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Marked increase in tumor transfection with a truncated branched polymer

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Abstract

Background: We previously determined that polyplexes formed by linear H2K peptides were more effective in transfecting tumors *in vivo* than polyplexes formed by branched H2K4b-20 peptides. Based on trypsin digest and salt displacement studies, the linear H2K polyplexes were less stable than the branched H2K4b-20 polyplexes. Because binding and release of the polymer and DNA from the H2K4b-20 polyplex may account for the ineffectiveness, we investigated whether four-branched histidine-lysine (HK) peptides with varying numbers of amino acids in their branches would be more effective in their ability to increase gene expression in tumors *in vivo*.

Methods: Linear and branched peptides with multiple -KHHK- motifs were synthesized by solid-phase synthesis. The branched H2K4b-20, -18, -14 and 12 peptides had 20, 18, 14 and 12 amino acids in their branches, respectively. These peptides were examined for their ability to carry luciferase-expressing plasmids to human breast cancer xenografts in a mouse model. With gel retardation and *in vivo* transfection, the incorporation of a targeting ligand and an endosomal lysis peptide into these polyplexes was also examined. A blocking antibody was pre-injected prior to the polyplexes to determine the role of neuropilin 1 in the uptake of these polyplexes by the tumor. The size of the polyplexes was measured by dynamic light scattering.

Results: Of the four negative surface-charge polyplexes formed by the branched carriers, the H2K4b-14 polyplex was determined to be the most effective plasmid delivery platform to tumors. The incorporation of a targeting ligand and an endosomal lysis peptide into H2K4b-14 polyplexes further enhanced their ability to transfect tumors *in vivo*. Furthermore, after pre-injecting tumor-bearing mice with a blocking antibody to the neuropilin-1 receptor (NRP-1), there was a marked reduction of tumor gene expression with the modified H2K4b-14 polyplexes, suggesting that NRP-1 mediated their transport into the tumor.

Conclusions: The present study established that branched peptides intermediate in length were very efficient in delivering plasmids to tumors *in vivo*.

Songhui Xu and Jiaxi He contributed equally to this work.

KEYWORDS
cancer, gene therapy, neuropilin-1 receptor, non-viral, peptide, plasmid

1 | INTRODUCTION

Diverse delivery systems have shown antitumor efficacy with nucleic acids, including plasmids, RNA interference and mRNA-directed therapies. One major approach has been the use of non-viral methods to deliver nucleic acids. Non-viral methods include electroporation,[1] hydrodynamic-injection,[2] and nanoparticle (NP) carriers such as liposomes,[3] lipopolymers,[4] liposome-polymers[5] and polymers.[6–8] Our laboratory has primarily focused on inhibiting tumor growth with pH-sensitive polymers and their polyplexes.

Most polyplexes are dependent on the enhanced permeability and retention effect (EPR) for accumulation in tumors. EPR results from leakiness of tumor blood vessels allowing influx of nanoparticles (NPs) from the blood into the tumor tissue in combination with reduced lymphatic tumor drainage, enhancing retention of NPs in the tumor. Although a few approaches may enhance EPR,[9–11] the accumulation of NPs in tumors is usually modest and about 30% greater than accumulation by normal tissues.[11] There are other disadvantages of NPs that depend on EPR for their entry into the tumor. Because blood vessel permeability may vary significantly within tumor tissue as well as between primary and metastatic tumors, the antitumor efficacy of NPs may be inconsistent[12] and lead to their reduced effectiveness in tumor treatments *in vivo*. An alternative pathway of entry that enables the accumulation of the NP in the tumor may circumvent this obstacle.

One approach of antitumor-directed NPs that is not dependent on EPR is targeted binding to the tumor vasculature, such as activating the NRP-1 pathway.[13] NRP-1 is frequently upregulated in tumor cells and endothelial cells of tumors.[14,15] Thus, even when the NRP-1 transport system of tumor cells is not increased, upregulation of NRP-1 in tumor endothelial cells ensures that targeted NPs traverse the vascular barrier of the tumor. The amino acid sequence, R/KXXR/K-OH, where R/K represents either arginine or lysine and X represents any amino acid, binds to and activates the NRP-1 receptor.[16,17] Moreover, the requirement of the K/RXXK/R sequences to have a C-terminal carboxyl end to bind NRP-1 is unconditional. The cyclic tumor-penetrating peptides such as iRGD, LyP-1 and iNGR, which contain the K/RXXK/R sequence, require limited digestion to expose the C-terminal end. To enhance specificity further, the tumor penetrating peptides initially bind to receptors expressed on tumor endothelial cells prior to binding to NRP-1. For example, the cyclic peptide, iRGD, first binds to the $\alpha_v\beta3/\beta5$ receptor on the endothelial cell surface with subsequent enzymatic digestion to bind and activate NRP-1.[16,17] Tumor-penetrating peptides have been conjugated to a wide variety of therapeutic and targeting agents such as small molecule chemotherapeutic agents and nanoparticles, resulting in their increased delivery to tumors in murine models.[18]

Although several studies have shown that tumor-penetrating peptides improved antitumor efficacy of NPs incorporating drugs,[19–21] there have been a limited number of studies using tumor penetrating peptides with nucleic acid NPs.[22,23] For example, Shen *et al.*[23] demonstrated that iRGD targeted NPs carrying both paclitaxel and a plasmid-expressed short hairpin RNA (shRNA) targeting survivin significantly inhibited the size of lung cancer xenografts. In another study, Li *et al.*[24] co-delivered a siRNA targeting PD-1 and a small molecule checkpoint inhibitor, methyl-dl-tryptophan, in micelles to reduce tumor size. The micelle was composed of an NRP-1 tumor-targeting peptide with a histidine-rich domain and cholesterol. Compared to either checkpoint inhibitor alone, co-delivery of the siRNA and 1-methyl-dl-tryptophan showed a marked reduction in the size of tumors.

