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Regulation of Cellular Signaling with an Aptamer Inhibitor to Impede Cancer Metastasis

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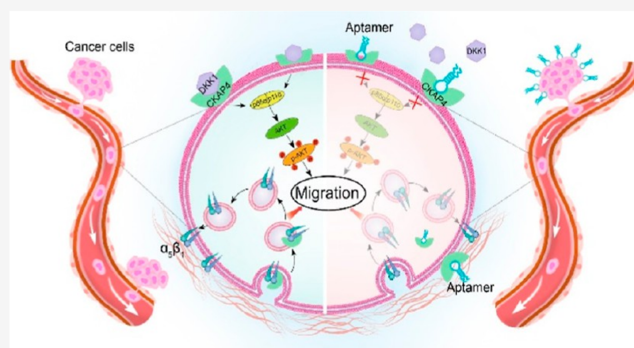
ABSTRACT: Tumor invasion and metastasis are the main causes of tumor progression and are the leading causes of death among cancer patients. In the present study, we propose a strategy to regulate cellular signaling with a tumor metastasis-relevant cytoskeleton-associated protein 4 (CKAP4) specific aptamer for the achievement of tumor metastasis inhibition. The designed aptamer could specifically bind to CKAP4 in the cell membranes and cytoplasm to block the internalization and recycling of $\alpha5\beta1$ integrin, resulting in the disruption of the fibronectin-dependent cell adhesion and the weakening of the cell traction force. Moreover, the aptamer is able to impede the interaction between CKAP4 and Dickkopf1 (DKK1) to further block the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which subsequently reduces AKT phosphorylation and inhibits the reorganization of the actin cytoskeleton in cell migration. The synergetic function of the designed aptamer in inhibiting cancer cell adhesion and blocking the PI3K signaling pathway enables efficient tumor cell metastasis suppression. The aptamer with specific targeting ability in regulating cellular signaling paves the way for cancer treatment and further provides a guiding ideology for inhibiting tumor metastasis.



INTRODUCTION

Malignant tumors are the most serious public health concern worldwide due to their systemic invasion, difficulty to conquer, and high mortality.[1] Tumor metastasis and invasion remain formidable challenges in the treatment of cancer.[2,3] Our quest to understand malignant tumors relies on the investigation of tumor metastasis-related molecular events for elucidating the mechanisms underlying tumorigenesis and deterioration, with the ultimate goal of developing effective therapies to prevent the spread of tumor cells. Steadily accumulating evidence proves that tumor metastasis involves a multistep mechanochemical process that is well-regulated by numerous proteins.[4−6] For example, cell-adhesion proteins of various functions and classes, including integrins, cadherins, and the hyaluronan receptor CD44, can modulate the adhesion in cell−cell and cell−matrix interactions, which provides the driving forces to disassemble adhesions at the cell rear and propel the cell forward.[7−10] Previous studies further showed that reorganization of the actin cytoskeleton plays a critical role in cell motility, and its regulatory proteins are crucial for cell migration in most cells. Small GTPases of the Rho family such as Rac1, Rho, and Cdc42 can remodel actin by transmitting extracellular chemotactic signals to the downstream effectors, including Wiskott−Aldrich syndrome protein and tyrosine kinase family proteins. Those proteins are central to actin cytoskeletal organization and serve as the key regulators of cell migration.[11,12] Thus, the control of cell migration via regulating the function of migration-related proteins provides the possibility of regulating cancer cell invasion and metastasis, which can provide opportunities for the development of effective clinical methods for cancer therapy.

Cytoskeleton-associated protein 4 (CKAP4) is a palmitoylated type II transmembrane protein that can anchor rough endoplasmic reticulum (ER) to microtubules to maintain the structure of the ER.[13,14] In recent years, there has been substantial accumulating evidence about the various oncogenic roles of CKAP4 localized outside ER, including CKAP4 in the cell surface or even in the nucleus.[15] These studies reported that CKAP4 is a protumor molecule to mediate the deterioration of cancers including esophageal and pancreatic tumors.[16,17] Also, a growing number of research studies prove that CKAP4 plays a prominent role in tumor progression and migration by modulating integrin-mediated adhesions and

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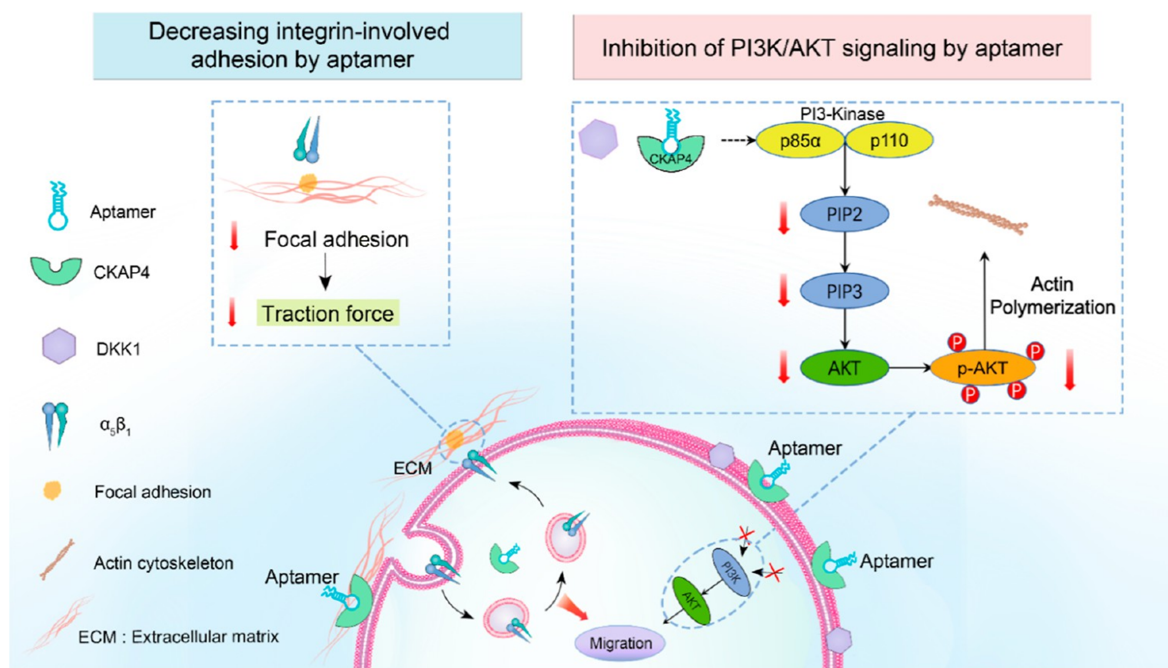
Scheme 1. Schematic Illustration Showing the Inhibition of Tumor Metastasis and Progression via Regulating Integrin-Involved Cell Adhesion and PI3K/AKT Signaling Pathways by CKAP4-Targeted Aptamers



participating in intracellular signaling pathways.[18−20] CKAP4 controls the trafficking of $\alpha5\beta1$ integrin through the interactions with $\beta1$ integrin, which further regulates the adhesion of tumor cells to fibronectin and enhances the metastatic potential of tumor cells.[18] Moreover, a previous study demonstrates that CKAP4 acts as a receptor for Dickkopf1 (DKK1).[21] DKK1-CKAP4 signaling promotes cell migration and proliferation by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway in the bladder, pancreas, and esophageal cancers.[18,22] The PI3K-AKT signaling pathways participate in the remodeling of the actin cytoskeleton and promote tumor metastasis-related cellular events.[23] Considering the multiple roles of CKAP4 in promoting tumor migration, CKAP4 is believed to be Pandora's box in tumor genesis and deterioration. Therefore, the targeted blocking of CKAP4 is highly promising in suppressing the migration and invasion of cancer cells for precision tumor therapy.

