene expression, we modified all six surface-exposed tyrosine residues on AAV3 capsids and substituted with phenylalanine residues (Y–F). Each of the single tyrosine-mutant vectors encapsidating scAAV2–CBAp–EGFP genomes could be successfully packaged. Vector titers for each of the mutants were determined by both quantitative DNA slot blots and quantitative PCR, and no significant differences in the packaging efficiency were observed (data not shown). The transduction efficiency of each of the tyrosine-mutant vectors was analyzed and compared with the WT scAAV3–CBAp–EGFP vector in both Huh7 (Figure 5b) and Hep293TT (Figure 5c) cells under identical conditions. From these results, it is evident that the transduction efficiency of the double and triple-mutant vectors (Y701F, Y705F and Y731F) is significantly higher compared with the WT scAAV3 vector. Specifically, the transduction efficiency of Y731F vector was approximately eightfold higher than the WT vector, followed by Y705F (approximately threefold) and Y701F (approximately twofold) vectors.

Multiple mutations in surface-exposed tyrosine residues further improve the transduction efficiency of AAV3 vectors

In our recently published studies with Y–F mutant AAV2 vectors, we observed that specific combinations of the most efficient single mutations of surface-exposed tyrosine residues further augmented the transduction efficiency of AAV2 vectors. To examine whether a similar enhancement could be achieved with AAV3 vectors, we generated the following double- and triple-mutant AAV3 vectors: Y701F+731F, Y705F+731F and Y731F. Each of these mutant vectors was packaged to similar titers, as determined by both quantitative DNA slot blots and quantitative PCR (data not shown). The transduction efficiency of these multiple mutants was compared with the WT and the Y731F single-mutant AAV3 vectors in Huh7 cells under identical conditions. These results are shown in Figure 6a. As can be seen, although the Y731F mutation significantly increased the transduction efficiency of AAV3 vectors, as observed before, only one of the double mutations (Y705F+731F) led to an additional significant increase in transgene expression. Interestingly, the transduction efficiency of both the double-mutant (Y701F+731F) and the triple-mutant (Y701F+705F+731F) vectors was reduced to levels similar to that of the WT AAV3 vector. The best performing single and multiple tyrosine mutants on human liver cancer cells were then evaluated for transduction of T47D and T74D+hHGFR cells (Figure 6b). Similar to human liver cancer cells, the tyrosine-mutant rAAV3 vectors led to high-efficiency transduction of both cell types, with or without hHGFR expression.

To examine the possibility whether the observed enhanced transduction efficiency of the Y–F mutant vectors was due to the involvement of one or more additional putative cellular receptor/co-receptor functions, the WT, Y731F and Y705F+731F mutant scAAV3–CBAp–EGFP vectors were used to transduce Huh7 cells in the absence or the presence of 5 μg ml⁻¹ hHGFR under identical conditions. These results are shown in Figure 6c. As is evident, the presence of hHGFR dramatically inhibited the transduction efficiency and transgene expression of all the three AAV3 vectors, which is consistent with the interpretation that the tyrosine-mutant vectors also utilize hHGFR as a cellular receptor/co-receptor for viral entry.

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AAV3 vectors transduce human liver tumors in murine xenograft models in vivo

It was important to obtain proof of principle whether AAV3 vectors could also transduce human HB and HCC tumors in a xenograft mouse model in vivo. To this end, ~5×10⁸ HCC (Huh7) or HB (Hep293TT) cells were injected subcutaneously in non-obese diabetic (NOD)/severe combined immunodeficiency disease gamma (NSG) mice. Four weeks later, when tumors were clearly visible and palpable in both groups of animals, ~2×10⁸ vgs of scAAV3–CBAP–EGFP vectors were injected directly into tumors. Four days after vector injections, tumors were excised, and thin sections were examined under a fluorescence microscope. These results, shown in Figure 7, indicate that AAV3 provides an effective means to transduce both human HCC (Figure 7a) and HB (Figure 7b) tumors in vivo.

Consistent with the in vitro data, the transduction efficiency of AAV3 vectors was higher in Hep293TT cell-derived tumors than that in Huh7 cell-derived tumors.