By forming ionic and non-ionic bonds with nucleic acids,[25] histidine-lysine (HK) peptides have been used by several groups, including ours as carriers of nucleic acids.[7,26–28] In a prior study, positive surface polyplexes formed by the branched H2K4b-20 peptide (wt:wt, peptide:DNA, 4:1 ratio) delivered luciferase-expressing plasmids effectively to malignant cells *in vitro*, whereas positive polyplexes formed by the linear H2K peptide were extremely poor carriers *in vitro*.[29] By contrast to the *in vitro* results, H2K or H2K4b-20 negative surface charged polyplexes made at low peptide to plasmid DNA ratios (wt:wt, ~1:2) demonstrated higher luciferase expression in tumor xenografts than the positive polyplexes.[29] Furthermore, compared to the branched H2K4b-20 peptide, the linear H2K peptide carrier was a significantly more effective carrier of luciferase-expressing plasmids to tumors *in vivo*.[29] Interestingly, the linear H2K peptides had the same number and sequence patterns of histidines and lysines as the branches of H2K4b-20 peptides. Similar to tumor-penetrating peptides and associated nanoparticles, we also determined that the transport of negative polyplexes formed by the linear H2K peptide to tumors was mediated by NRP-1.[29,30] Consequently, these H2K polyplexes required limited enzymatic digestion to expose a C-terminal end group with the KHHK sequence pattern. Moreover, adding endosomal disrupting H3K-33 peptides to H2K polyplexes significantly increased tumor transfection *in vivo*. The H3K-33 peptide showed marked membrane lysis in an acidic environment using an *in vitro* endosomal model.[30] Tumor accumulation and specificity of the H2K polyplexes was further increased by the addition of the cRGD peptide ligand, which targeted the $\alpha v\beta3/\beta5$ integrins of the tumor endothelial cells.[29,30]

Based on previous trypsin digest and salt displacement studies, polyplexes formed by the linear H2K peptide were less stable than those formed by the branched H2K4b-20. These findings support that the linear H2K peptide on the surface of the polyplex may be more susceptible to enzymatic digests, enabling increased activation of NRP-1.[29,30] As a result, the present study explored whether polyplexes formed with shorter branches of H2K4b (-12, 14 or -18) would be less stable than those formed with H2K4b-20 and enable greater gene expression in tumors in a mouse model. With the

branched carriers containing multiple -KHHK- motifs, we also examined whether tumor uptake of these polyplexes was mediated by the neuropilin-1 receptor.

2 | MATERIALS AND METHODS

2.1 | Animals

Female athymic mice (Balb/c, 4–8 weeks old, weighing ~20 g) were purchased from Envigo (Indianapolis, IN, USA). The experiments were performed following regulations by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

2.2 | Cell lines

The human breast cancer cell line, MDA-MB-231, was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 20 mM glutamine.

2.3 | Plasmids

Preparation of pCpG-Luc (luciferase-expressing) plasmids was prepared as described previously.[31] The plasmids were purified with an EndoFree Maxi kit (Qiagen, Valencia, CA, USA). To confirm that the plasmid was primarily supercoiled and had no evidence of bacterial chromosomal DNA, each sample preparation was analyzed by gel electrophoresis.

2.4 | Peptides

Linear and branched HK peptides were synthesized on a Ranin Voyager synthesizer (Tucson, AZ, USA) by the biopolymer core facility at the University of Maryland or by Genscript (Piscataway, NJ) as described previously.[32] To ensure purity of 90% or greater,[32] peptides were analyzed by high-performance liquid chromatography (Beckman Coulter, Fullerton, CA, USA) and by electrospray ionization mass spectrometry (LCMS-2020; Shimadzu Corporation, Kyoto, Japan) (see Supporting information, Figure S1). The c(RGDfV)-PEG-H3K4b conjugate was synthesized with an average of four c(RGDfV) ligands per H3K4b molecule as determined by amino acid analysis.[33]

Nomenclature of polymers: (1) for H2K4b, the dominant repeating sequence in its terminal branch is -(K)HHK-, thus "H2K" is part of the name; the "4b" refers to the number of terminal branches. (2) H2K4b-20, H2K4b-18, H2K4b-14 and H2K4b-12 are four-branched polymers with 20, 18, 14 and 12 amino acids in each branch, respectively. For these branched H2K4b peptides, the four branches are covalently bonded to a three-lysine core. (3) The linear peptide, H2K, contained multiple -(K)HHK-sequences, whereas the linear peptide, H3K-33, contained multiple -(K)HHK- and -(K)HHHK- sequences. The sequences of the HK peptides are shown in Table 1.

2.5 | *In vitro* transfection

Several branched HK peptides were examined for their ability to carry a luciferase-expressing plasmid into MDA-MB-231 cells (America Type Tissue Culture, Manassas, VA, USA). In brief, 3×10^4 cells were plated into a 24-well plate containing 500 μl of DMEM and 10% FCS (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h, when the cells were 60%–80% confluent, the media in each well was changed to fresh DMEM/10% FCS (500 μl). To prepare HK polyplexes, pCpG-Luc plasmids (1 μg) in 46 μl of Opti-MEM (Thermo Fisher Scientific) were briefly mixed well with one of the HK peptides (0.5 or 4 μg, total volume 4 μl) and maintained at room temperature for 30 min. This polyplex (50 μl) was then added dropwise to the cells. Twenty-four hours later, the cells were lysed (Passive Lysis Buffer; Promega Corporation, Madison, WI, USA), and the luciferase activity (expressed as relative light units, RLU) was measured[34] (Promega Corporation).