Nucleic acid nanodrugs have shown great promise in cancer therapy and vaccine development.[24] A variety of nucleic acid drugs (e.g., DNA, antisense oligonucleotides, small interfering RNA [siRNA], mRNA, CRISPR-Cas9 systems, and miRNA) are emerging as valuable inhibitors against various diseases.[25] Aptamers, also known as chemical antibodies, show great potential in both cancer diagnosis and therapy due to their high binding specificity, strong binding affinity, low immunogenicity, reproducible manufacturing, and deep tissue penetration.[26] Aptamers have been explored as therapeutic molecules to regulate signaling pathways to combat a variety of diseases including cancer and cardiovascular diseases.[27] Previous studies reported that aptamers can block the function of targeted proteins by interfering with the ligand−receptor interaction to realize tumor therapy.[28−30] Based on the function mechanism of the aptamer in inhibiting ligand-dependent receptor activation, aptamers targeting CKAP4 can be used for cancer therapy by specifically blocking the function of CKAP4. In this study, we proposed a CKAP4-targeted aptamer that can inhibit cell migration by simultaneously blocking the internalization/recycling of $\alpha5\beta1$ integrin and down-regulating the PI3K/AKT signaling pathway (Scheme 1). This chemically designed aptamer can bind to CKAP4 on the cell surface and block the binding of CKAP4 to integrins, which slows the endocytosis and recycling of integrin. The dysfunction of integrin metabolism ultimately weakens cell adhesion and the driving forces of migration. Further, the aptamer also downregulates the PI3K/AKT signaling pathway by inhibiting the binding of DKK1 to CKAP4. The disruption of the PI3K/AKT signaling pathway affects the reorganization of the actin cytoskeleton, which further impedes tumor cell migration. Additionally, the designed CKAP4-specific aptamer achieves efficient cell and tumor growth inhibition. The regulation of a tumor-metastasis-relevant signaling pathway with a specifically designed multifunctional aptamer offers a strategy to achieve cancer treatment and further provides instructions to hinder tumorigenesis and deterioration. This work provides important instructions for elucidating the biological functions of chemically synthesized aptamers and the design of efficient aptamer-based drugs.

■ RESULTS AND DISCUSSION

Targeted Recognition of CKAP4 by the spl3c Aptamer. CKAP4 is highly expressed or hyperactivated in multiple cancers, which makes CKAP4 a valuable molecular target for cancer therapy.[18,31] In this study, the influence of spl3c, an aptamer with hairpin structure that can specifically target CKAP4,[32] on function of CKAP4 was investigated. In particular, EJ and T24 bladder cancer cells were employed as models to study the influence of spl3c on the function of CKAP4 in oncogenesis and metastasis. The capability of the spl3c aptamer to target bladder cancer cells was evaluated using confocal microscopy and flow cytometry (Figure 1a). The confocal microscopy images show that the red AF647-

