Systemic delivery of HK Raf-1 siRNA Polyplexes Inhibits MDA-MB-435 Xenografts

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Abstract

Our past research has focused on identifying an effective carrier composed of histidine and lysine for delivery of nucleic acid into cells. For this purpose, we developed histidine-lysine-rich (HK) polymers with specific sequences and branching. We have found that branched HK polymers in complex with Raf-1 siRNA markedly decreased Raf-1 mRNA and induced apoptosis in cell lines *in vitro*. The primary focus of the present study was to determine an effective carrier to deliver siRNA systemically to tumor xenografts. After comparing HK:Raf-1 polyplexes for their *in vivo* efficacy, we investigated in greater detail whether one of these polymers, H3K(+H)4b, in complex with Raf-1 siRNA, inhibited the growth of MDA-MB-435 xenografts. H3K(+H)4b is a four-branched HK peptide whose predominant repeating sequence within the terminal arm is -HHHK-. After the first tail vein injection in a mouse model, there was a statistically significant reduction in tumor size between the H3K(+H)4b:Raf-1 siRNA treated and the control groups (P<0.01). By the third injection, there was nearly a 50% reduction in the Raf-1 siRNA-treated group compared to the control siRNA-treated or untreated group. Consistent with a significant effect of the HK:Raf-1 polyplex on the tumor, there were marked histological changes, increased apoptosis, and fewer vessels in the Raf-1 siRNA-treated group. Raf-1 protein within the tumor was significantly decreased after treatment with the HK:Raf-1 siRNA polyplex compared to the control treatment groups. Despite the striking effect on the tumor by the HK Raf-1 siRNA, there was little evidence of toxicity in normal tissues with this therapy. By harnessing the ability to modify the amino acid sequence and branching of HK polymers, we expect continued development of HK polymers as *in vivo* carriers of siRNA.

Keywords

Histidine; lysine; peptides; Raf-1; tumor xenograft

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Introduction

The ability to down-regulate target genes by using double-stranded RNA interference (RNAi) has revolutionized basic science research on signal transduction and gene function [1]. RNAi also has tremendous therapeutic potential for treating diseases such as cancer in which an oncogene or angiogenic growth factor is overexpressed. Despite the promise of siRNA therapy, it shares the classic delivery problem of antisense and gene therapies: nucleic acids are highly negatively charged and cannot easily be transported through the blood stream to their cytosolic targets. A host of carriers for siRNA are now being tried in several animal disease models including cholesterol conjugates [2], liposomes (e.g., stable nucleic acid lipid particles), atelocollagen [3], cyclodextrin [4–6], polyethylenimine [7,8], and fusion protamine-antibody carriers [9].

We have focused on developing histidine-lysine (HK) polymers as carriers of nucleic acids including siRNA. To this end, we developed a series of such polymers with specific sequences and branching [10,11]. Whereas the histidine component is essential for buffering and lysing endosomes, thereby releasing the nucleic acid *in vitro*, the lysine component is important in binding the HK polymer with the negatively charged phosphates of the nucleic acids. Even though HK polymers can deliver both siRNA and DNA, the requirements for successful delivery of siRNA usually differ from those for delivering DNA. For example, we have found that H2K4b with a predominant repeating pattern of -HHK- was an effective carrier of plasmids, but was ineffective as a carrier of siRNA *in vitro*. For delivery of siRNA to SVRbag4 cells expressing β-galactosidase, the most effective polymer was the eight-branched peptide, H3K8b, although this polymer was not particularly effective as a carrier of plasmids [12]. The predominant pattern in H3K8b within the terminal branch is the repeating amino acid sequence of -HHHK- (e.g., H3K8b). In addition to being an effective carrier of siRNA into SVRbag4 cells, H3K8b proved effective in several cell lines as an *in vitro* carrier of Raf-1 siRNA and resulted in reduced Raf-1, decreased cell number, and induced apoptosis in several cell lines [13]. Selection of the optimal HK polymer for nucleic acid delivery appears to depend on various patterns within the HK polymer that have a role in binding and release of different forms of nucleic acids. Although the eight-branched polymer, H3K8b, was an effective carrier of siRNA *in vitro*, the four-branched HK polymer, H3K4b, was significantly more effective carrier of siRNA when injected intratumorally [13]. HK carriers with fewer than four branches were less effective as carriers of nucleic acids (DNA or RNA) unless combined with another carrier (e.g., liposomes) [14–16]. Thus, together with different amino acid patterns, the degree of branching and transport medium may be important in determining the most effective HK carrier of siRNA.

To determine whether one or more of several HK siRNA polyplexes can effectively reduce tumor growth, Raf-1 was targeted. We and other laboratories have found that targeting Raf-1 through a variety of therapeutic strategies can reduce the growth of tumor xenografts [13,17–20]. Raf-1 also has a central role in tumor angiogenesis. In endothelial cells, oncogenic Ras or its activated substrate, Raf-1, is associated with increased levels of vascular endothelial growth factor (VEGF), which is the primary growth factor responsible for inducing tumor angiogenesis [21]. Furthermore, because it is differentially phosphorylated at separate sites by VEGF or bFGF signal transduction pathways, Raf-1 protects endothelial

cells from apoptosis [22]. Finally, Raf-1 has also been shown to increase cancer metastases [23]. Thus, Raf-1 is an attractive therapeutic target for siRNA since its down-regulation affects multiple pathways, including tumor angiogenesis, tumor cell growth, and formation of metastases.

Our current goal is to develop an effective HK carrier for systemic delivery of Raf-1 siRNA to inhibit the growth of tumors *in vivo*. Consequently, several systemically injected HK peptides, differing in their branching and their terminal amino acid patterns, were compared for their ability to reduce the growth of tumor xenografts. We found that systemically delivered HK polymers in complex with Raf-1 siRNA can effectively reduce MDA-MB-435 xenografts in a mouse model.

MATERIALS AND METHODS

Cell line

MDA-MB-435 cells, a human malignant cell line, and SVRbag4, a mouse transformed endothelial cell line, were obtained from the American Type Culture Collection and maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal calf serum (FCS) and 20 mM glutamine. Primary human umbilical vein endothelial cells (HUVEC, Clonetics) were grown in EGM-2 Bullet Kit medium (Clonetics).

Polymers

The biopolymer core facility at the University of Maryland synthesized HK polymers on a Ranin Voyager synthesizer (PTI) as previously described. Three polymers (H3K8b, H3K(+H)4b), H3K(+G)4b) were used as siRNA carriers in this study. H3K8b has 8 terminal branches and a molecular weight of 22 922; H3K(+H)4b and H3K(+G)4b have 4 terminal branches and molecular weights of 10 191 and 10 539, respectively. See Table 1 for structure of the three HK polymers. Nomenclature of HK polymers: 1) for H3K4b, the dominant repeating sequence in its terminal branch is -HHHK-, thus "H3K" is part of the name; the "4b" refers to the number of terminal branches; 2) for H3K(+H)4b, four-branched analog of H3K4b in which 1 extra histidine is inserted in the terminal branch of H3K4b; 3) for H3K(+G)4b, four-branched analog of H3K4b in which 2 glycines are interspersed within the dominant pattern of the terminal branch; 4) for H3K8b, an eight-branched HK polymer with a dominant terminal sequence of –HHHK-.

SiRNA

The siRNA sequences were screened for lack of homology to other genes within the mouse and human transcriptome to minimize cross-hybridization and inadvertent knockdown of unrelated genes by off-target effects.

Raf-1 siRNA—The Raf-1 siRNA duplex is as follows: Sense, 5′-UGU CCA CAU GGU CAG CAC C-dTdT-3′; Antisense, 5′ GGU GCU GAC CAU GUG GAC AdTdT-3′

Control siRNA—Sense, 5′-CAG UUG CGC AGC CUG AAU GdTdT-3′; Antisense, 5′-CAU UCA GGC UGC GCA ACU GdTdT-3′; (no target, Qiagen)

Fluorescent labeled siRNA—Alexa Fluor 555 Allstars siRNA (no target, Qiagen)

β-galactosidase siRNA—Sense 5′-CAG UUG CGC AGC CUG AAU G dTdT-3′; Antisense, 5′-CAU UCA GGC UGC GCA ACU GdTdT-3′.

In vitro siRNA Transfection

Two cell lines, MDA-MB-435 and HUVEC, were examined for the ability of HK in complex with Raf-1 siRNA to inhibit cell growth and to induce apoptosis. The SVRbag4 cell line, which expresses β-galactosidase, was also examined for the ability of the HK polymers in complex with β-gal siRNA to reduce expression of the intracellular marker. In brief, 3×10^4 cells were plated into a 24-well plate containing 500 μl of DMEM and 10% serum. After 24 h, when the cells were 40% confluent, transfection polyplexes were added to the media. To prepare polyplexes of HK:siRNA, siRNA (2 μg) in 50 μl of OptiMEM was briefly mixed well with HK (16 μg) and maintained at room temperature for 30 min. This polyplex was then added dropwise to the cells in 0.5 ml of DMEM/10% serum; 24–48 h after transfection, cell number or β-galactosidase activity was determined as described previously [12,13]. Data are represented as percent of untreated control.