2.6 | Stability of HK polyplexes to enzymatic degradation

Polyplexes were formed at two peptide: plasmid ratios [w/w: 4:1; peptide (4 μg):plasmid (1 μg) or 1:2 (0.5 μg):plasmid (1 μg)] with the four polymers (H2K4b-12, H2K4b-14, H2K4b-18 and H2K4b-20). To

TABLE 1 Sequences and structure of HK peptides

HK polymers	Sequence	Number of amino acids in linear or terminal branch	Structure
H2K	K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-K [a]	20-mer (8K, 12H)	Linear
H3K-33	K-H-K-H-H-K-H-H-K-H-H-H-K-H-H-H-K-H-H-H-K-H-H-H-K-H-H-H-K-H-H-K-H-H-K-H-K	33-mer (11K, 22H)	Linear
H2K4b-12	[K-H-H-K-H-H-K-H-K-H-H-K]$_4$LYS	12-mer (5K, 7H)	4-Branched
H2K4b-14	[K-H-H-K-H-H-K-H-H-K-H-H-K]$_4$LYS	14-mer (5K, 9H)	4-Branched
H2K4b-18	[K-H-H-K-H-H-K-H-H-H-K-H-H-K-H-H-H-K]$_4$LYS	18-mer (6K,12H)	4-Branched
H2K4b-20	[K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-K]$_4$ LYS	20-mer (8K,12H)	4-Branched

H2K4b-12, -14, -18 and -20 are four-branched peptides with branches emanating from a 3-lysine core (LYS).
[a]Single letter amino acid code.

distinguish susceptibility of the four polyplexes at the two ratios to enzymatic degradation, optimization of the trypsin concentration and incubation times were done. After trypsin and incubation times were established, these polyplexes were then placed at 37°C with the following concentrations of trypsin and times: 0.025%, 30 min, 4:1 ratio or 0.01%, 10 min, 1:2 ratio. The HK polyplexes were then loaded on a 1% agarose gel (Sigma-Aldrich, St Louis, MO, USA; 10× BlueJuice Gel loading buffer; Thermo Fisher Scientific) and electrophoresis was carried out at 75 V for 30–60 min in Tris acetate-ethylenediaminetetraacetic acid buffer (TAE) (Quality Biological, Gaithersburg, MD) buffer containing ethidium bromide (0.5 μg/ml; Thermo Fisher Scientific). The gel containing the stained DNA was exposed to UV light, and the images were acquired with a gel documentation system (ChemiDoc Touch Imaging System; Bio-Rad, Hercules, CA, USA).

2.7 | Polyplex formation for *in vivo* use

To the plasmid DNA (36 μg in 150 μl of water), branched H2K4b (15 μg in 100 μl of water) or branched H2K4b/H3K-33 (12.5/2.5 μg) peptides were added quickly and mixed by pipetting. Forty-five minutes after mixing, cRGD-PEG-H3K4b (1.63 μg in 30 μl of water) or water (30 μl) was added to polyplexes for 15 min before the intravenous injection of the polyplex (250 μl). After intravenous injection, the remainder (30 μl) of the polyplex preparation was analyzed by gel electrophoresis (10 μl) and by size and zeta potential (20 μl).

2.8 | Transfection *in vivo*

After MDA-MB-231 tumor cells were injected (8×10^5 cells) into the mammary fat pad and the tumors 7 days later were approximately 100–150 mm^3 in size, the mice were treated by intravenous injection in the tail vein of mice with plasmid polyplexes (250 μl). After 24 h, the tumors and major organs were homogenized, and luciferase activity was measured with a direct current TD 20/20 luminometer (Turner Design, Sunnyvale, CA, USA). In selected cases, mice were administered intravenously with NRP-1 antibody [R&D Systems Minneapolis, MN, USA; 6 μg in 200 μl of phosphate-buffered saline (PBS)] or an immunoglobulin (Ig)G antibody control (6 μg in 200 μl of PBS) 30 min prior to injection of HK polyplexes. The number of tumor-bearing mice per group differed with the transfection experiment: (1) polymer alone as the carrier; effect of various components; and H2K4b-14 compared to linear H2K ($n = 4$); (2) tumor specificity of optimal polymer formulations ($n = 12$–14); and (3) NRP-1 dependent luciferase expression of optimal H2K4b-14 polyplex ($n = 3$).

2.9 | Gel retardation assay

After HK carriers with plasmid DNA were mixed (HK/DNA, w/w, ~1:2) for *in vivo* injections, a sample of these polyplexes (~1 μg of plasmid DNA) was loaded onto the gel (1% agarose; 10× BlueJuice Gel loading buffer). Electrophoresis was carried out at a constant voltage of 75 V for 30 min in TAE buffer containing ethidium bromide (Thermo Fisher Scientific). The gel was then exposed to the UV imager (ChemiDoc Touch).

2.10 | Particle size, polydispersity index (PDI) and zeta potential

The size, PDI and zeta potential were determined with the Zetasizer (Malvern, Westborough, MA, USA) and analyzed with the instrument manufacturer's software (Zetasizer, version 6.2). Using dynamic light scattering at a 90° angle, particle sizes were reported as the *Z*-average diameter from the intensity-weighted size distribution. Calibration with controls (50, 100, 300 and 500 nm) was conducted to validate the size of the polyplexes. The particle size, PDI and zeta potential data points represent the mean ± SD of three measurements. After mixing HK peptides (15 or 16.3 μg) and plasmids (36 μg) in 280 μl (water) for 1 h, 20 μl was removed and diluted with water to measure the size and PDI of the polyplexes (180 μl, final volume). After the size measurements, the polyplex sample (180 μl) was further diluted with water (final volume of 900 μl) to determine the zeta potential.

2.11 | Statistical analysis

The results are reported as mean ± SD and represent three or more separate data measurements. The results were analyzed using a two-tailed *t* test or one-way analysis of variance (ANOVA) with multiple comparisons. $p < 0.05$ was considered statistically significant, Analyses were conducted using SigmaPlot (San Jose, CA, USA).

3 | RESULTS

3.1 | H2K4b-14 peptide was the most effective carrier *in vivo*

In vitro transfection studies (at higher peptide:DNA ratios; 4:1) demonstrated that the H2K4b-14 peptide was a poor carrier of plasmids for MDA-MB-231 cells compared to the branched H2K4b peptides with longer branches (i.e., H2K4b-18 and H2K4b-20) (see Supporting information, Figure S2). At both the lower or higher peptide:plasmid DNA ratio (wt:wt, 1:2 or 4:1), the H2K4b-18 peptide was the most effective carrier for transfection *in vitro*. Interestingly, the remarkably high transfection *in vitro* observed with the H2K4b-18 carrier at the higher ratio may correlate with its instability as observed with the trypsin digest assay (see Supporting information, Figure S3). Nonetheless, differences in the stability to partial trypsin digests were not observed between the four polyplexes at the lower peptide:DNA ratio despite testing various incubation times and trypsin concentrations (see Supporting information, Figure S3).