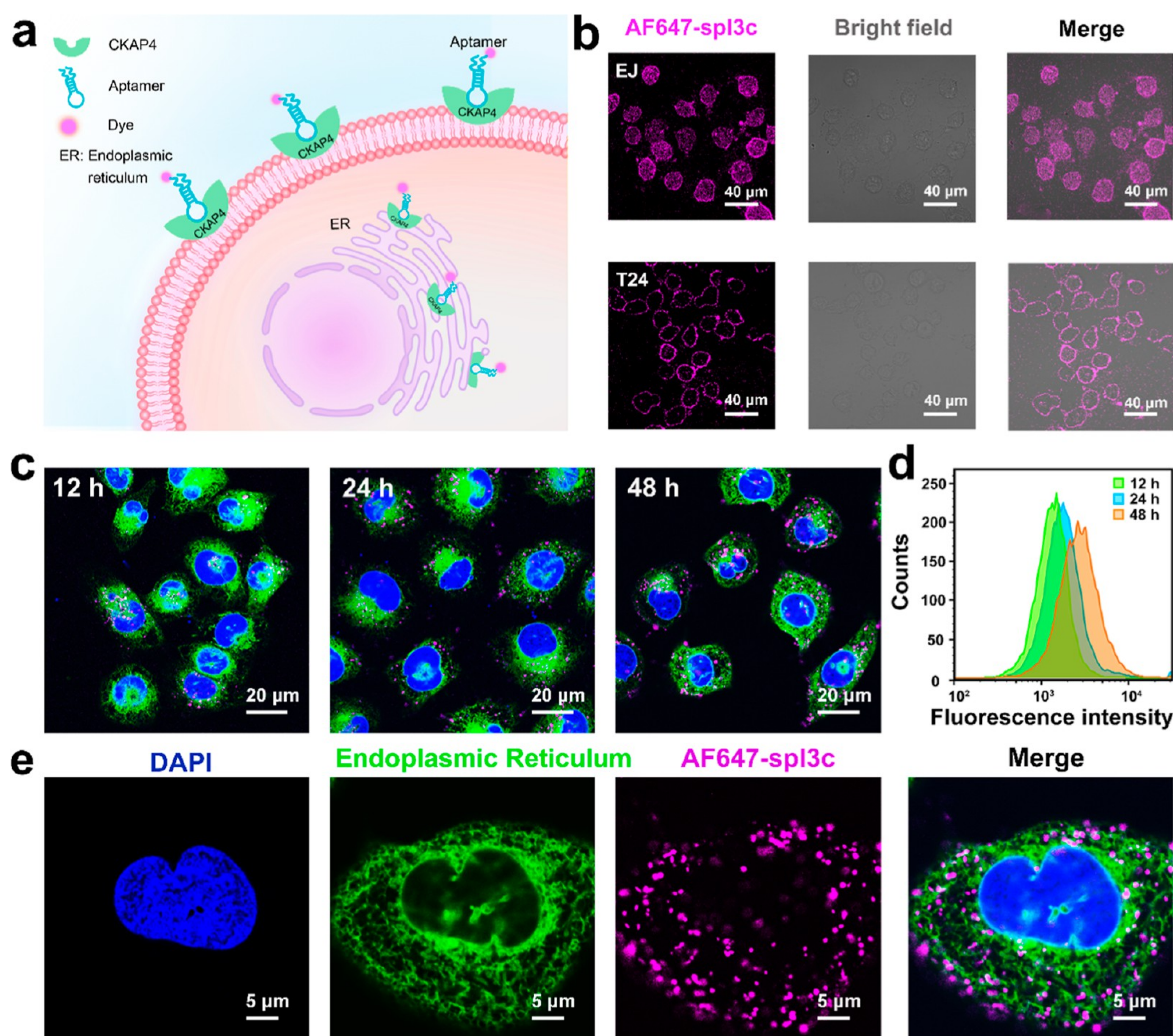


Figure 1. Spl3c aptamer binding to CKAP4 in the cell membrane and cytoplasm. (a) Schematic illustration showing spl3c aptamer binding to CKAP4 in the cell membrane and cytoplasm. (b) Laser confocal microscopy images of EJ cells and T24 cells after incubation with the AF647-spl3c aptamer. (c) Laser confocal microscopy images of EJ cells after treatment with AF647-spl3c aptamers for 12, 24, and 48 h. (d) Flow cytometry results of EJ cells after incubation with 500 nM spl3c aptamers for 12, 24, and 48 h. (e) Magnified microscopy images showing the location of spl3c aptamers on the endoplasmic reticulum.

spl3c signals were localized on the surface of bladder cancer cells, suggesting that the spl3c aptamer possesses a high binding affinity to CKAP4 in the membrane of the bladder cancer cells. Moreover, the signal of AF647-spl3c is apparently located on the surface of EJ bladder cancer cells (Figure 1b), whereas the aptamer signal is along the periphery of the T24 bladder cancer cells (Figure 1b). Such different signals can be attributed to the heterogeneous expression of CKAP4 on the surface of EJ and T24 cells (Figures S1 and S2). The control DNA with random sequences shows weak binding to EJ cells (Figures S3 and S4). To further quantitatively evaluate the binding affinity of the spl3c aptamer to bladder cancer cells, EJ and T24 cells were incubated with different concentrations of AF647-spl3c, and the labeled cells were then analyzed by flow cytometry. The fluorescence intensity of the labeled cells first increases and then reaches a plateau value as the concentration of aptamer increases. The average mean fluorescence intensity corresponding to varying concentrations of aptamers was plotted to determine the dissociation constant K_d (Figures S5 and S6). The K_d values of the spl3c aptamer to EJ and T24 cells were determined to be 79 and 103 nM, respectively, indicating the strong binding affinity of the spl3c aptamer to both EJ and T24 cells. With prolonged incubation, the spl3c aptamer localizes to the endoplasmic reticulum (Figure 1c), where CKAP4 is known to reside and is abundantly expressed (Figure S1). Moreover, the aptamers binding to CKAP4 on the endoplasmic reticulum increases with further increasing the incubation time (Figure 1c,d), indicating the ability of the spl3c aptamer to penetrate cells and recognize cytoplasmic CKAP4. The internalization of the spl3c aptamer into cancer cells can be ascribed to receptor-mediated endocytosis.[30] The serum stability of the spl3c aptamer was investigated, and the aptamer signal was kept at half after 24 h (Figure S7). After 48 h of incubation, the signals of the spl3c aptamer are colocalized well with the endoplasmic reticulum stained with a green endoplasmic reticulum (Figure 1e), which is consistent with the fact that CKAP4 shows a cluster distribution on the endoplasmic reticulum.[17] Taken together, these results indicate

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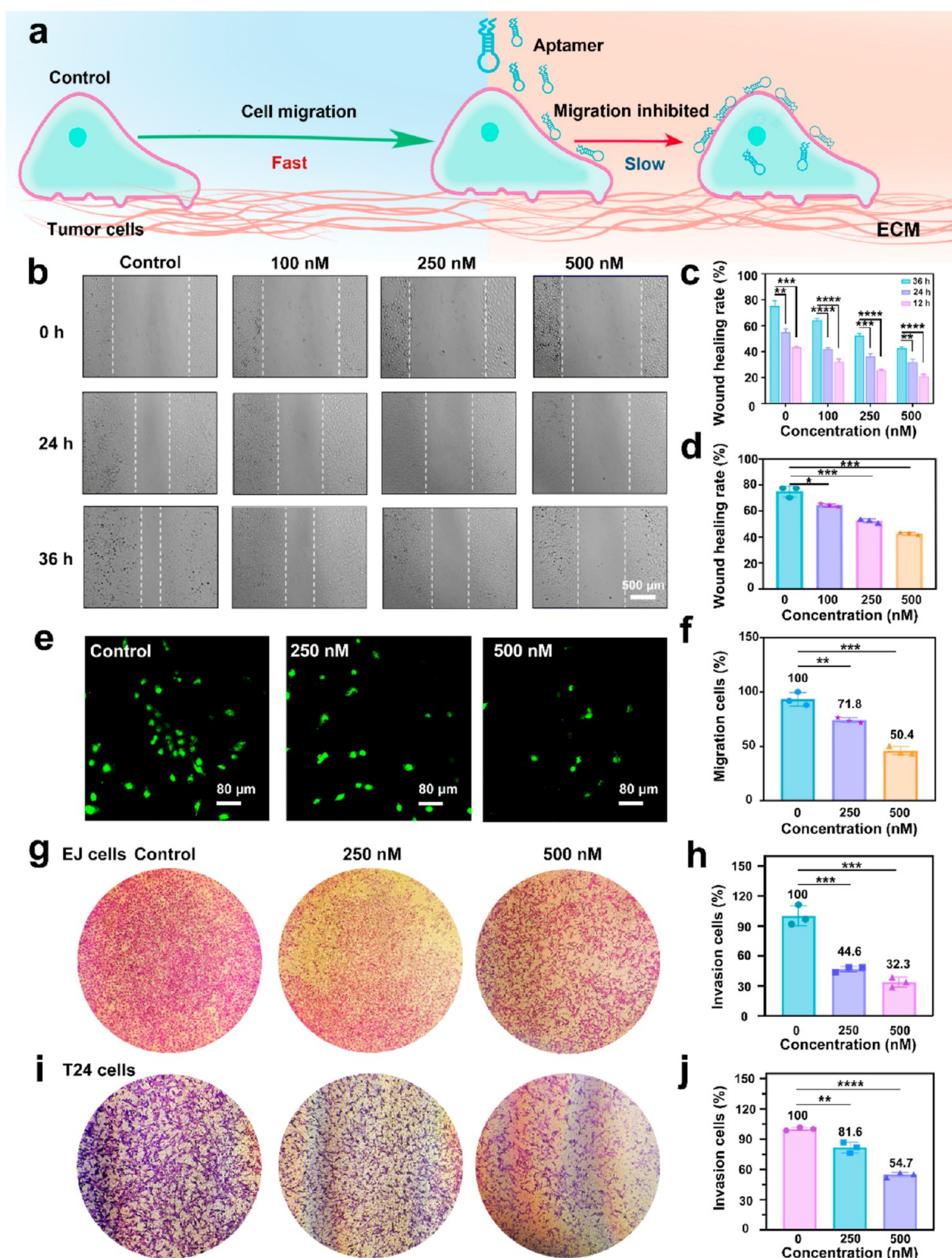