Optimized tyrosine-mutant AAV3 vectors are highly efficient in transducing human liver tumors in a murine xenograft model in vivo

Next, the best performing double tyrosine-mutant AAV3 vectors were further evaluated in vivo for xenograft human liver tumor gene transfer. In the first set of experiments, ~5×10¹⁰ vgs of either the WT scAAV3– or Y705+731F–AAV3–CBAP–EGFP vectors were intratumorally injected in NSG mice bearing human HB (Hep293TT) tumors. Four days after vector injections, tumors were excised, and thin sections were examined under a fluorescence microscope (Figure 7c). As can be seen, tumors injected with the WT AAV3 vectors exhibited detectable levels expression of EGFP. The transduction efficiency of the optimized tyrosine-mutant AAV3 vectors (Figure 8c), once again, was significantly higher compared with the WT AAV3 vectors, which is consistent with our in vitro data.

In the second set of experiments, ~5×10¹¹ vgs of either the WT scAAV3– or the Y705+731F–scAAV3–CBAP–EGFP vectors were injected via the tail vein in NSG mice bearing human HB (Hep293TT) tumors. Phosphate-buffered saline injections were used as an appropriate control. As can be seen in Figure 8, although little transgene expression occurred in tumors from mice injected with phosphate-buffered saline (Figure 8a), direct tumor targeting could be achieved following systemic administration of AAV3 vectors. The transduction efficiency of the optimized tyrosine-mutant AAV3 vectors (Figure 8c), once again, was significantly higher than that of the WT AAV3 vectors (Figure 8b). These data suggest that the observed increased transduction
efficiency of tyrosine-mutant AAV3 vectors is independent of viral administration route. Additional studies are warranted with the Y705+731F mutant scAAV3 vectors containing a therapeutic gene to further evaluate their safety and efficacy for the potential gene therapy of HB and HCC.

**DISCUSSION**

Recombinant vectors based on AAV serotype 2 are currently in use in a number of gene therapy clinical trials, and have recently shown remarkable efficacy in the treatment of Leber's congenital amaurosis. However, concerns have been raised with reference to the...
humoral response to AAV2 vectors based on the high prevalence of seropositivity in the general population (approximately 80–90%). The discovery of many novel AAV serotypes has prompted the development of AAV vectors to circumvent this potential problem. For example, recombinant AAV8 vectors were recently reported to be therapeutic in a mouse model of liver cancer. However, several groups have described various strategies to target human liver cancer cells in murine models using AAV2 vectors. In our pursuit to identify the most efficient AAV serotype to target human liver cancer cells, we observed that three different human liver cancer cell lines could be transduced extremely efficiently by AAV3 vectors. We subsequently identified hHGFR as a cellular co-receptor for AAV3 infection. However, the precise role of hHGFR, especially the role of tyrosine kinase activity associated with the intracellular domain of hHGFR, in AAV3-mediated transduction remained unclear. Here, we present a more detailed account of the AAV3–hHGFR interactions, and describe the development of an optimal AAV3 vector for its potential use in targeting human liver cancer cells.

Figure 7 Transduction efficiency of AAV3 vectors in vivo following direct intratumoral injections. Transduction efficiency of WT AAV3 vectors in (a) Huh7- and (b) Hep293TT-derived tumors in NOD/severe combined immunodeficiency disease gamma (NSG) mice. Transduction efficiency of (c) WT- and (d) Y705+731F–AAV3 vectors in Hep293TT-derived tumors in NSG mice. EGFP fluorescence (green) and 4',6-diamidino-2-phenylindole staining (blue) of two representative tumor sections from each set of mice is shown.

Figure 8 Transduction efficiency of WT- and Y705+731F–AAV3 vectors in Hep293TT-derived tumors in NSG mice following tail-vein injections. EGFP fluorescence (green) and 4',6-diamidino-2-phenylindole staining (blue) of tumor in three representative tumor sections from each set of mice injected with (a) phosphate-buffered saline or (b) WT AAV3 or (c) Y705+731F–AAV3 vectors are shown.
HGFR is a transmembrane receptor tyrosine kinase, and binding of its ligand HGF results in dimerization of the receptor and inter-
molecular transphosphorylation of multiple tyrosine residues in the
intracellular domain.63 Although it is clear that AAV3 capsid interacts
with the extracellular domain of hHGFR, it is less clear whether AAV3
binding to hHGFR also triggers its activation and phosphorylation of
the downstream target proteins. Our data do indeed demonstrate that
suppression of the hHGFR–PTK activity leads to a modest increase in
AAV3 vector-mediated transgene expression. In this context, it is of
interest to note that the transduction efficiency of AAV3 vectors is
significantly higher in a more recently established human HB cell line
Hep293TT compared with that in a HB cell line Huh6, which was
established nearly three decades ago. Although subtle differences
might exist between the two cell lines, we have identified specific
mutations in the tyrosine kinase domain of hHGFR in Hep293TT
cells, which render it inactive, and that the hHGFR-specific kinase
inhibitor BMS-777607, which augments the transduction efficiency in
Huh6 cells, has little effect on AAV3 transduction efficiency in
Hep293TT cells (data not shown).