By contrast to the *in vitro* transfection results, the H2K4b-14 peptide proved to be the most effective carrier of plasmids to tumor xenografts *in vivo* (Figure 1). Based on prior studies, a low peptide: DNA ratio (wt:wt, ~1:2) was selected in which the surface charge of the H2K4b polyplexes was negative[29] (Table 2). Whereas negative charged H2K4b-14 polyplexes were the most efficient in targeting tumors, the four polyplexes (H2K4b-12, -14, 18 and -20) gave similar luciferase expression in the lungs, liver, and spleen (Figure 1). The rank of tumor transfection by the negative polyplexes formed by the four polymers was: H2K4b-14 > > -20 > -12 > -18.

We then compared negative and positive polyplexes of H2K4b-14 for their ability to transfect tumors *in vivo* (wt:wt, peptide:DNA ratio, ~1:2 or 4:1) (Table 2; see also Supporting information, Figure S4). Similar to the H2K4b-20 polyplexes previously reported[29] and used as a control for this experiment, the negative H2K4b-14 polyplexes (wt:wt, ~1:2) gave higher luciferase levels in tumor xenografts than the positive H2K4b-14 polyplexes (wt:wt, 4:1) (see Supporting information, Figure S4). The background values for tumors, lungs, liver and spleen were 3.9, 4.6, 2.3 and 6.5 RLU/mg-protein, respectively.

3.2 | Components enhanced tumor transfection *in vivo*

We next investigated whether the addition of an endosomal lysis peptide (H3K-33) and a targeting cRGD peptide (cRGD-PEG-H3K4b) enhanced delivery of branched H2K4b polyplexes (Figure 2). The amount of cRGD and H3K-33 peptides incorporated into HK polyplexes was previously optimized using a tumor-bearing mouse model (unpublished data; J. He, Q. Leng, A.J. Mixson). Increasing the H3K-33 peptide above 30% of the total peptide in the polyplex enhanced luciferase expression in the lungs, whereas greater amounts of the cRGD ligand (3.2 μg) did not further increase luciferase levels in tumors.

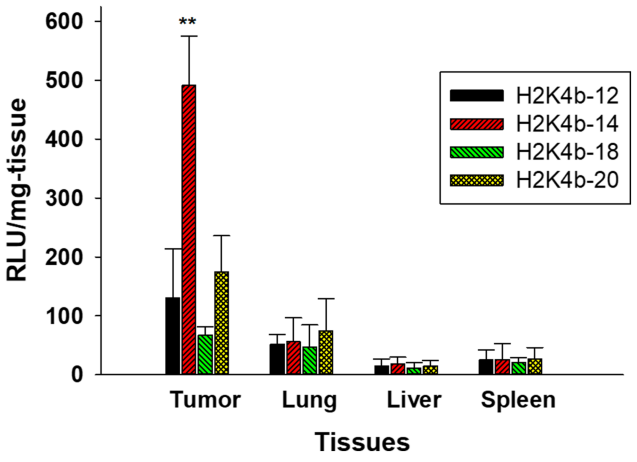

FIGURE 1 *In vivo* transfection with branched HK peptides in a MDA-MB-231 tumor mouse model. At a low peptide: DNA ratio, several branched peptide polyplexes (H2K4b-12, -14, -18 and -20) were compared to transfect tumors and tissues. After tumors had grown to 100–150 mm^3, the branched HK peptides (15 μg) in complex with luciferase-expressing plasmids (36 μg) were injected intravenously (250 μl) into mice. Twenty-four hours later, tissues including tumors were removed, and luciferase activity was measured. One-way ANOVA with Holm-Sidak method. $^{**}p < 0.01$; H2K4b-14 vs. H2K4b-12, H2K4b-18, H2K4b-20 ($n = 4$)

We determined that incorporation of the first component, H3K-33, into the branched polyplexes increased their tumor transfection *in vivo* (polyplexes with the added H3K-33 endosomal lysis component were referred to as H2K4b-12E, H2K4b-14E, H2K4b-18E and H2K4b-20E) (Figure 2). The H3K-33 peptide enhanced tumor transfection with H2K4b-14 polyplexes by approximately 41%, whereas the peptide enhanced transfection with the H2K4b-18 polyplexes the most ($p < 0.05$, H2K4b-18E vs. H2K4b-18). Nevertheless, the H2K4b-14E polyplexes appeared more effective than the other polyplexes modified by H3K-33 (H2K4b-12E, -18E, -20E).

These polyplexes were further modified by adding a second component, an endothelial targeting ligand cRGD-PEG-H3K4b peptide (polyplexes with the two added components were labeled H2K4b-12EL, H2K4b-14EL, H2K4b-18EL, H2K4b-20EL). The two incorporated components did not significantly affect the Z-A size (Table 2) or intensity size distribution (see Supporting information, Figure S5) of the polyplexes. The cRGD-PEG-H3K4b peptide, which was positively charged, bonded by ionic interactions to the negative-charged surfaces of H2K4b polyplexes. With the incorporation of the targeting ligand peptide, the surface charge of the polyplex was modestly less negative (Table 2). Tumor expression with H2K4b-14EL had a 63% higher luciferase level with the addition of the targeting ligand ($p < 0.05$, H2K4b-14EL vs. H2K4b-14E; $p < 0.01$, H2K4b-14EL vs. H2K4b-14). To varying degrees, the two components added to the branched polyplexes improved transfection *in vivo* (Figure 2). Although addition of the H3K-33 component to the polyplex had greater variability, augmenting tumor transfection between 41% and 507%, the addition of the cRGD-PEG-H3K4b ligand peptide showed less variability, increasing tumor transfection between 22% and 63%.

3.3 | Gel migration of polyplexes with the different branched polymers

At low peptide to plasmid DNA ratios (wt:wt, ~1:2, 15 or 16.3 μg; 36 μg) used for *in vivo* experiments, we examined the migration of the plasmid (Figure 3). The four-branched peptides alone (15 μg) retarded the plasmid DNA, indicating polyplex formation. Similarly, by incorporating one or both components, H3K-33 and RGD-PEG-H3K4b, into the polyplexes, the plasmid was also significantly retarded.

3.4 | H2K4b-14EL is the optimal plasmid carrier to tumors *in vivo*

Although the component experiment suggested that H2K4b-14EL was the optimal carrier, we further confirmed that this carrier was

Polymer(s)	Ratio[a]	Z-A size[b] (nm)	Zeta potential (mv)	PDI
H2K4b-20	1/2	147 ± 21.6[c]	−19.2 ± 8.5	0.30 ± 0.043
H2K4b-20 + H3K-33	1/2	130 ± 25.1	−19.1 ± 6.3	0.27 ± 0.072
H2K4b-20 + H3K-33 + cRGD	1/2	145 ± 15.0	−14.0 ± 4.9	0.28 ± 0.041
H2K4b-18	1/2	164 ± 24.0	−17.2 ± 6.1	0.26 ± 0.053
H2K4b-18 + H3K-33	1/2	136 ± 17.0	−15.8 ± 2.6	0.27 ± 0.046
H2K4b-18 + H3K-33 + cRGD	1/2	156 ± 16.6	−14.5 ± 4.9	0.27 ± 0.028
H2K4b-14	1/2	150 ± 22.5	−17.9 ± 3.4	0.26 ± 0.014
H2K4b-14 + H3K-33	1/2	123 ± 18.4	−17.5 + 3.7	0.29 ± 0.035
H2K4b-14 + H3K-33 + cRGD	1/2	150 ± 23.4	−16.2 ± 2.7	0.28 ± 0.042
H2K4b-12	1/2	149 ± 21.1	−19.6 ± 3.2	0.30 ± 0.018
H2K4b-12 + H3K-33	1/2	133 ± 17.9	−16.6 ± 1.5	0.27 ± 0.040
H2K4b-12 + H3K-33 + cRGD	1/2	156 ± 16.2	−15.3 ± 1.8	0.29 ± 0.032
H2K4b-20	4/1	116 ± 12.4	+24.3 ± 6.9	0.28 ± 0.062
H2K4b-14	4/1	98 ± 14.5	+25.6 ± 7.4	0.22 ± 0.032

TABLE 2 Biophysical properties of HK polyplexes

[a]Ratio of HK:DNA (w/w).
[b]Z-A is the Z-average size of the polyplex in water.
[c]Data are the mean ± SD.

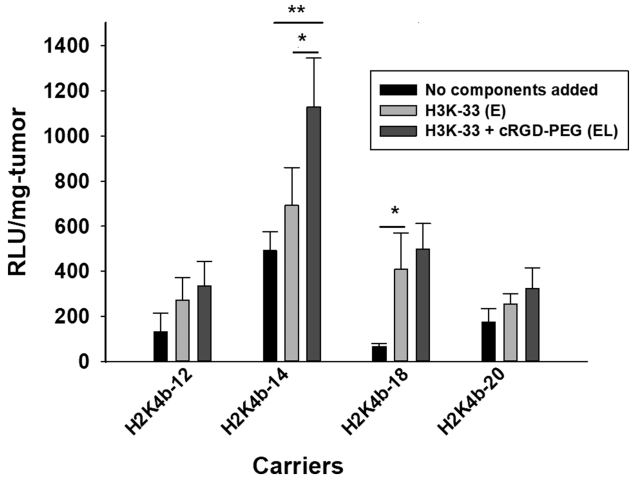

FIGURE 2 Effect of different component peptides on tumor transfection *in vivo*. Different formulations of the branched peptides (H2K4b-12, H2K4b-14, H2K4b-18 and H2K4b-20) were investigated for their ability as carriers of luciferase-expressing plasmids to MDA-MB-231 tumors in a mouse model. These HK luciferase-expressing polyplexes (peptide:DNA ratio, ∼1:2, wt:wt, 36 μg of plasmid) included three different carrier formulations: (1) branched polymers alone (H2K4b-12, -14, -18, -20; 15 μg); (2) branched polymers mixed with the H3K-33 peptide (H2K4b-12E, -14E, -18E, -20E; 15 μg); and (3) branched polymers mixed with both H3K-33 and endothelial targeting cRGD-PEG-H3K(+H)4b peptides (H2K4b-12EL, -14EL, -18EL, -20EL; 16.3 μg). Twenty-four hours after the intravenous injection of polyplexes (250 μl), the luciferase levels were measured from lysates of tumors. *t* test, **p* < 0.05; H2K4b-14EL vs. H2K4b-14E; H2K4b-18E vs. H2K4b-18; ***p* < 0.01, H2K4b-14EL vs. H2K4b-14 ($n = 4$)

superior *in vivo* to the other carriers. Whereas the polyplexes of H2K4b-12EL and H2K4b-20EL had similar tumor transfection, they had modestly lower tumor transfection than polyplexes of H2K4b-18EL. Nevertheless, these three polyplexes had significantly lower tumor transfection compared to the H2K4b-14EL polyplexes ($p < 0.01$). The rank of tumor transfection by these branched peptide polyplexes was: H2K4b-14EL > > -18EL > -20EL, -12EL ($p < 0.01$, H2K4b-14EL vs. H2K4b-12EL, H2K4b-18EL, H2K4b-20EL) (Figure 4). The H2K4b-14EL polyplexes also had a significantly higher tumor specificity than the other H2K4b polyplexes. Because HK polyplexes in prior studies gave higher luciferase levels in the lungs (compared to other tissues),[31] we were particularly interested in the tumor/lung ratio of the various branched H2K4b formulations. The tumor/lung ratio of H2K4b-14EL polyplex was approximately 13, the ratios of H2K4b-18EL and H2K4b-12EL were approximately 6, and the ratio of H2K4b-20EL was approximately 4. Moreover, the H2K4b-14EL polyplex demonstrated greater tumor gene expression compared to the linear H2K-EL polyplex ($p < 0.05$, H2K4b-14EL vs. linear H2K-EL) (Figure 5).