Measurement of particle size of HK siRNA polyplexes

H3K8b, H3K(+H)4b and H3K(+G)4b, (300 μg) in complex with siRNA (75 μg) (4:1 wt:wt, polymer: siRNA) were prepared similarly to the transfection polyplexes described previously. After the polyplexes were prepared in D_5W (5% Dextrose; 375 μl) for 30 min, water (625 μl) was added. Particle size was determined by measurement of light scattering at a 90-degree angle on an N4 Submicron Particle Size Analyzer (Beckman Coulter, Hialeah, FL), and is reported as the average size obtained from a Unimodal analysis carried out using the software provided by the instrument manufacturer. Each data point represents the mean ± S.D. of three measurements.

Preparation of HK:siRNA polyplexes for *in vivo* experiments

To the siRNA (50 μg in 125 μl of D_5W), HK (100, 200, 300, or 400 μg in 125 μl D_5W) was added quickly and mixed briefly with a Vortex mixer. After the polyplexes were maintained for 30 min at room temperature, 250 μl of each were injected into the tail vein of each mouse.

In vivo xenograft experiments

Female adult nude mice (4–6 weeks) were obtained from NCI Frederick. MDA-MB-435 cells (7×10^5 cells/injection) were injected bilaterally into the mammary fat pads of each mouse. Ten days later, when the subcutaneous tumors were established, the mice were separated into groups of four mice to determine efficacy of the HK in complex Raf-1 siRNA. Depending on the experiment, the mice were given two or seven intravenous injections of polymer-siRNA complex, each separated by 4 days (50 μg of siRNA/tumor/injection and various amounts of HK). Polyplexes were administered every three days based on down-regulation of the target gene and maximum tumor response and recovery from therapy. Tumor size was assessed with caliper measurements of the tumors in two dimensions before

each injection and 3 days after the last injection; the volume was calculated by formula $1/2 \times$ length×width^2. Animal care and treatment were conducted in accordance with established guidelines and protocols approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee.

Tumor localization and distribution

H3K(+H)4b in complex with fluorescently-labeled (AlexaFluor 555) siRNA was used to validate siRNA delivery. After implanted MDA-MB-435 tumor xenografts reached 50 mm^3, H3K(+H)4b in complex with AlexaFluor-555 siRNA was administered by i.v. injections; mice were euthanized 6 h later. Frozen sections were prepared from several organs (lungs, liver, kidney) and tumor xenografts. Images of these tissues were obtained with a Diaphot-TMD fluorescence microscope fitted with a Z-motor and deconvolved with 3-D Volocity Restoration software.

Immunohistochemical detection of Raf-1, Ki67, and CD31

Tumors were fixed with 4% formalin overnight, after which tumor sections (5 μm) were deparaffinated with xylene and hydrated with ethanol. Antigens were retrieved in 10 mM citrate, pH 6.0, by boiling for 20 min, then cooling to room temperature. Endogenous peroxidase activity was blocked by 3% H_2O_2 in 100% methanol for 10 min, and the sections were then incubated with 3% goat serum for 30 min at room temperature. Rabbit anti-human polyclonal antibody (Raf-1, diluted 1:100, Genscript, Ab-259; Ki67, 1:200 dilution, Chemicon; CD31, 1:100 dilution, Cell Signal) was added to the tumor sections for 1 h at room temperature and the secondary horseradish peroxidase-labeled antibody was applied to the sections for 30 min at room temperature (Histoscan HRP universal rabbit kit; Biomeda, cat. No.06-602). The chromagen diaminobenzene (DAB) was incubated with the tumor sections for 10 min to permit color development, and the tumor sections were dehydrated and mounted with glass coverslips. A brown color indicated positive staining.

In vivo apoptosis as determined by TUNEL assay

The TUNEL (terminal deoxynucleotidyl transferase) assay was done according to the manufacturer's instructions in the FragEL™ DNA Fragmentation Detection Kit (Calbiochem). The tumors were first fixed in 4% formalin, embedded in paraffin, and tissue sections were then deparaffinated. After the sections were treated with proteinase K (20 mg/ml) for 20 min at room temperature, endogenous peroxidase was inhibited with 3% H_2O_2. The TUNEL reaction mixture (deoxynucleotidyl transferase) was incubated with the tumor section for 90 min at 37°C in a humidified chamber. The labeling reaction was then stopped and conjugated buffer was added to the sections for 30 min at room temperature. After the DAB substrate was applied to the tumor sections for 10 min, the slides were mounted with glass coverslips and examined by light microscopy.

Apoptotic and proliferation Index

To determine the apoptotic and proliferation indices, four randomly chosen fields (100×) of stained slides were examined and the total numbers of cells and positively stained cells in each field were then counted. The percentages of apoptotic and proliferating cells were

calculated by the following formula: (number of positively stained cells/total tumor of cells in a field) × 100.

Statistical analysis

When multiple groups were compared, results were analyzed using one-way analysis of variance with multiple comparisons (Bonferroni's t-test); when only two groups were compared, results were analyzed using a two-tailed Student's t-test. Values of P < 0.05 were considered significant.

Results

In vitro comparison of HK polymers as carriers of siRNA

We had previously found that H3K8b was an effective carrier of siRNA *in vitro*. Thus, in SVRbag4 cells expressing β-galactosidase, the H3K8b siRNA polyplex reduced β-gal by nearly 75% compared to untreated cells. In a similar study (Fig 1A), we compared the efficacy of H3K8b with two other polymers, H3K(+H)4b and H3K(G)4b, for their ability to inhibit β-gal in SVRbag4 cells. As shown in Fig. 1, the H3K8b siRNA polyplex was markedly more effective than H3K(+H)4b, reducing β-gal by 71% (P<0.001), compared with 43% for the latter polyplex (P<0.01); H3K(+G)4b, a polymer which contained glycine in its terminal branches, was the least effective.

Surprisingly, in two cell lines, H3K8b was not the optimal carrier of siRNA. In complex with Raf-1 siRNA, H3K(+H)4b was more effective in reducing the number of MDA-MB-435 cells compared to H3K8b (Fig. 1B). H3K(+H)4b:Raf-1siRNA polyplex reduced cell numbers by about 60% while the H3K8b polyplex reduced the cell number by 35%. In HUVEC, H3K8b: and H3K(+H)4b:Raf-1 siRNA polyplexes were comparable in reducing cell number by 53% and 64%, respectively (Fig. 1C). We also examined the toxicity of these polymers. Compared to the H3K8b polyplex, H3K(+H)4b in complex with the control siRNA had moderately increased toxicity (Fig. 1B, 1C). Whereas the H3K8b control siRNA polyplex exhibited little to no toxicity compared to untreated cells, the H3K(+H)4b control siRNA polyplex showed 11% and 18% reduction in number of HUVEC and MDA-MB-435 cells, respectively.

In a previous study, we had found that plasmids delivered by HK polyplexes were markedly affected by conditions used for their preparation; in particular, salt conditions that altered the HK polyplex from spherical to needle-like morphology were associated with higher transfection activity [24]. In contrast, the efficacy of the three HK-siRNA polyplexes used in this study was not affected by salt conditions during their formation. We found no evidence of needle-like morphology of HK siRNA polyplexes despite their formation in medium of different salt concentrations; indeed, HK siRNA polyplexes had a spherical morphology when they were formed in different media. Not surprisingly, regardless of whether the HK-siRNA polyplex was formed in water, isotonic salt, or sequential water and isotonic salt conditions, reduction of its mRNA targets was similar (data not shown).

H3K(+H)4b and H3K8b are comparable carriers of Raf-1 siRNA *in vivo*

We then screened the three HK polymers to determine which was the most effective carrier of Raf-1 siRNA in reducing the growth of tumor xenografts. After the tumors reached 20 mm^3, we injected HK polymers (200 μg) in complex Raf-1 siRNA (50 μg) into the tail vein of a mouse tumor xenograft model. After the second injection, we assessed the antitumor efficacy of each of these HK polyplexes. H3K(+H)4b and H3K8b Raf-1 siRNA polyplexes reduced the growth of tumors to a comparable degree (Fig. 2A) (P<0.02, untreated vs. H3K(+H)4b, H3K8b). Similar to the situation *in vitro*, H3K(+G)4b was an ineffective carrier of Raf-1 siRNA *in vivo* (Fig. 2A). The size of the polyplexes could not explain differences in their antitumor efficacy, because the H3K(+H) K4b (230.2 ± 129 nm) and H3K(+G)K4b (274.3 ±128) polyplexes were comparable in size, while H3K8b polyplexes were smaller (137±56 nm) (Table I).

Systemically delivered H3K(+H)4b:Raf-1 siRNA markedly reduce tumor growth

Because the four-branched H3K(+H)4b was markedly easier to synthesize than is the eight-branched H3K8b peptide, we selected this polymer to study in greater detail. We first examined the optimal ratio of H3K(+H)4b to siRNA. We examined four ratios (w/w; 2:1, 4:1, 6:1, 8:1) and found that the 4:1 ratio was the most effective in reducing tumor growth (Fig. 2B) (P<0.05, untreated vs 4:1 ratio). To examine uptake by the tumor and normal tissues of the polyplex, the HK peptide in complex with Alexafluor-labeled siRNA was injected into tumor-bearing mice. Six hours later, frozen sections of the tumor and tissues were prepared. Intratumoral accumulation of fluorescently-labeled H3K(+H)4b/polyplex was evident (Fig. 3) and there was widespread deposition in other tissues of these polyplexes with the greatest accumulation in the kidneys.