Despite the utilization of two distinct cellular growth factor
receptors as co-receptors by AAV2 (hFGFR1) and AAV3 (hHGFR),
the two serotypes appear to share certain post-receptor entry and
intracellular trafficking pathways. For example, both capsids become
phosphorylated at tyrosine residues by EGFR–PTK, presumably in
late endosomes, followed by ubiquitination, which leads to protea-
some-mediated degradation.35 However, although six of seven surface-
exposed tyrosines in AAV2 are conserved in AAV3, the patterns of
behavior of the corresponding Y–F mutants are somewhat divergent.
For example, Y730F (for AAV2) and Y731F (for AAV3) are the most
efficient simple mutants, followed by Y444F (for AAV2) and Y705F (for
AAV3); the transduction efficiency of Y444F (for AAV3) remains
unaltered. Similarly, although the transduction efficiency of the
Y730+444F double mutant (for AAV2) is not significantly different
from that of Y730F, the transduction efficiency of the Y705+731F
double mutant (for AAV3) is significantly higher than Y731F. Further-
more, the Y730+500+444F triple mutant (for AAV2) is the most
efficient, the Y731+501+705F triple mutant (for AAV3) is the most
efficient, the Y501 residue having already been mutated in the WT
AAV3 capsid. The molecular basis for these observed differences are
not readily apparent, and further studies are warranted to gain a better
understanding of the underlying molecular mechanisms. Interesting-
ly, even the WT AAV3 vectors were able to transduce human liver tumors
reasonably well in a mouse xenograft model in vivo following
intratumoral injection. However, we showed evidence that the
tyrosine-mutant vector resulted in higher gene transfer efficiency in
vivo. It is tempting to speculate that the use of the optimized tyrosine-
mutant AAV3 vectors containing a proapoptotic gene would lead to a
therapeutic effect in human liver tumors in vivo.

Human liver cancer, especially HCC, is one of the most aggressive
malignant tumors. The major obstacle to survival with HCC is
recurrence after HCC resection.64 Thus, transduction of 100% of
target cells is desirable to totally eliminate the tumor. In our previous
studies, we have observed that melittin, a toxic peptide derived from
bee venom, inhibits the viability and motility of HCC cells both
in vitro and in vivo via the suppression of Rac1-dependent pathway65
and upregulation of mitochondria membrane protein 7A6.66 We have
also demonstrated that melittin can induce apoptosis of HCC cells
potentially by activating CaMKII/TAK1/JNK/p38 signaling pathway.67
On the basis of our previous studies with recombinant adenovirus
vectors containing the melittin gene driven by a liver cancer cell-specific
promoter to achieve specific killing of liver cancer cells both

in vitro and in vivo,68 we now wish to develop optimized tyrosine-
mutant AAV3–melittin vectors under the control of a liver cancer
cell-specific promoter to selectively target both primary and metastatic
liver cancer.

MATERIALS AND METHODS

Cell lines and cultures

Human cervical cancer (HeLa) and HCC (Huh7) cell lines were purchased
from American Type Culture Collection (Manassas, VA, USA), and maintained
in complete Dulbecco’s modified Eagle’s medium (Mediatech Inc., Manassas,
VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-
Aldrich, St Louis, MO, USA), 1% penicillin and streptomycin (Lonza,
Walkersville, MD, USA). A newly established human HB (Hep293TT) cell
line69 was generously provided by Dr Gail E Tomlinson, University of Texas
Health Science Center at San Antonio (San Antonio, TX, USA), and was
maintained in complete RPMI medium 1640 (Invitrogen, Camarillo, CA, USA)
supplemented with 15% heat-inactivated fetal bovine serum (Sigma-Aldrich)
and 1% penicillin and streptomycin (Lonza). Cells were grown as adherent
cultures in a humidified atmosphere at 37 °C in 5% CO2 and were subcultured
after treatment with trypsin–versex mixture (Lonza) for 2–5 min at room
temperature, washed and resuspended in complete medium. A human breast
cancer cell line, T47D, and T47D cells stably transfected with a hHGFR
expression plasmid (T47D+hHGFR) were maintained in complete Dulbecco’s
modified Eagle’s medium (Mediatech Inc.), with or without 600 µg ml−1
of G418, supplemented with 10% heat-inactivated fetal bovine serum
(Sigma-Aldrich) and 1% penicillin and streptomycin (Lonza).