Figure 2. Inhibition of the migration and invasion of tumor cells by the spl3c aptamer. (a) Schematic diagram illustrating the spl3c aptamer inhibiting cell migration. (b) Evolution of EJ cell wound healing in the presence of the spl3c aptamer at different concentrations. Scale bar = 500 μm. (c) Quantitative analysis of the wound healing rate of EJ cells in the presence of different concentrations of the spl3c aptamer. Gap areas were quantified with ImageJ software. (d) Quantitative analysis of the wound healing rate of EJ cells when treated with the spl3c aptamer for 36 h. (e,f) Fluorescence images of migrated cells stained with CFSE and the corresponding quantification of migrated EJ cells in a transwell assay after incubation with different concentrations of the spl3c aptamer. (g) Crystal violet staining images showing the invasion abilities of EJ cells in the presence of the spl3c aptamer at different concentrations. (h) Quantitative analysis of the EJ cell invasion abilities. (i) Crystal violet staining images showing the T24 cells in the transwell invasion assay treated by the spl3c aptamer at different concentrations. (j) Quantitative analysis of the T24 cells in the transwell invasion assay. Data are presented as mean values ± SD (n = 3). Statistical significance was calculated by Student's t-test (unpaired, two-tailed): $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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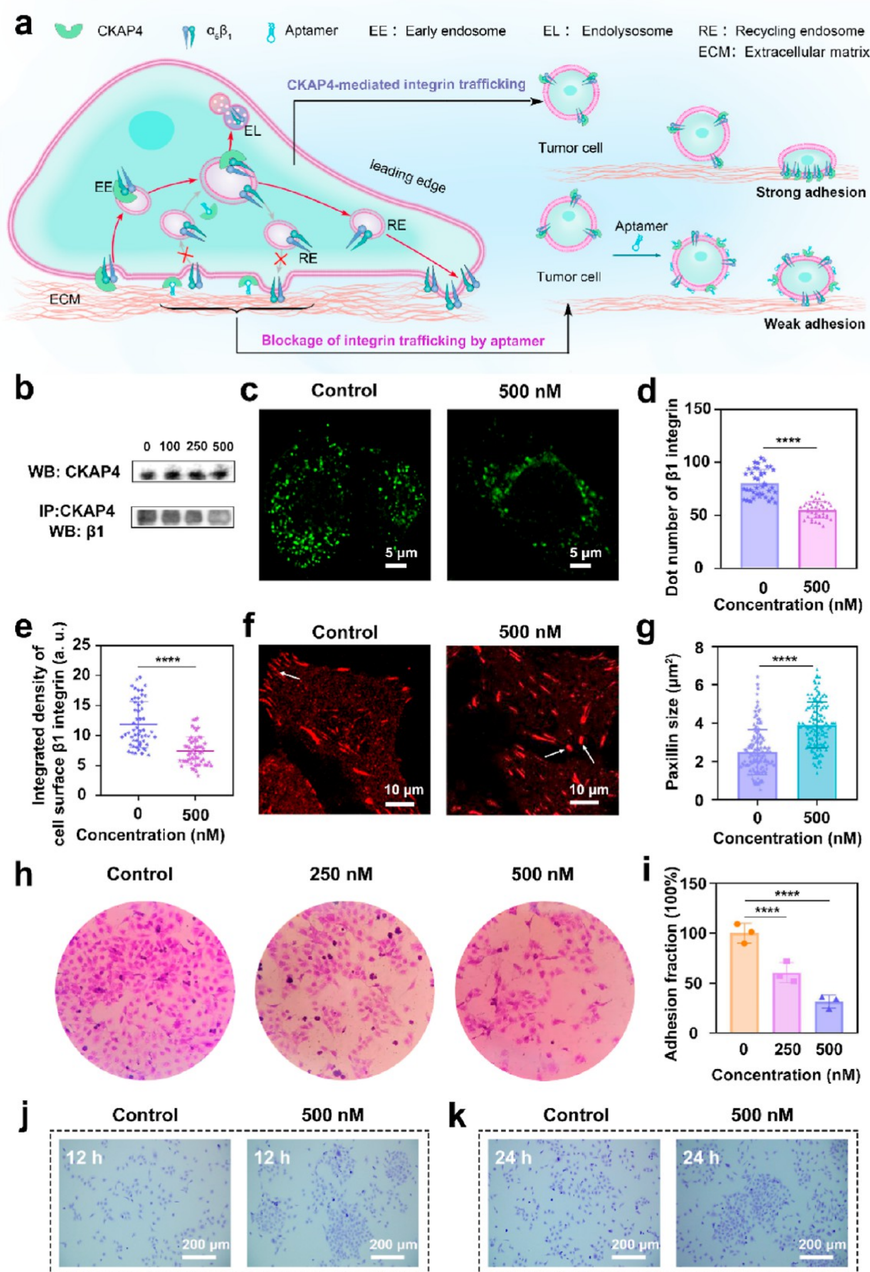

Figure 3. Inhibition of the integrin trafficking and cell adhesion by the spl3c aptamer. (a) Schematic diagram showing the spl3c aptamer influencing the CKAP4-mediated integrin trafficking and cell adhesion. (b) Co-IP and western blot assays showing the interactions between CKAP4 and $\beta 1$ integrin in the absence and presence of the spl3c aptamer. (c) Confocal microscopy images of internalized $\beta 1$ integrin. Scale bar = 5 μm. (d) Dot number plot of internalized $\beta 1$ integrin. (e) Integrated density of $\beta 1$ integrin on the cell surface measured using ImageJ software. (f) Confocal microscopy images of paxillin. (g) Paxillin-staining sizes measured with ImageJ software. (h) Cell adhesion results of EJ cells treated with different concentrations of the spl3c aptamer. (i) Adhesion fraction of EJ cells treated with different concentrations of the spl3c aptamer. (j,k) Microscopy imaging of EJ cells stained with crystal violet in the absence and presence of 500 nM spl3c aptamer at 12 and 24 h, respectively. Scale bar = 200 μm. Data from three replicates are combined and mean values ± SD [(d,e,g), n = 25; (i), n = 3]. Statistical significance was calculated by Student's t-test (unpaired, two-tailed): $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

that the spl3c aptamer is able to target CKAP4 both in the cell membrane and cytoplasm.