With the optimal HK:siRNA ratio, we evaluated in greater detail by continuing for 7 injections the H3K(+H)4b :Raf-1 siRNA polyplex in reducing tumor xenografts. After the tumors became visible, H3K(+H)4b in complex with Raf-1 siRNA (50 μg) was injected every 4 days for seven injections. After the first injection, there was a 40% reduction in tumor size between the HK/Raf-1 polyplex and the untreated control group (Fig. 4, P<0.01, untreated, control siRNA vs. Raf-1 siRNA). After the third injection, there was nearly a 50% difference between the control and Raf-1 siRNA treatment groups (Fig. 4, P<0.01, untreated, control siRNA vs. Raf-1 siRNA). This difference in tumor size between the control and Raf-1 treatment groups was maintained through the last (seventh) injection.

H3K(+H)4b:Raf-1 siRNA polyplex induces marked histological and immununochemical changes within the tumor

Histology and immunological staining studies showed marked antitumor effects in the H3K(+H)4b:Raf-1 siRNA treatment group (Fig. 5). Three days after the last treatment, mice were euthanized, and tumors and tissues were removed and examined histologically. Tumors from mice treated with H3K(+H)4b-Raf-1 siRNA polyplex showed marked cell death within the tumor compared to control siRNA polyplex or the untreated groups. Furthermore, in viable areas of the tumor, there was marked reduction of Raf-1, suggesting that therapeutic Raf-1 siRNA reduced its desired target. Because Raf-1 has a significant role in cellular mitosis, tumor angiogenesis, and preventing apoptosis, we examined whether the Raf-1

siRNA polyplex affected these pathways in the tumor. As evidenced by CD31 immunohistochemistry, the number of tumor vessels was decreased. Moreover, decreased proliferation and increased apoptosis were observed in the Raf-1 siRNA-treated group (Fig. 6). Proliferation and apoptotic indices of tumors validated the impression from the immunohistochemical images (Fig. 6). While the proliferation marker, Ki67, was decreased by nearly 50% within tumor sections, apoptosis increased by nearly 700% (P<0.001, untreated, control siRNA vs. Raf-1 siRNA). We also examined the histology of a variety of tissues (heart, lungs, liver, and kidneys; Fig. 7); there was no toxicity observed despite seven injections of the H3K(+H)4b siRNA polyplexes.

Discussion

Prior to studying the effects of amino acid patterns of HK on siRNA delivery, we examined conditions that might increase the efficacy of such delivery systems, because we had previously found that changes in the medium during the preparation of the HK:plasmid polyplexes were associated with a needle-like morphology and a marked increase in gene expression [24]. Unlike plasmids delivered by HK peptides, however, perturbations in the salt conditions during the HK polyplex formation failed to affect morphology or efficacy of siRNA polyplexes (data not shown). This likely indicates significant differences between the binding of plasmids and siRNA to HK peptides.

As a result, it perhaps is not surprising that there were differences in the ability of various HK peptides to transport siRNA and plasmids *in vivo* [12]. We considered a number of sequence and branching patterns of the HK polymer that might improve transport of siRNA. Although the H2K4b polymer with a repeating pattern of -HHK- was effective as a carrier of plasmids *in vitro* and *in vivo* [11,25], it was not an effective carrier of siRNA under either condition (data not shown). Thus, in this study we focused on HK polymers with a repeating pattern of -HHHK-, which we had previously shown to be effective for transporting siRNA *in vitro*. Of the polymers tested for siRNA delivery in a screening assay to silence β-gal expression *in vitro*, H3K8b was the most effective [12]. This is a highly branched polymer with a histidine-rich domain and terminal branches with a repeating pattern of –HHHK-. Initially, H3K8b was synthesized because its histidine-rich domain was expected to buffer the acidic endosomal compartment and thereby enable nucleic acid to escape from endosomes. This strategy was only partially successful in that the polymer augmented siRNA delivery but not plasmid delivery. In our tumor xenograft model, H3K8b was an effective carrier *in vivo* and did not exhibit significant toxicity. For several reasons, however, we sought other polymers that might be effective carriers of siRNA. Compared to H3K(+H)4b, H3K8b is expensive, more challenging to synthesize, and more difficult to modify by pegylation at specific sites. We therefore screened a number of polymers that had only four branches, but with the repeating patterns of -HHHK-.

In general, four-branched polymers with this pattern were effective carriers of siRNA *in vivo* except for H3K(+G)4b. Because glycine has the smallest side group and may increase the flexibility of the terminal branch, we tested the effect of adding glycine to the -HHHK- pattern. Insertion of glycines within the terminal branches of H3K(+G)4b decreased the efficacy of siRNA delivery significantly both *in vitro* and *in vivo* compared to the

H3K(+H)4b carrier. Perhaps, the mechanism for the difference in these two peptides for their ability to carry siRNA into cells is due to the reduced α-helical structure of H3K(+G)4b. Indeed, preliminary data with circular dichroism spectroscopy established that H3K(+G)4b has less α-helical structure in a hydrophobic environment compared to its H3K(+H)4b counterpart. The differences in the structure between these two polymers may affect interaction and release of siRNA. We are continuing our biophysical and structural studies to understand why H3K(+H)4b and H3K(+G)4b differ as carriers of siRNA.

Although HK siRNA polyplexes appear to have significant tumor-inhibitory activity with low toxicity *in vivo*, developing therapies such as this one often fail because toxicity reduces the therapeutic index window. To identify polymers with increased efficacy with lower toxicity, one strategy is to examine a variety of HK polymers with different amino acid sequences. For instance, preliminary studies suggest that H3K(+H)4b and a close analog H3K4b in complex with Raf-1 siRNA have comparable antitumor activity when given intravenously. Nevertheless, there were differences in toxicities between these two HK polymers *in vitro* even though their amino acid sequences differ by only a single histidine in each branch; the H3K(+H)4b polyplex is moderately more toxic *in vitro* compared to the H3K4b siRNA polyplex (data not shown). Although H3K(+H)4b siRNA showed no toxicity *in vivo* at dosages used in this study, the *in vitro* findings raise the possibility that there may be differences in toxicity among polymers and their polyplexes *in vivo* at higher dosages. A complementary strategy that our lab is developing to improve HK carriers with reduced toxicity is to increase the specificity and delivery of HK siRNA polyplexes to the tumor target. Although toxicity of HK siRNA polyplexes appears minimal, their wide distribution in a variety of tissues suggests that modification of HK with PEG and with a ligand may increase the specificity, increase antitumor efficacy, and decrease potential toxicity of the polyplexes. At least for luciferase-expressing plasmids, modification of HK peptides with PEG and a tumor-selective ligand has improved the efficacy and specificity of these polyplexes (data not shown). Other laboratories have reported similar findings [7,26–28]. There are some distinguishing characteristics in pegylating the HK polymer compared to other polymers. By incorporating cysteines within the polymer, the precise location of pegylation is known and the number of PEGs/molecule is the same. In contrast, many polymers such as polyethylenimine are randomly pegylated and an average number of PEGs/molecule is obtained.

In summary, we screened several HK polymer candidates for their ability to carry Raf-1 siRNA systemically to tumor xenografts in a mouse model. Although the eight-branched polymer was an effective carrier, the easier to synthesize and less expensive four-branched carrier was equally effective. In future studies, further modification of the four–branched HK peptide either through its amino acid sequence and/or by addition of PEG/ligand is required to improve its ability to carry siRNA systemically.