Recombinant AAV plasmids and vectors

Recombinant AAV3 packaging plasmid and recombinant AAV2–CBAP–EGFP
vector plasmid were generously provided by Drs R Jude Samulski and Xiao
Xiao, respectively, University of North Carolina (Chapel Hill, NC, USA). Highly
purified stocks of scAAV2 and scAAV3 vectors containing the EGFP gene driven
by the CBAP were packaged by the calcium phosphate triple-plasmid transfec-
tion protocol described previously.69,70 The physical particle titers of recombi-
nant vector stocks were determined by quantitative DNA slot-blot analyses.70

Construction of surface-exposed tyrosine residue mutant AAV3
capsid plasmids

A two-stage procedure, based on QuikChange II site-directed mutagenesis
(Stratagene, Santa Clara, CA, USA), was performed by using plasmid pAAV
as described previously.29,30 Briefly, in stage one, two PCR extension reactions
were performed in separate tubes for each mutant. One tube contained the
forward PCR primer and the other contained the reverse primer (Table 2).
In stage two, the two reactions were mixed and a standard PCR mutagenesis
assay was carried out according to the manufacturer’s instructions. PCR primers
were designed to introduce changes from tyrosine to phenylalanine residues
and a silent change to create a new restriction endonuclease site for screening
purposes (Table 2). All mutants were screened with the appropriate restriction
enzyme and were sequenced before use.

AAV vector transduction assays

Huh7 or HeLa cells were seeded in 96-well plates at a concentration of
5000 cells per well in complete Dulbecco’s modified Eagle’s medium.
AAV infections were performed in serum- and antibiotic-free Dulbecco’s
modified Eagle’s medium. Hep293TT cells were seeded in 96-well plates at
a concentration of 10000 cells per well in complete RPMI medium. The
infections were performed in serum- and antibiotic-free RPMI medium.
The expression of EGFP was analyzed by direct fluorescence imaging at 72 h after
transduction.

Western blot analyses

Cells were harvested and disrupted in a radioimmunoprecipitation assay lysis
buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25%
sodium deoxycholate and 1 mM EDTA with protease inhibitor cocktail, 1 mM
NaF and 1 mM Na2VO4). Total protein concentration was measured using a
Bradford reagent (Bio-Rad, Hercules, CA, USA) and equal amounts (50 µg) of
whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis. After electrophoresis, samples were electro-transferred to a nitrocellulose membrane (Bio-Rad), probed with relevant primary antibodies at 4 °C overnight, incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) and detected with an enhanced chemiluminescence substrate (Amersham, Piscataway, NJ, USA). Antibodies against phospho-c-Met (Y1234/1235), total c-Met, phospho-Akt (S473) and phospho-ERK (T202/Y204) were purchased from Cell Signaling (Danvers, MA, USA), and anti-β-actin (AC-74) antibody was obtained from Sigma (St Louis, MO, USA).

Recombinant AAV3 vector transduction studies in mouse xenograft models in vivo

Groups of 6-week-old NSG mice (Jackson Laboratories, Bar Harbor, ME, USA) were injected subcutaneously with 5 × 10⁶ Hep293TT or HuH7 cells. Four weeks after injection, indicated numbers of AAV3 vgs were administered either intratumorally or through tail vein. Four days after vector administration, tumors were resected, cross-sectioned and evaluated for EGFP expression using a fluorescent microscope. Sections were also stained with 4¢,6-diamidino-2-phenylindole to visualize the cell nucleus. All animal experiments were conducted in accordance with the University of Florida Institutional Animal Care and Use Committee guidelines.

Statistical analysis

Results are presented as mean ± s.d. Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student’s t-test. P-values <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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