Inhibition of Cell Migration and Invasion by the spl3c Aptamer. The capability of the spl3c aptamer to inhibit the migration and invasion of bladder tumor cells was then investigated with scratch wound healing and transwell migration/invasion assays (Figure 2a). As shown in Figure 2b, the wounds in EJ cells without aptamer treatment are almost 50% healed after 24 h of incubation. In contrast, a significant delay in wound healing is observed after treating cells with 500 nM spl3c aptamer, and large wound gaps in EJ cells still exist after 36 h of incubation. The derived percentages of gap areas are positively correlated with the concentration of the spl3c aptamer (Figure 2c). EJ cells treated with the spl3c aptamer at a concentration of 500 nM show only a half closure percentage compared to the untreated cells after 36 h of incubation (Figure 2d). A similar delayed wound-healing phenomenon is also found in the aptamer-treated T24 and

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

5637 bladder cancer cell lines (Figures S8−S12), and A549 lung cancer cell lines (Figures S12 and S13), demonstrating that the spl3c aptamer is capable of inhibiting the migration of cancer cells. Further, the transwell migration assay was performed to assess the effect of the spl3c aptamer on cell migratory capabilities. As shown in Figures 2e and S15−S19, a significant decrease in migratory cell numbers on outer chambers is observed after treating cells with the spl3c aptamer. The migratory cells show a reduction of proximately 28 and 50% in the presence of 250 and 500 nM spl3c aptamer, respectively (Figure 2f). We further tested the impact of the spl3c aptamer on cell invasion through the Matrigel envelope in the transwell invasion assay, which simulates the entire process of basement membrane cell invasion. Matrigel invasion shows that cell invasive capability is significantly blunted in the presence of the spl3c aptamer in the EJ cell line (Figure 2g). After treating EJ cells with the spl3c aptamer at concentrations of 250 and 500 nM, the invasion speed of EJ tumor cells is reduced by approximately 55% and 78%, respectively (Figure 2h). A similar delayed Matrigel invasion phenomenon is also found in the T24 bladder cancer cell lines (Figure 2i,j). Cell invasion of T24 bladder cancer cell lines was significantly inhibited by the spl3c aptamer in a concentration-dependent manner; that is, the number of invading cells decreases as the aptamer concentration increases (Figures S20−S22). Together, the wound healing and transwell migration/invasion assays demonstrate that the spl3c aptamer can significantly reduce the migratory and invasive capabilities of bladder tumor cells.

Blockage of CKAP4-Mediated Integrin Trafficking by the spl3c Aptamer. The underlying molecular mechanism by which the spl3c aptamer inhibits cell migration and invasion was investigated (Figure 3a). Cell migration is a highly integrated multistep process that involves cellular protrusion, adhesion, pulling from the front, and squeezing from the rear.[33] The formation of cell adhesion sites is supported by integrins and adaptor proteins. Integrins are the main cell adhesion transmembrane receptors that are critical for the maintenance of diverse cellular processes, including adhesion, spreading, migration, and cancer invasion.[34] Integrins undergo a dynamic exocytic−endocytic cycle to sense the environment for adhesive and migratory cues and to adapt their morphological properties for migration.[35] $\alpha5\beta1$ integrin trafficking potentiates signaling to induce metastasis and is essential for sustained cell migration. Previous studies show that CKAP4 interacts with $\beta1$ integrin and specifically regulates the balance of degradation and recycling of $\alpha5\beta1$,[18] thereby governing the adhesion sites and promoting the invasion of tumorigenic keratinocytes. Immunoprecipitation (IP) of CKAP4 and western blotting assay was carried out to determine whether the spl3c aptamer could disturb the interplay between CKAP4 and integrins. The coimmunoprecipitation (co-IP) results reveal the physical interactions between endogenous CKAP4 and the $\beta1$ integrin, indicating the formation of the CKAP4/$\beta1$ complex. After treatment with the spl3c aptamer, the $\beta1$ integrin level in the CKAP4/$\beta1$ complex decreases and is negatively correlated with the concentration of the aptamer (Figure 3b), indicating the blockage of binding between $\beta1$ integrin and CKAP4 by the spl3c aptamer. The influence of the spl3c aptamer on the CKAP4-mediated integrin internalization and recycling was further tested. As shown in Figures 3c and S23, obviously reduced levels of internalized $\beta1$ integrin into intracellular vesicles were observed in the cytoplasm after treating the cells with the spl3c aptamer. Quantitative analysis in Figure 3d,e further confirms that the internalized level of $\beta1$-integrin is reduced by about 30% with 500 nM spl3c aptamer exposure, indicating that the internalization process of integrin was inhibited. The downregulation of integrin internalization can be attributed to impaired formation of the CKAP4/$\beta1$ complex by the spl3c aptamer. The recycling of $\beta1$ integrin back to the cell surface was reduced with aptamer exposure (Figures S24 and S25), indicating that spl3c had a significant inhibitory effect on the integrin recycling. $\alpha5\beta1$ is known to be transported to lysosomes for degradation after the integrin internalization, whereas CKAP4 interferes with the binding of SNX17 to $\beta1$ integrin in the early endosome and suppresses the degradation of $\alpha5\beta1$. After spl3c aptamer stimulation, an increased $\alpha5$ integrin level is observed in western blotting results (Figure S26), suggesting that the binding of spl3c to CKAP4 can upregulate the degradation of $\alpha5\beta1$ integrin. The above results indicate that the binding of the spl3c aptamer to CKAP4 can significantly inhibit the binding of CKAP4 to $\beta1$ integrin, which further interrupts the internalization and recycling of integrins.