Acknowledgments

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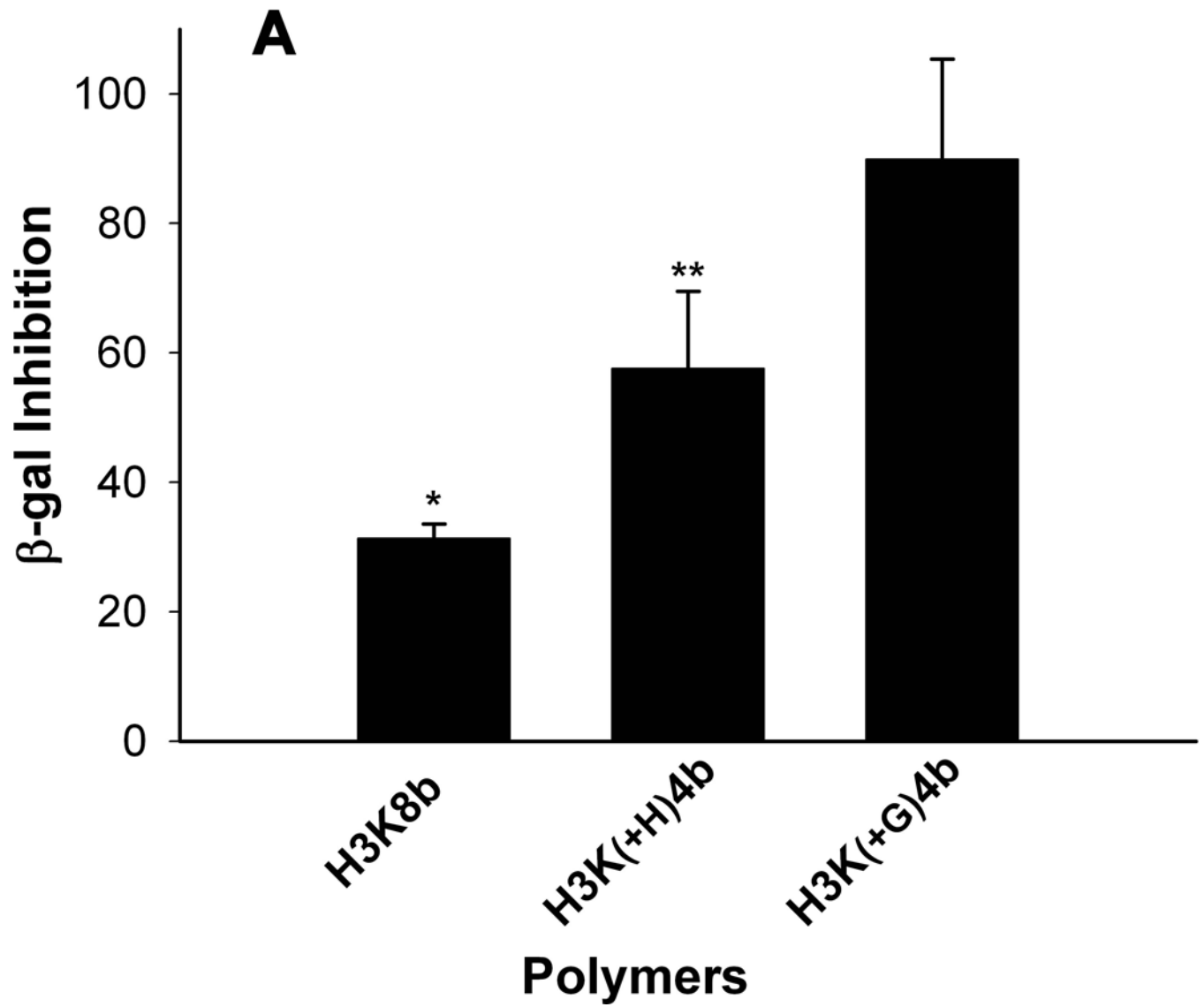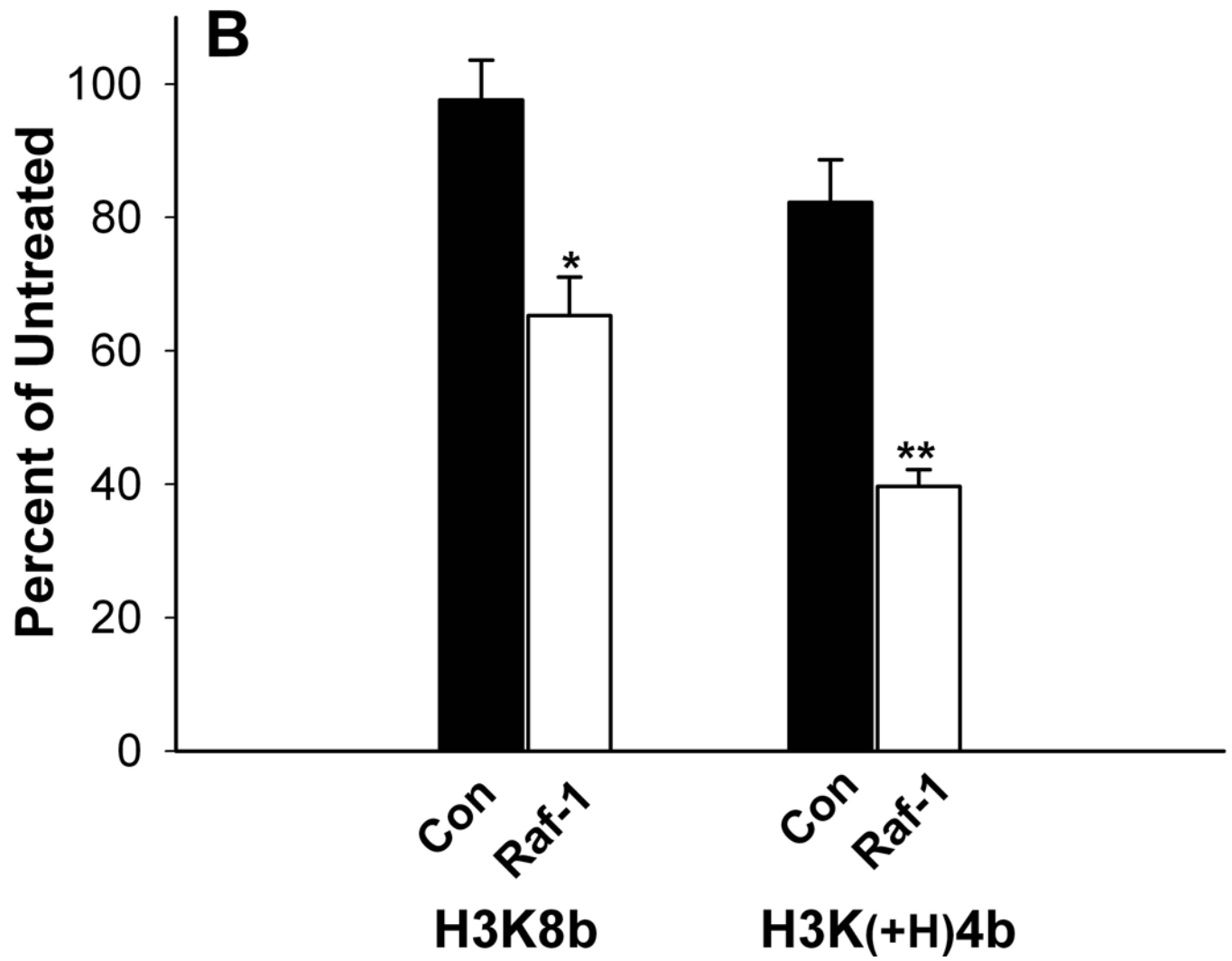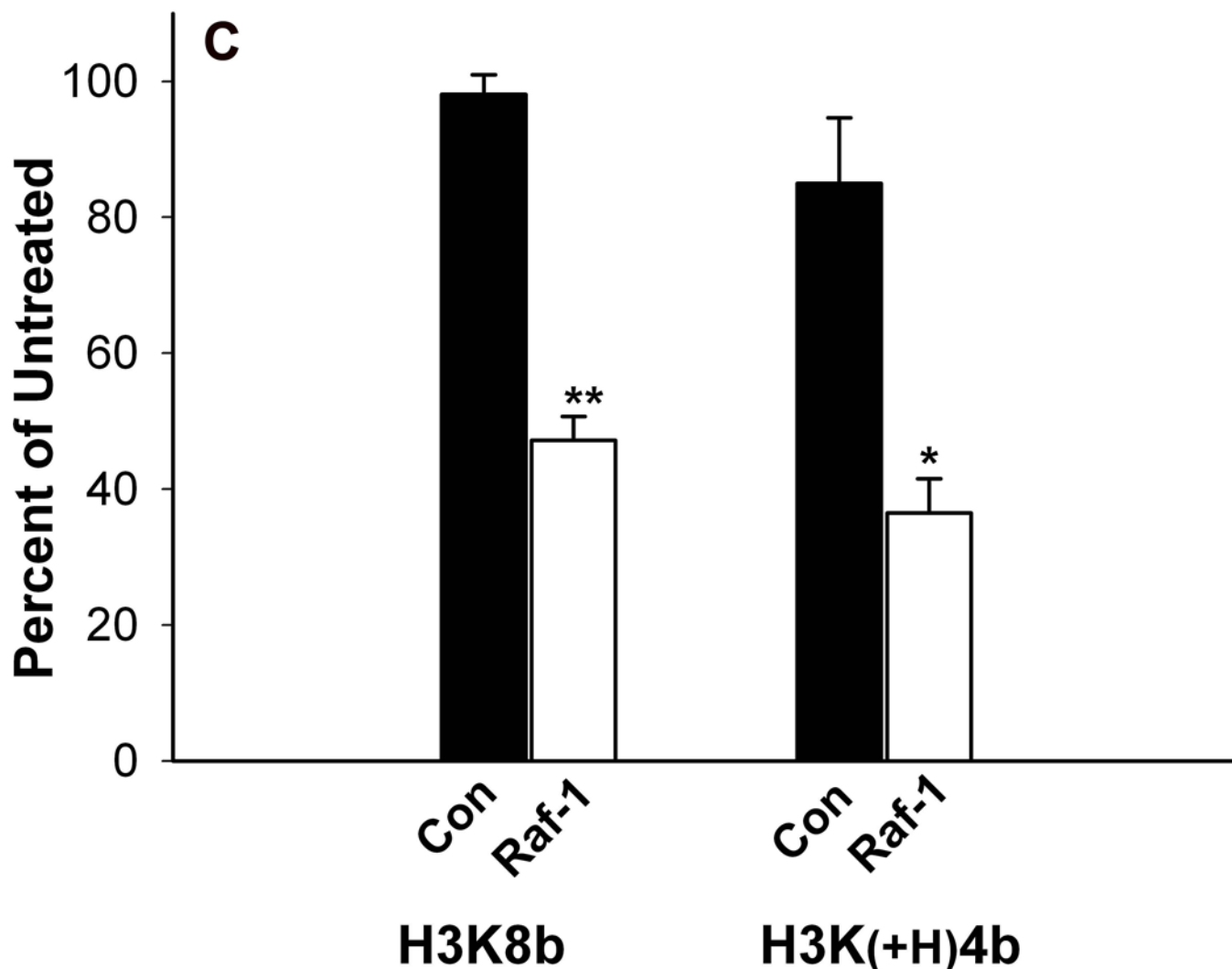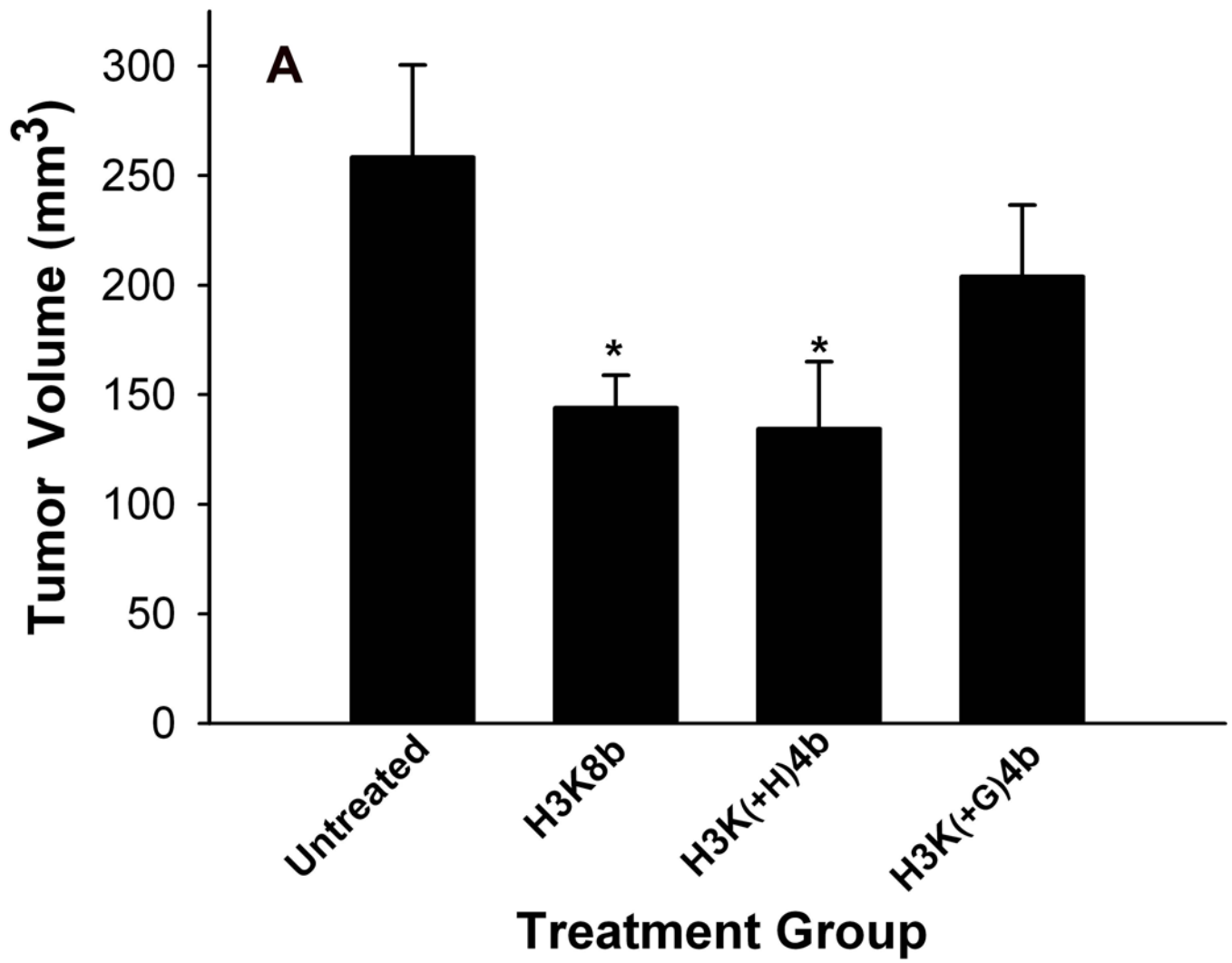
Figure 1.
Comparison of HK polymers as carriers of siRNA in cell lines. Cells (5×10^4 cells per well; A, SVRbag4; B, MDA-MB-435; C, HUVEC) were plated into 24-well plates. After 24 h, when the cells were 50% confluent, 2 μg of siRNA in complex with an HK polymer was prepared and added to the cells as described under "Method and Materials." A) Three carriers (H3K8B, H3K(+H)4B, H3K(+G)4B) of β-gal siRNA were tested for their ability to inhibit β-galactosidase expression in SVRbag4 cells. *, P<0.001, untreated vs. H3K8b; **, P<0.01, untreated vs. H3K(+H)4b (Multiple Comparisons versus Control Group (Bonferroni t-test). B, C) Forty-eight hours after transfection with HK: Raf-1 siRNA, MDA-MB-435 (B) or HUVEC (C) cells were treated with trypsin-EDTA and counted in a Coulter apparatus. *, P<0.001; **, P=0.002, Raf-1 