3.5 | NRP-1 mediated transport of polyplexes

We investigated whether the gene expression of H2K4b-14 polyplexes was mediated by NRP-1. Endothelial and most tumor cell lines, including MDA-MB-231 cells, express NRP-1.[35,36] Thirty minutes after injection of a NRP-1 blocking antibody (Ab), H2K4b-14 polyplexes carrying luciferase-expressing plasmids were injected intravenously into MDA-MB-231 tumor-bearing mice; twenty-four hours later, mice were killed, and gene expression in tumors/tissues was analyzed. Although luciferase activity in the lungs was slightly decreased by 20% compared to the control IgG groups, its activity in tumors was reduced by approximately 98% ($p < 0.01$, NRP-1 vs. IgG Ab) (Figure 6). These findings provided further support that transport of H2K4b-14EL polyplexes into tumors was mediated by the NRP-1 receptor.

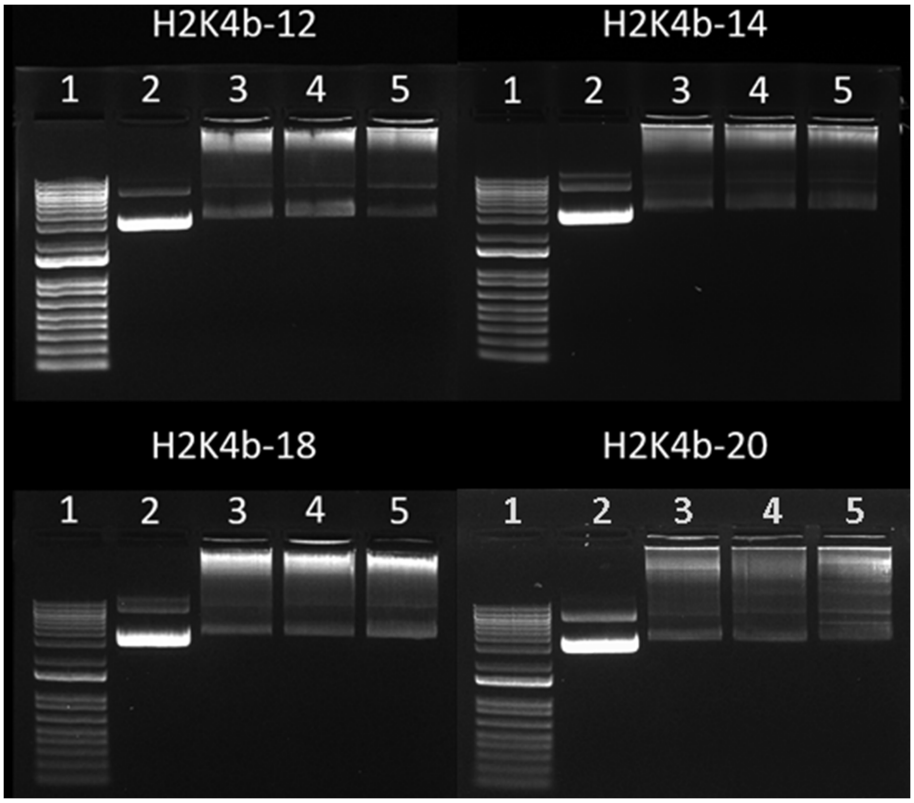

FIGURE 3 Gel migration of the four polyplexes. After HK carriers with plasmid DNA were mixed (peptide:DNA, w:w, ~1:2; peptide,15 or 16.3 μg; plasmid 36 μg) for *in vivo* use, a sample of these polyplexes (~1 μg of plasmid DNA) was loaded onto the gel. Electrophoresis was then carried out in TAE buffer containing ethidium bromide. Peptide:DNA, w:w, ~1:2; 15 or 16.3 μg, HK; 36 μg, plasmid. Lane 1, molecular weight marker; lane 2, plasmid control; lane 3, H2K4b-12, -14, -18 and -20 polyplexes; lane 4, H2K4b-12E, -14E, -18E and -20E polyplexes; lane 5, H2K4b-12EL, -14EL, -18EL and -20EL polyplexes

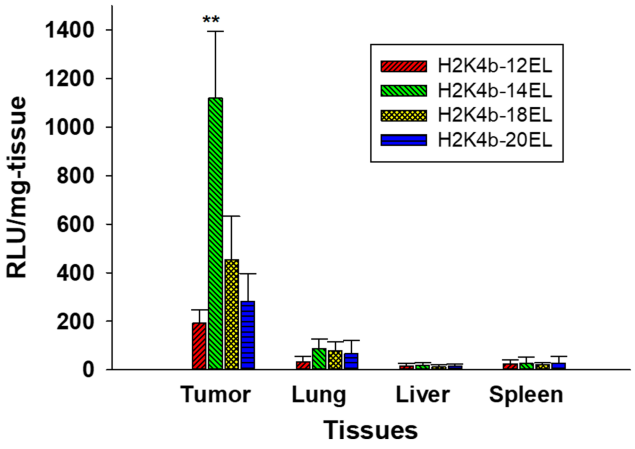

FIGURE 4 Comparison of different HK polyplexes to transfect MDA-MB-231 tumors. After tumor xenografts were established, several polyplexes formed by branched peptides were assessed for their ability to transfect the xenografts and tissues. Twenty-four hours after intravenous injection of the polyplexes (250 μl), lysates of tissues including tumors were measured for luciferase activity. H2K4b-12EL, -14EL, -18EL and -20EL represent polyplexes comprised of respective branched polymers (12.5 μg), H3K-33 (2.5 μg), cRGD ligand (1.63 μg) and luciferase-expressing plasmids (36 μg). One-way ANOVA with the Holm–Sidak method. $^{**}p < 0.01$; H2K4b-14EL vs. H2K4b-12EL, H2K4b-18EL, H2K4b-20EL ($n = 12$–14)

4 | DISCUSSION

Because binding and release of the polymer and DNA from the polyplex may account for variation in transfection *in vivo*, we investigated whether four-branched HK peptides with shorter branch lengths (H2K4b-12, H2K4b-14, H2K4b-18) than H2K4b-20 would be more effective in their ability to increase gene expression in tumors *in vivo*. The tumor transfection and tissue specificity of the negative surface-charged H2K4b-14 polyplexes, particularly the H2K4b-14EL polyplex, was remarkable. Still, the tumor specificity of the other negative-surface charged polyplexes (H2K4b-12, H2K4b-18 and H2K4b-20) was quite good compared to earlier reported positive-surface charged H2K4b-20 polyplexes.[31] The enhanced tumor specificity of these polyplexes may partly be a result of targeting the αvβ3 and NRP-1 receptors, both upregulated in tumor blood vessels.[14,37]

Most NPs, including polyplexes, accumulate in tumors by the EPR mechanism, whereas alternative routes of entry such as the activation of the neuropilin-1 receptor offer the potential for increased uptake in the tumor of these therapeutic particles. If the tumor cells express NRP-1, which occurs in 50% or more of several cancers including gastric and pancreatic cancers, then there may be widespread distribution of the molecule.[14,15] The increased tumor accumulation of NRP-1 mediated transport of tumor penetrating peptides and polyplexes is not well understood,[38,39] although two mechanisms have been

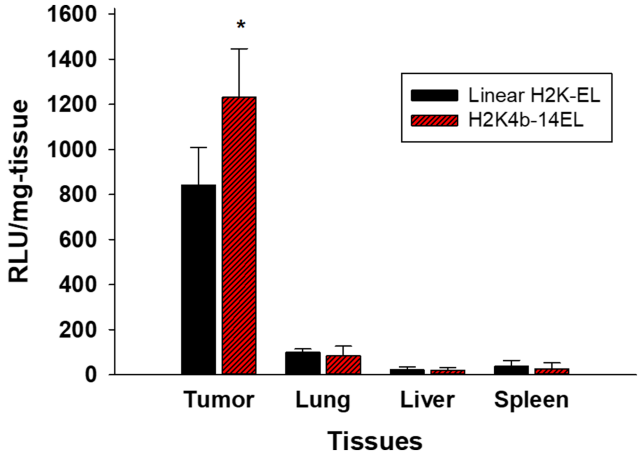

FIGURE 5 Comparison of linear H2K-EL and branched H2K4b-14EL polyplexes *in vivo*. To compare these two polyplexes (peptide: plasmid, ~1:2), luciferase levels in tumors were measured 24 h after injection of polyplexes (250 μl). H2K and H2K4b-14EL polyplexes represent polyplexes comprised of respective polymers (12.5 μg), H3K-33 (2.5 μg), cRGD ligand (1.63 μg) and luciferase-expressing plasmids (36 μg). $^{*}p < 0.05$, H2K4b-14EL vs. H2K-EL ($n = 4$)

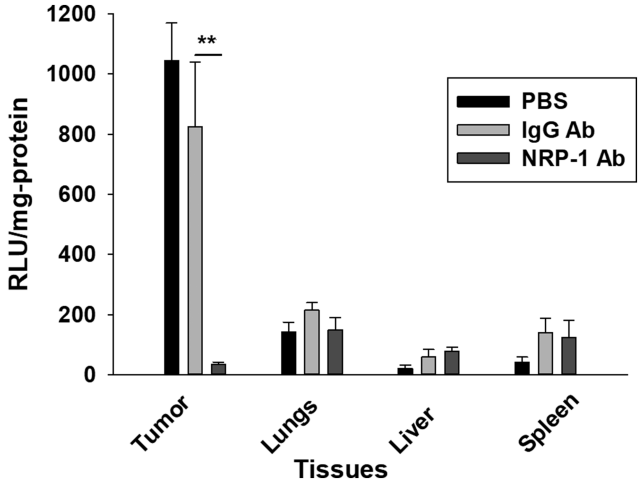

FIGURE 6 Effects of blocking NRP-1 antibody on transfection with HK polyplexes. MDA-MB-231 tumor-bearing mice were injected with IgG (6 μg in 200 μl of PBS) or NRP-1 Ab (6 μg in 200 μl of PBS); 30 min later, the mice were injected intravenously with H2K4b-14EL plasmid polyplexes (250 μl, H2K4b-14EL; 16.6 μg; plasmid, 36 μg). After 24 h, luciferase activity was measured in lysates of tumors and normal tissues. Values are the mean ± SD from three experiments. $^{**}p < 0.01$, *t* test, control ab vs. NRP-1 ab for H2K4b-14EL polyplexes ($n = 3$)

proposed. One is increased vascular permeability, enabling more extravasation of the peptide or peptide-tagged macromolecules into the tumor matrix. A second potential mechanism of NRP-1 mediated transport of molecules may be transcytosis through the endothelium into the tumor matrix.[39] Note that neither mechanism is mutually exclusive, and thus both may be active.

Because the NRP-1 receptor binds to peptides with a -KXXK- amino acid sequence, the branched H2K4b-14 and linear H3K-33 peptides with multiple -KHHK- sequences have the potential to activate this receptor (Table 1). Since none of the peptide sequences in the H2K4b polyplexes (including H2K4b-14EL) contained a lysine with a C-terminal carboxyl group (-KHHK-CO_2), limited enzymatic digest of the surface peptides on the polyplex must occur for NRP-1 mediated uptake into the tumor. Multiple enzymes, such as matriptase, within the tumor matrix have trypsin-like enzymatic activity that can expose the C-terminal carboxyl group once the H2K4b polyplex is bound to endothelial cells of the tumor.[13,40–42] The marked decrease in gene expression of H2K4b-14EL polyplexes in tumors of mice pretreated with the NRP1 antibody strongly supports that these polyplexes activated the neuropilin-1 receptor.