Integrin-mediated cell migration relies on the assembly of adhesion sites. There is an intimate relationship between the size of cell adhesion sites and cell motility: the larger the cell adhesion sites, the slower the cell migration.[33,36] The area of paxillin was quantified using confocal microscopy and ImageJ software to determine if the spl3c aptamer impacts the state of cell adhesion sites. Figures 3f,g and S27 show that the area of adhesion sites in cells treated with the spl3c aptamer is significantly larger than that in the untreated cells, showing that the spl3c aptamer can inhibit cell migration by enlarging the adhesion sites. Further, adhesion sites that anchor stress fibers provide cells with firm substrate attachment and mechanical support for spreading, and the cell adhesion strength reflects the force transmission on the substrate and migration ability. The changes in cell-to-substrate adhesion and cell-to-cell adhesion were further investigated to test if the spl3c aptamer affects the adhesion behaviors of bladder cancer cells. Figure 3h reveals a significantly diminished attachment of EJ cells to substrates in the presence of the spl3c aptamer. Moreover, the ratio of cell attachment to substrates shows a negative correlation with the concentration of the aptamer (Figure 3i). The adhesion assays in the Matrigel substrates also exhibited a substantial decrease in cell attachment in the presence of the spl3c aptamer compared with those cultured without an aptamer (Figure S28). In addition to roles in cell-to-substrates adhesion, integrins play pivotal roles in cadherin-dependent cell−cell adhesion.[37] The influence of the spl3c aptamer on cell−cell adhesion strength was assessed. Figure 3j,k shows the formation of cell clusters and the presence of large gaps between cell clusters after treating the cells with the spl3c aptamer. The clustering of cancer cells increases with an increase in the concentration of the aptamer (Figure S29), indicating that spl3c treatment enhanced the cell−cell contacts. E-cadherin mainly dominates the adherents' junctions to form strong cell−cell adhesion, and the expression level of cadherin correlates with the adhesion strength between neighboring cells. Western blotting results show that the level of E-cadherin increases by 35% after 250 nM spl3c aptamer treatment (Figure S30), suggesting that the aptamer facilitates strong restoration of cell−cell adhesions. These findings show that the spl3c aptamer can interfere with the strength of CKAP4-related cellular adhesions, which affects the motility as well as attenuates the invasiveness and metastasis of cancer cells.

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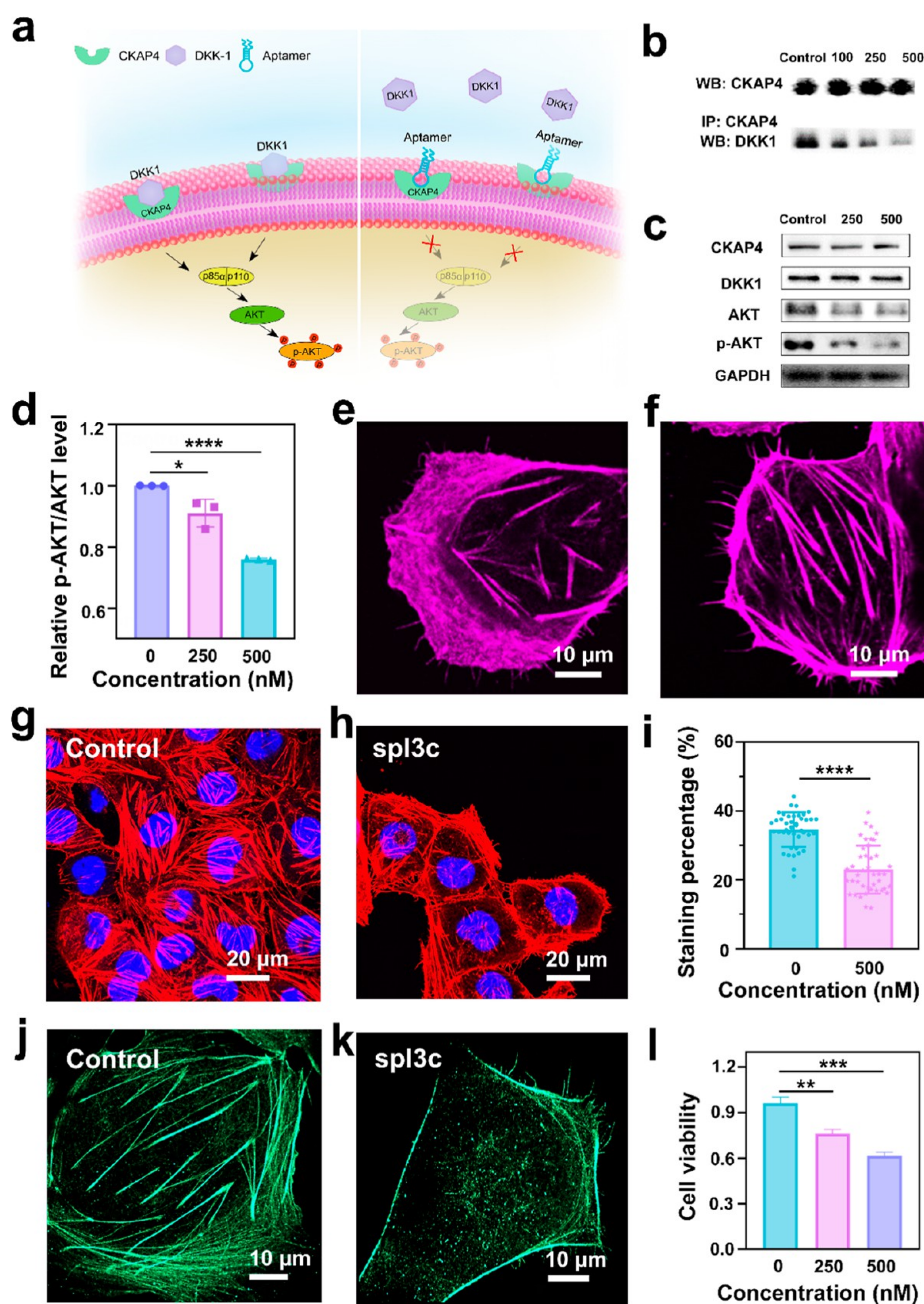

Figure 4. Inhibition of PI3K/AKT signaling. (a) Schematic illustration showing the spl3c aptamer impeding the CKAP4/DKK1-mediated PI3K/AKT signaling pathways. (b) Co-IP assay showing the interactions between CKAP4 and DKK1 integrin in the absence and presence of the spl3c aptamer. (c) Expression levels of CKAP4, DKK1, PI3K, AKT, and p-AKT measured by western blotting. (d) Relative p-AKT/AKT protein expression in the absence and presence of the spl3c aptamer. (e) Alexa Fluor 647 phalloidin-labeled F-actin showing the leading lamellipodium. (f) Confocal microscopy images of Alexa Fluor 647 phalloidin-labeled F-actin. (g,h) Confocal microscopy images of the F-actin cytoskeleton in collective cells in the absence and presence of the spl3c aptamer. (i) Statistical analysis of the F-actin cytoskeleton staining percentage near the nucleus. (j,k) dSTORM super-resolution imaging of the cytoskeleton in the absence and presence of the spl3c aptamer. (l) Cell viability of EJ cells treated with different concentrations of the spl3c aptamer. Scale bars in (e,f,j,k) are 10 μm. Scale bars in parts (g) and (h) are 20 μm. Data are mean values ± SD [(d,l), $n = 3$; (i), $n = 25$]. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (by two-tailed unpaired Student's t-test).