siRNA vs. Control siRNA in MDA-MB-435 cells or HUVEC cells (Student t-test). H3K(+H)4b in complex with the control siRNA showed an 11% and 18% reduction in HUVEC and MDA-MB-435 cells, respectively, compared to untreated cells. Results represent percent of untreated controls and are expressed as the mean ± S.D. of three independent experiments.
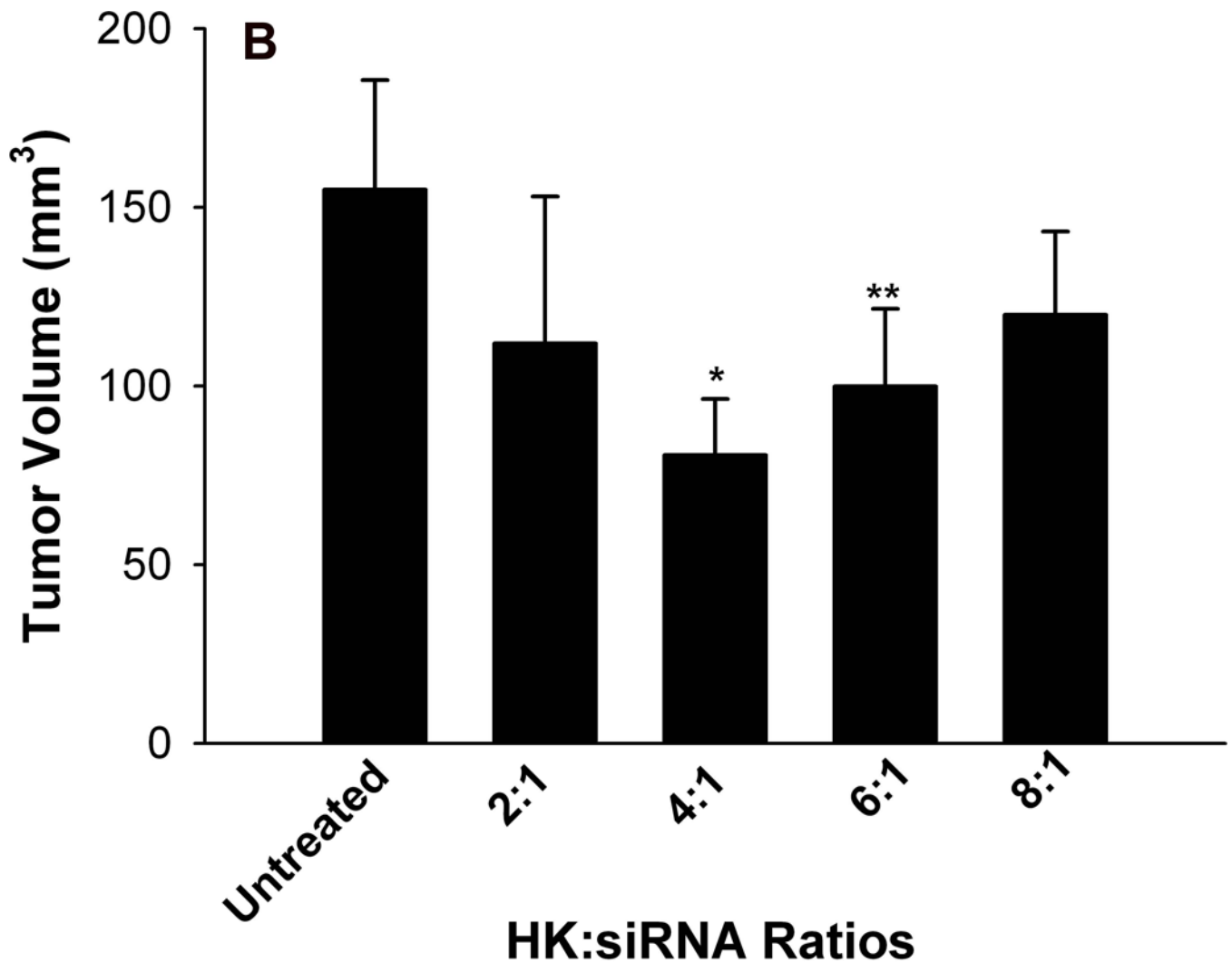
Figure 2.
Selection and optimization of HK polymer for systemic delivery of siRNA. A) *In vivo* screening of HK polymers to determine effective carrier of Raf-1 siRNA. Ten days after the injection of MDA-MB-435 cells into the mammary fat pad, mice with visible tumors were separated into treatment groups (untreated, H3K(+G)4b/Raf-1 siRNA, H3K(+H)4b/Raf-1 siRNA, H3K8b/Raf-1 siRNA). Each group had 4 mice with 8 tumors and tumor size and treatment efficacy were evaluated after the second injection. Results of tumor size per treatment group 3 days after the second injection are expressed as the mean ± S.D. *, P<0.02: H3K(+H)4b, H3K8b vs untreated. B) Determination of the optimal ratio of H3K(+H)4b/siRNA polyplex that inhibits tumor growth. After MDA-MB-435 xenografts became visible, different ratios of H3K(+H)4b to Raf-1 siRNA were administered to determine the optimal ratio (2.0, 4.0, 6.0, 8.0: 1). After two injections, tumor size was assessed in the treatment and untreated groups. P<0.05, untreated vs. 4:1 ratio, one-way analysis of variance with multiple comparisons versus untreated group (Bonferroni t-test)
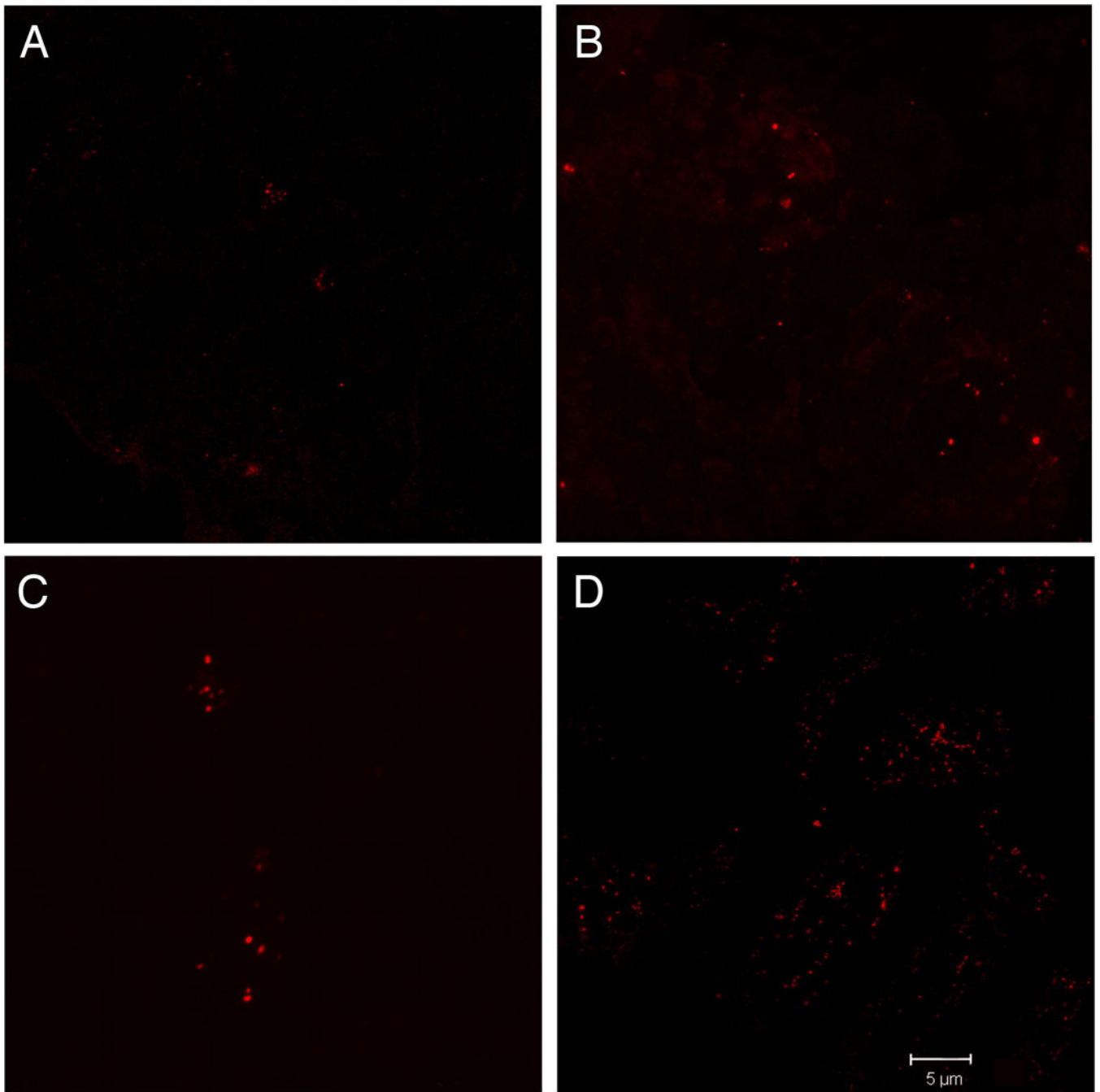
Figure 3.
Distribution of HK siRNA polyplex in tissues after systemic injection. H3K4b in complex
with fluorescently-labeled (AlexaFluor 555) siRNA was used to validate siRNA delivery.
After implanted MDA-MB-435 tumor xenografts reached 50 mm^3, H3K4b in complex with
AlexaFluor-555 siRNA was administered by i.v. injection; mice were euthanized 6 h later.
Frozen sections were prepared from several organs (lungs, liver, kidney) and tumor
xenografts. Images of these tissues were obtained with a Diaphot-TMD fluorescence

microscope fitted with a Z-motor and deconvolved with 3-D Volocity Restoration software. A, lung; B, tumor; C, liver; D, kidney.
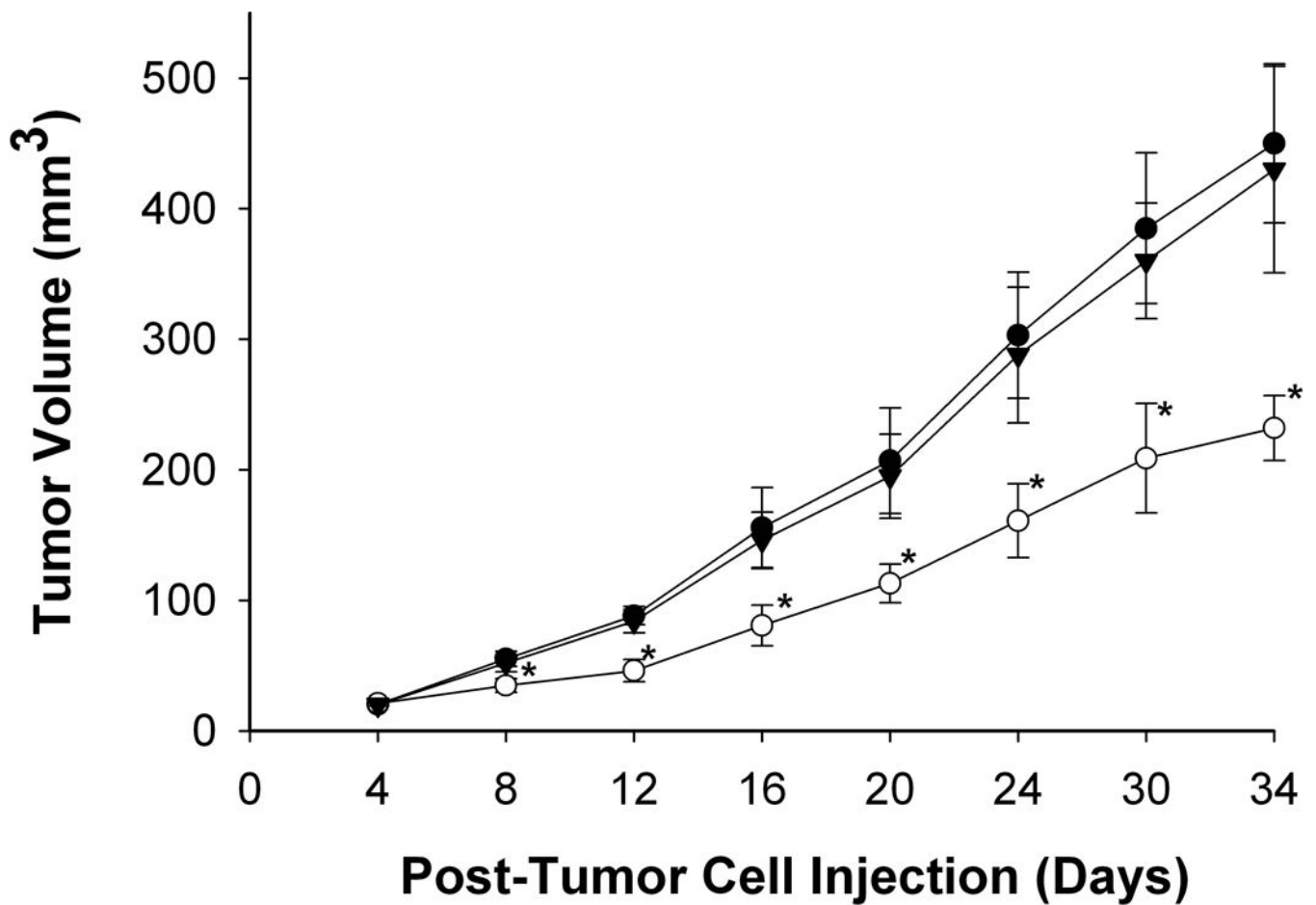
Figure 4.
Systemic injection of H3K(+H)4b: Raf-1 siRNA polyplex into athymic nude mice bearing MDA-MB-435 tumors. Tumor volume (mm^3) was measured in the groups (untreated-close circles, control siRNA-close inverted triangles, or Raf-1 siRNA-open circles) before to each treatment injection time. After the tumors became visible, H3K(+H)4b (200 µg) in complex with siRNA (50 µg) were injected every 4 days for seven injections. By the second injection, there was a significant difference between the control siRNA and Raf-1 siRNA groups. *, $P<0.01$. One-way analysis of variance with multiple comparisons versus control siRNA or untreated group (Bonferroni's t-test).
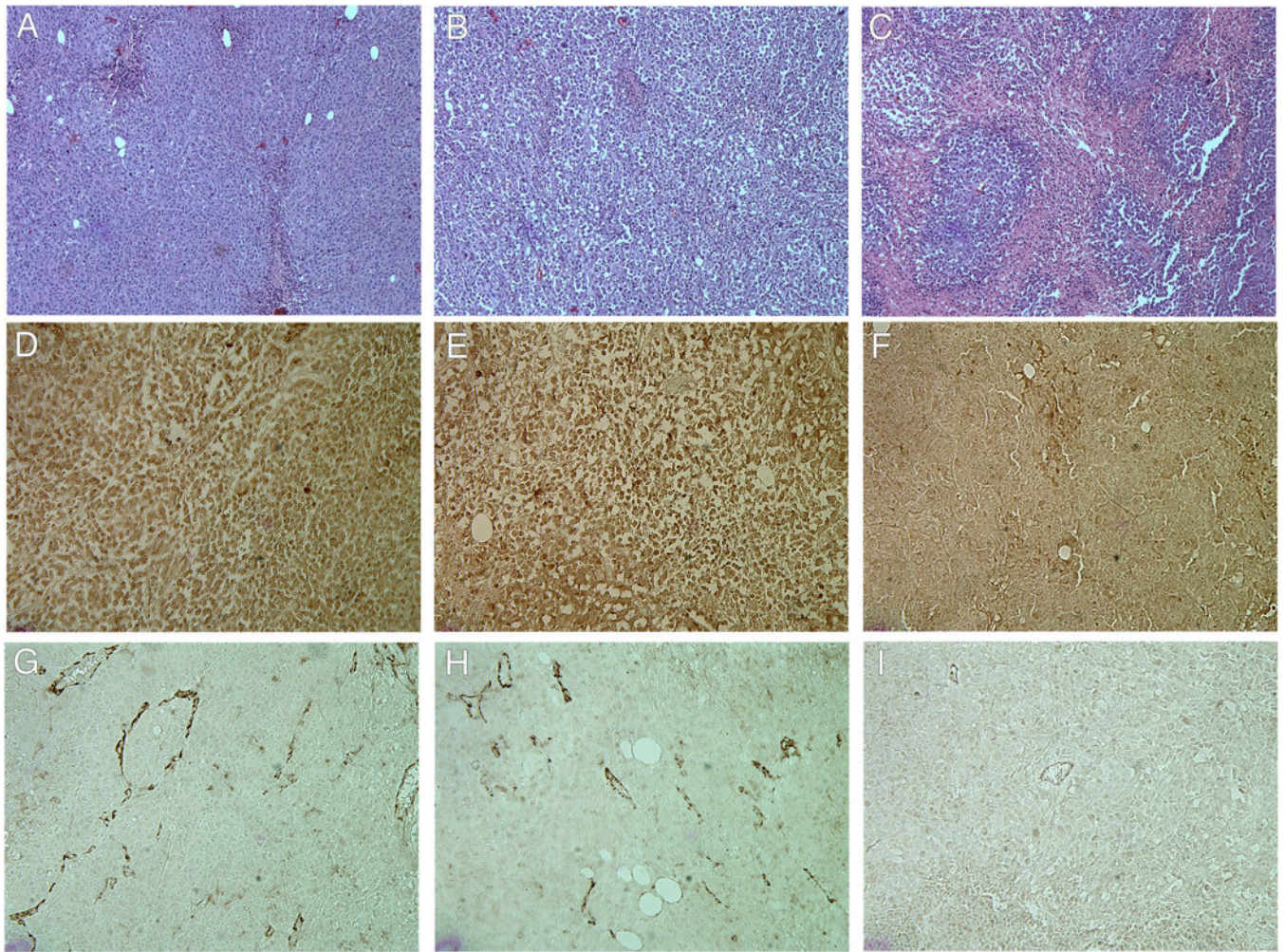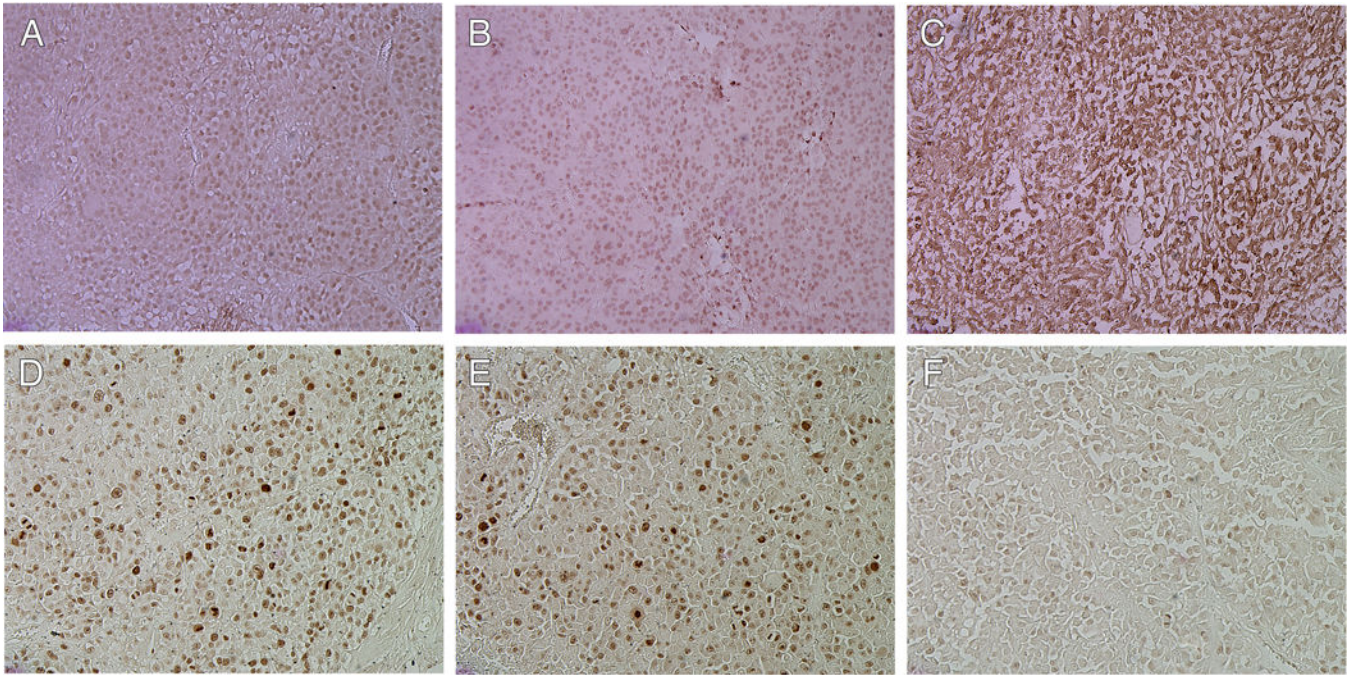
Figure 5.
Histologic and immunohistochemical analysis of CD31 and Raf-1 content of tumor xenografts. Hematoxylin and eosin staining of tumors excised from mice after various treatments (A, untreated; B, control siRNA; C, Raf-1 siRNA). Immunohistochemical staining detection of Raf-1 expression (D, untreated; E, control siRNA; F, Raf-1 siRNA). A diaminobenzidine substrate was used to visualize antibody binding to Raf-1 and is shown as the dark brown precipitate. Immunohistochemical staining detection of CD31 to determine vessel content within treatment groups (G, untreated; H, control siRNA; I, Raf-1 siRNA). For further details, see "Materials and Methods."