The uptake of tumor penetrating peptide, iRGD, and associated nanoparticles into the tumors is mediated by the NRP-1 receptor.[13] Similar to H2K4b-14EL polyplexes, pretreating the mice with the NRP-1 antibody substantially reduced the entry of these iRGD peptides into the tumor. There are other functional parallels between H2K4b-14EL polyplexes and the cyclic tumor penetrating iRGD peptides and their nanoparticle conjugates. Both have cyclic RGD ligands that can target the $\alpha v\beta_3/\beta_5$ integrin receptors on mitogenic endothelial cells of tumors.[37] Because both integrin and NRP-1 receptors are on the surface of mitogenic endothelial cells, the cRGD ligand (and associated nanoparticle) is assumed to bind initially to the integrin receptor, facilitating limited digestion and later interaction of the enzymatically exposed -KXXK-CO_2^- ligand with the NRP-1 receptor.[18] Similarly, the H2K14-EL polyplex may have enhanced tumor targeting by interacting initially with the integrin and then with the NRP-1 receptors. The iRGD and H2K4b-14EL polyplex nanoparticles differ in that both the cRGD and NRP-1 ligands are located within the cyclic iRGD peptide, whereas these ligands are located on different peptides for H2K4b-14EL polyplexes. For the polyplex, the cRGD ligand is located on the cRGD-PEG-H3K polymer, whereas trypsin digest of H2K4b-14 or H3K-33 could yield sequences which can bind to NRP-1.

Incorporation of H3K-33 and RGD peptides within the H2K4b-14EL polyplexes enhanced tumor gene expression by approximately 130% compared to the H2K4b-14 polyplexes. Although the H3K-33 peptide, together with the H2K4b, may have a role in activating the NRP-1 receptor, H3K-33 was initially added because of its potential to enhance endosomal lysis based on a prior study.[30] The cRGD ligand, integrated within the cRGD-PEG-H3K peptide, enhanced gene expression of the H2K24b-14 polyplex by approximately 60% in the tumor xenograft. Because the addition of PEG can enhance the half-life of HK polyplexes,[43,44] both PEG and the cRGD ligands may act in concert. A longer half-life of the RGD-PEG-HK containing polyplexes increases the likelihood that these polyplexes will interact with the integrin receptors on endothelial cells. Because cRGD-PEG-H3K4b peptide does not contain a -KHHK- sequence, it likely has no direct role in binding to NRP1. Together with the H2K4b peptides, the H3K-33 and cRGD-PEG-H3K4b peptides increased the specificity of the polyplex toward the tumor.

In the present study, several methods (*in vitro* transfection assays, gel retardation assays, trypsin digests and size and surface-charge) evaluated the efficacy of these negative surface charge plasmid polyplexes. Some of these *in vitro* assays, including gel retardation and

the size of the polyplexes, did not show significant differences with branch length. Interestingly, *in vitro* transfection assays for these HK polyplexes were not predictive of the *in vivo* transfection. By contrast, *in vitro* transfection assays with other polyplexes such as with the polyethylenimine (PEI) carrier have been found to be more predictive of *in vivo* efficacy. This higher correlation may be a result of the high density of ethyleneimine subunits in PEI, which have buffering, cationic charges and non-ionic interaction properties.[45,46] Similar to PEI polyplexes, *in vitro* transfection assays of other polyplexes, stabilized with oxidized cysteines and hydrophobic domains, were predictive of their efficacy *in vivo*.[47,48] Further stabilization of HK polyplexes may increase the correlation between *in vitro* and *in vivo* studies, but whether tumor transfection is improved by a stabilized H2K4b-14 polyplex remains to be demonstrated. Future planned studies include stabilization of HK with oxidized cysteines and perhaps hydrophobic domain,[47,48] although new strategies and methods for *in vitro* and *in vivo* characterization of stabilized and non-stabilized polyplexes are required to understand the potential of nucleic acids delivery systems for human therapy.

The reason for the higher tumor transfection with negative charge H2K4b-14 polyplex is not clear from the assays conducted in the present study. In addition to the differences in the length of the branches of the four polymers, the sequence patterns were slightly different. Instead of varying the length of the branches, it may be informative to vary the sequence patterns of one of the polymers. Relatively minor modifications in sequence patterns, such as the addition of a single histidine in different locations of the branches of HK peptides, have been observed to significantly affect the delivery of mRNA *in vitro*.[49] H2K4b-14 would be a particularly interesting polymer with respect to varying the sequence patterns in that it has a high tumor transfection efficiency *in vivo* (Figure 1) and a low transfection *in vitro* (see Supporting information, Figure S2) compared to H2K4b-18. For example, by exchanging the -KHHH- with a later -KHH- sequence on the branches of H2K4b-14, the modified peptide with a branch sequence, KHHKHHKHHHKHHK, would have the same 14 amino acids from the N-terminal end as H2K4b-18 (Table 1). Such modifications may enable greater insight not only regarding the transfection profile of H2K4b-14, but also that of the H2K4b-18 peptide.

Although our laboratory and other investigators have shown that non-viral carriers of tumor-inhibitory plasmids with and without chemotherapy reduced tumor size,[31,50] gene expression within tumors by carriers including polymers has been low.[31] To improve the ability of the plasmids to reduce tumor size, we screened carriers for enhanced transfection of tumors. In the present study, we determined that minor modifications in the lengths and sequences of peptides can have profound differences in their efficacy as carriers of nucleic acids *in vivo*. Similar to the linear H2K carriers, the branched H2K carriers of gene-expressing plasmids was enhanced in tumor xenografts by an NRP-1 dependent mechanism. Of the four branched polyplexes, the H2K4b-14EL polyplex at low peptide:plasmid ratios showed the most promise as a systemic carrier of antitumor genes (i.e., shRNA targeting oncogenes) to primary and metastatic cancers.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SX, JH, ZI, AKA, MCW and AJM were responsible for the experiments and their design. MCW, ZI and AJM wrote the manuscript. All authors approved the final version of the manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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