Blockage of PI3K/AKT Signaling by the spl3c Aptamer. Besides acting as a receptor of β1 integrin to participate in $\alpha 5\beta 1$ integrin internalization and recycling, CKAP4 also functions as a DKK1 receptor to form the CKAP4/DKK1 complex at endogenous levels, which promotes cancer cell migration and proliferation by activating the PI3K/AKT signaling pathway (Figure 4a). The potential influence of the spl3c aptamer on the CKAP4/DKK1-mediated PI3K/AKT signaling was investigated. The co-IP results reveal that CKAP4 is coimmunoprecipitated with DKK1, indicating the formation of the CKAP4/DKK1 complex. In contrast, the presence of the spl3c aptamer resulted in a significant reduction of DKK1 level within the CKAP4/DKK1 complex, and this decrease was observed to be concentration-dependent (Figure 4b), suggesting the blockage of binding between DKK1 and CKAP4 by the spl3c aptamer. After binding to DKK1, CKAP4 interacts with the p85α subunit of PI3K to activate PI3K/AKT signaling, which further promotes the

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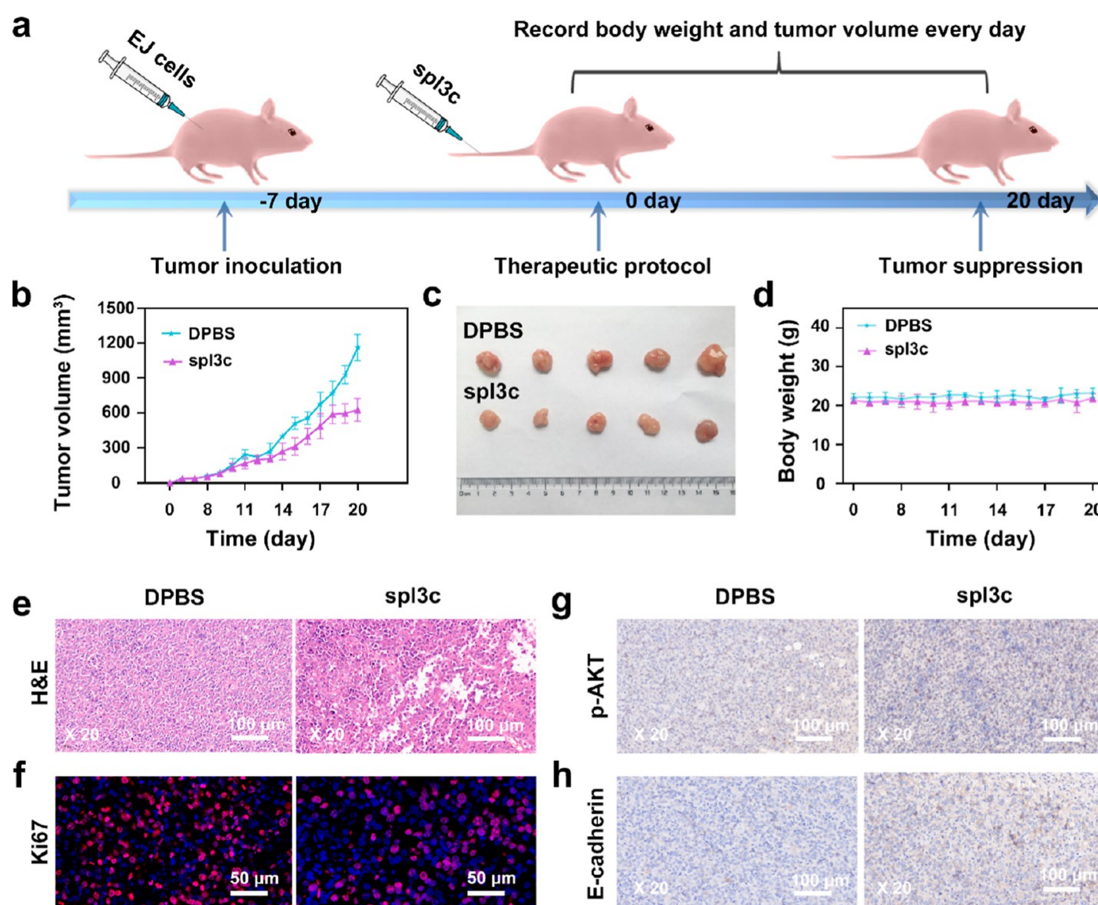

Figure 5. In vivo tumor treatment efficacy of the spl3c aptamer. (a) Schematic diagram of the timeline of in vivo tumor treatment. (b) Relative tumor growth curves of tumor-bearing mice after intravenous injection of the spl3c aptamer. (c) Digital photographs of tumors from mice receiving DPBS and the spl3c aptamer. (d) Body weight of tumor-bearing mice treated by DPBS and the spl3c aptamer. (e) H&E-staining analysis of tumor slices. Scale bars are 100 μm. (f) Confocal fluorescence images of Ki67 protein in tumor tissue. Scale bars are 50 μm. Immunohistochemical staining of (g) p-AKT and (h) E-cadherin protein levels in tumor tissue from mice with different treatments. Scale bars are 100 μm.

phosphorylation of AKT to regulate the downstream signaling for cancer cell migration.[21] Western blotting was employed to test the influence of spl3c aptamer treatment on the total protein levels of CKAP4, DKK1, and AKT and the phosphorylation levels of AKT. The expression of CKAP4 and DKK1 remains unchanged in the total lysates of EJ cells after spl3c aptamer treatment. By contrast, the ratios of key downstream effector proteins p-AKT/AKT are significantly reduced after incubating the cells with the spl3c aptamer (Figure 4c). Additionally, the protein expression ratio of p-AKT/AKT decreases with an increase of spl3c aptamer concentration (Figure 4d). About a 25% drop in the expression ratio of p-AKT/AKT is observed after incubating the cells with 500 nM spl3c aptamer for 24 h. The western blotting assay thus suggests that the binding of the spl3c aptamer to CKAP4 does not affect the expression of CKAP4 and DKK1 proteins, but can downregulate the downstream phosphorylation of AKT to disrupt PI3K/AKT signaling. Similarly, the fluorescent microscopy images of EJ and T24 cells also reveal that the p-AKT/AKT signals on the cell membranes are reduced after incubation with the spl3c aptamer (Figures S31−S33). Collectively, the above results demonstrate that the spl3c aptamer blocks the interaction between CKAP4 and DKK1, which results in the suppression of the PI3K/AKT signaling pathway transduction to inhibit cell migration and invasion.