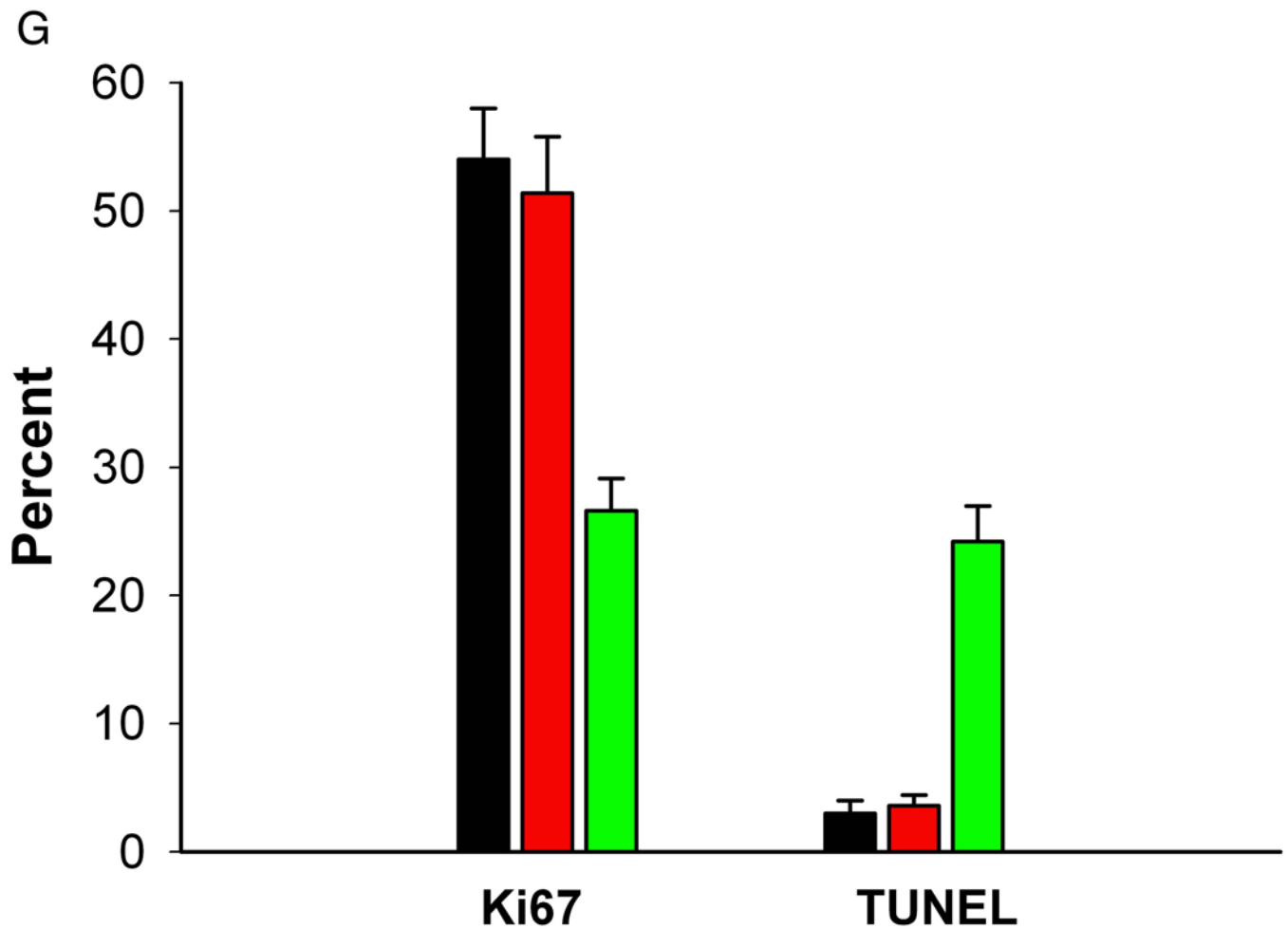
Figure 6.
Proliferation and apoptosis of tumors. Apoptosis of tumor induced by Raf-1 siRNA (C), control siRNA (B), or untreated (A) as determined by TUNEL assay. Detection of Ki67 as an indicator of proliferation of tumor cells within various treatment groups (D, untreated; E, control siRNA, F, Raf-1 siRNA) × 100 magnification. Proliferation and apoptotic indices were calculated by counting positive cells in four random fields at ×100 magnification from untreated (black), control siRNA-treated (red), and Raf-1 siRNA-treated (green) mice (G) ($P<0.001$, untreated, Control siRNA vs. Raf-1 siRNA, One-way analysis of variance with multiple comparisons versus control siRNA and untreated groups (Bonferroni's t-test)).
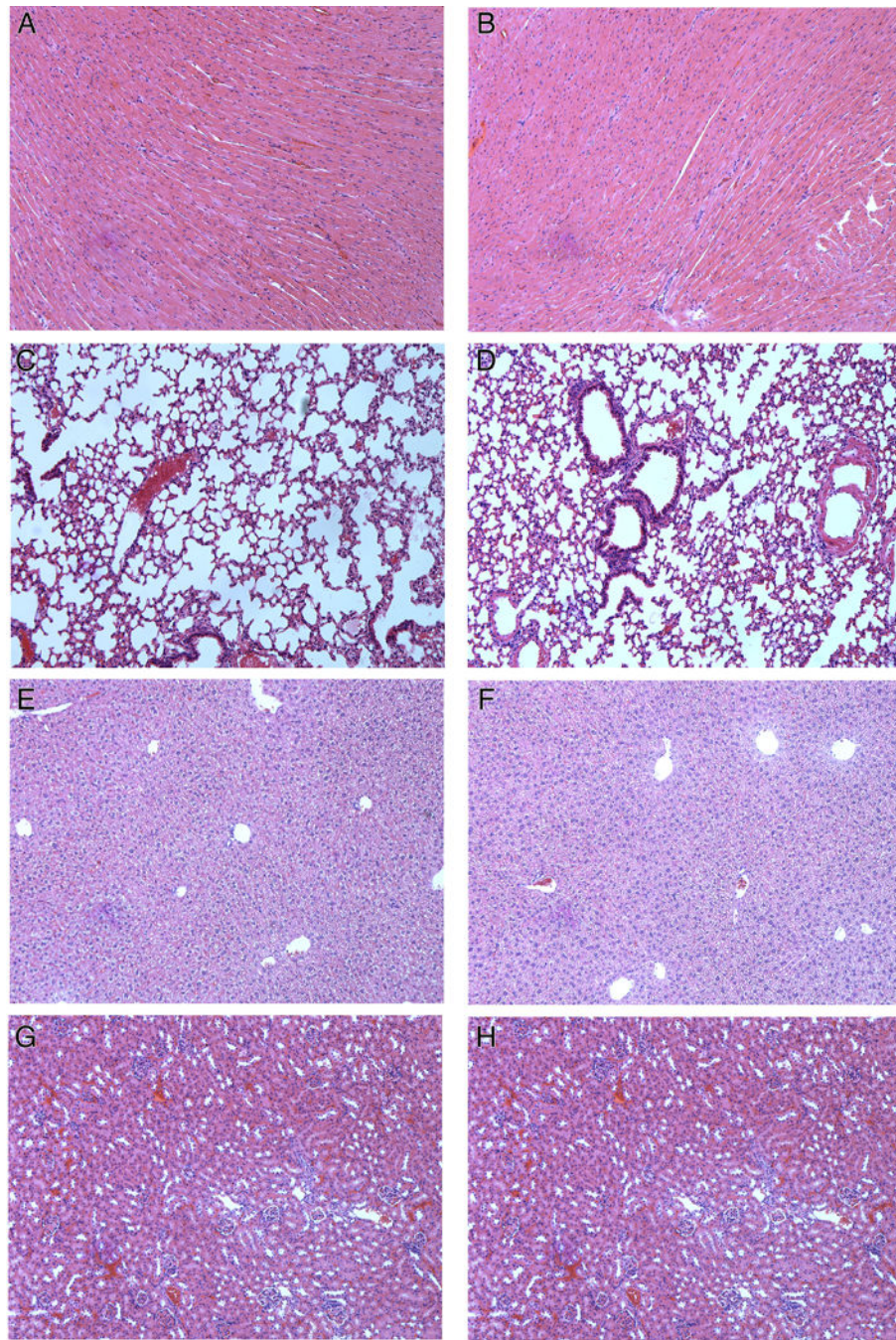
Figure 7.
Hematoxylin and Eosin staining of several normal tissues (A,B, heart; C,D, lung; E,F, liver; G,H, kidney) of untreated and Raf-1 siRNA treatment groups. There was no toxicity in the control (A,C,E,G) or Raf-1 siRNA (B,D,F,H) treatment groups.

Table 1

Sequence and Size of HK Polyplexes

Polymer	Structure	Terminal Sequence	Molecular Weight	Size (nm)_
H3K(+H)4b	R R R	R=KHHHKHHHKHHHHKHHHK	10 191	230±129
H3K(+G)4b	R- K- K- K	R=KHHHKHHHKGHHHKHHHKG	10 035	274±128
H3K8b		R=HHHKHHHKHHHKHHHK	22 908	137±56