Collective cell migration relies on the synchronization of actin cytoskeleton polymerization and organization, which is regulated by Rho GTPases including Rho, Rac, and Cdc42.[38] Since AKT plays a critical role in actin cytoskeleton remodeling,[39] the blockage of the PI3K/AKT signaling pathway by the aptamer may also influence the cytoskeleton reorganization. To test this hypothesis, F-actin was labeled with Alexa Fluor 647 phalloidin, and the organization of F-actin in the presence of the spl3c aptamer was directly monitored with confocal microscopy and super-resolution microscopy. Before the treatment with the spl3c aptamer, the EJ cells extend the broad leading lamellipodium at the leading edge and spike-like filopodia at the cell periphery (Figures 4e,f, and S35). The broad lamellipodia and spike-like filopodia formation driven by the polymerization of actin filaments are both considered highly aggressive phenotypes of tumor cells. Figure 4g shows the polarity toward the direction of migration in cell populations without spl3c treatment, which are recognized to be indicators of metastasis. Also, F-actin forms longitudinal fibers around the nucleus and are parallel to the direction of movement, showing the "ready" state of the untreated EJ cells to migrate.[40] After incubating EJ cells with the spl3c aptamer, the cell filopodia become fewer and the stress fibers appear far away from the nucleus (Figure 4h). The statistical analysis of F-actin shows that the cytoskeleton staining percentage near the nucleus decreases after spl3c

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

aptamer treatment (Figure 4i). The remodeling of F-actin represents the poor migration and attacking ability of the aptamer-treated EJ cells. Direct stochastic optical reconstruction microscopy (dSTORM) was further used to obtain high-resolution images of the F-actin cytoskeleton. The dSTORM image in Figure 4j exhibits an actin cytoskeleton with heightened precision and resolution. Actin filaments oriented along the longitudinal axis of the cell are observed, and distinct stress fibers are evident in untreated EJ cells (Figures 4j and S36). After treatment with the spl3c aptamer, the actin filaments in EJ cells were found to be disorganized and truncated instead of being aligned with the long axis of the cell (Figures 4k and S37). Additionally, a dense network of actin filaments is observed throughout the periphery of the cells, which can hinder cell migration. The above findings demonstrate that the spl3c aptamer can disrupt the cytoskeletal rearrangement to inhibit the migratory ability of tumor cells. Taken together, our aptamer possesses combined applicability in combating the CKAP4-related adhesion and PI3K/AKT signals, making the aptamer efficient in inhibiting cell migration and invasion.

Previous studies have reported that DKK1/CKAP4 signaling activated AKT by forming a complex between the proline-rich domain of CKAP4 and the Src homology 3 domain of PI3K, which resulted in the proliferation of cells. Further, EJ cells were employed to examine the inhibitory effects of the spl3c aptamer on PI3K/AKT signaling and cell proliferation. After incubating EJ cells with the spl3c aptamer, fewer cells adhere to the wall and a slower proliferation rate is observed compared to the untreated EJ cells (Figure 4l), which suggests that the spl3c aptamer can inhibit cell proliferation by influencing the PI3K/AKT signaling.

Treatment of Tumor by the spl3c Aptamer. The therapeutic potential of the spl3c aptamer in treating bladder tumors was further explored by using the nude mice xenograft tumor models in which the spl3c aptamer was injected every day through the tail vein for tumor treatment (Figure 5a). The Cy5 labeled-spl3c aptamer effectively accumulates in tumors (Figure S38), showing the in vivo tumor cell targeting capability of the aptamer. Compared to the tumor-bearing mice receiving Dulbecco's phosphate-buffered saline (DPBS), a remarkable decrease in tumor volume is observed in spl3c aptamer-treated mice (Figure 5b,c). Moreover, the body weight of mice receiving the spl3c aptamer at the end of treatment is close to that of the mice before treatment (Figure 5d), indicating the efficient inhibition of tumor deterioration by the aptamer. H&E staining shows that the spl3c aptamer induces the apoptosis of tumor cells (Figure 5e), whereas the cells in normal tissues are not affected (Figure S39), demonstrating the highly specific tumor cell-killing ability and the excellent biocompatibility of the spl3c aptamer. The immunofluorescence results show the lower Ki67 in spl3c-treated tumors compared to those of tumors in mice treated by DPBS (Figures 5f and S40), suggesting that the inhibition of tumor growth is derived by the spl3c aptamer. The tumor-suppressing functions of the spl3c aptamer were further demonstrated by immunohistochemical staining of tumor tissues. The xenograft tumors from the spl3c-treated mice show a significant decrease in p-AKT compared to the control group (Figure 5g), suggesting the apoptosis of EJ cells via the inhibition of the PI3K/AKT signaling pathway by the spl3c aptamer. These results thus suggest that the spl3c aptamer is a valid therapeutic option for tumors by targeted inhibition of the PI3K/AKT signaling pathways. E-cadherin acts as a key component of the adherents' junctions that play essential roles in tissue formation and the maintenance of tissue structures. The E-cadherin binding between cells is important in mediating contact inhibition of proliferation. After treatment by the spl3c aptamer, the E-cadherin expression in cancer tissue increases (Figure 5h), showing the therapeutic ability of spl3c on tumors. E-cadherin also acts as a suppressor of tumor invasion and metastasis. The re-expression of E-cadherin in cancer tissue with spl3c aptamer treatment indicates the weak migration of cancer cells in tissue. Conclusively, the spl3c aptamer is expected to be tumor therapeutic and has the potential to impair the migration of cancer cells in vivo. It is worth noting that since the free aptamer is vulnerable to nuclease digestion in vivo, strategies that can enhance the in vivo stability of the spl3c aptamer and promote the accumulation of the spl3c aptamer in tumors such as site-specific chemical modifications, carrier protection, and multivalence recognition could be employed in the future to improve the therapeutic efficiency of the spl3c aptamer.[41]

CONCLUSIONS

In this study, we reported the prominent role of the spl3c aptamer in modulating CKAP4-associated signaling pathways and the accompanied inhibitory effect on tumorigenic progression. The spl3c aptamer binds specifically to CKAP4 in both the cell membrane and the cytoplasm of bladder cancer cells. Upon binding to CKAP4, the spl3c aptamer disrupts the integrin-mediated adhesion process by reducing cell adhesion ability and changing the cell−substrate/cell−cell adhesion behavior, which finally leads to the efficient impeding of tumor metastasis. Moreover, the spl3c aptamer inhibits the binding of CKAP4 to DKK1 and results in the reduced expression of p-AKT/AKT. The downregulation of AKT and p-AKT weakens the PI3K/AKT signaling transmission, which further interferes with the cytoskeletal reorganization and suppresses cell migration. The xenograft tumor model demonstrates the potential of the spl3c aptamer in inhibiting tumor growth. Our findings clarify the role of the spl3c aptamer in impeding CKAP4-involved cellular processes, and this work can provide valuable instructions for therapeutic innovations in combating cancer metastasis and tumorigenic progression.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c09091.

Reagents and materials, experimental section including details of the cell culture, sample characterization, the results obtained using confocal microscopy imaging, flow cytometric analysis, wound healing assay, cell migration, cell invasion, and cell adhesion assays, super resolution imaging, integrin internalization and recycling assays, western blot analysis, immunoprecipitation, proliferation assay, in vivo tumor imaging, antitumor performance of the spl3c aptamer, and H&E staining and analysis of tissues (PDF)

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Notes

The authors declare no competing financial interest.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX