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Targeted RNA Interference of Cyclin A_2 Mediated by Functionalized Single-Walled Carbon Nanotubes Induces Proliferation Arrest and Apoptosis in Chronic Myelogenous Leukemia K562 Cells

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Cyclin A_2 plays critical role in DNA replication, transcription, and cell cycle regulation. Its overexpression has been detected and related to many types of cancers including leukemia, suggesting that suppression of cyclin A_2 would be an attractive strategy to prevent tumor progression. Herein, we apply functionalized single wall carbon nanotubes (f-SWNTs) to carry small interfering RNA (siRNA) into K562 cells and determine whether inhibition of cyclin A_2 would be a potential therapeutic target for chronic myelogenous leukemia. The results show functionalized SWNTs can facilitate the coupling of siRNA specifically targeting human cyclin A_2 to form cyclin A_2 siRNA–f-SWNTs complexes. These functionalized SWNTs readily enter K562 cells, resulting in suppression of cyclin A_2 expression. We demonstrate that depletion of cyclin A_2 in this manner inhibits cell proliferation and promotes apoptosis, and cyclin A_2 can serve as a novel therapeutic target. siRNA against cyclin A_2 delivered by functionalized single wall carbon nanotubes may be a useful therapeutic strategy for chronic myelogenous leukemia cells. This would provide new insights on additional therapeutic options for chronic myelogenous leukemia beyond chemotherapy in light of increasing multidrug resistance.

Introduction

Cyclin A_2 is a member of the cyclin family which may be of particular interest as it is involved in two steps in the cell cycle,[1] both in the entry of G_1 cells into S phase and in the G_2–M transition.[2,3] It also participates in DNA replication and transcription by regulating the activity of some replication and transcription machinery components.[4–6] The timely appearance and disappearance of cyclin A_2 is crucial: excessive cyclin A_2–CDK activity can cause S phase defects and chromosomal instability.[7] Increased expression of cyclin A_2 has been detected in many types of cancers including leukemia.[8–16] Moreover, a high level of cyclin A_2 expression may be a poor prognostic marker for patients with some tumors.[11–15] In haematological malignancies, cyclin A_2 is associated with the proliferation rate of these disorders and can be used for molecular diagnostics as a proliferation marker.[15–16]

Cyclin A_2 and/or the cyclin A_2–CDK2 complex is a promising anticancer target with a high therapeutic index.[17] Small molecule inhibitors have been developed to suppress the activity of the cyclin A_2–CDK2 complex, leading to increased unphosphorylated transcription factors E2F, very high intracellular E2F activity, and ultimately apoptotic cell death.[18–19] However, these compounds are ATP competitive inhibitors and have several alternative targets that compromise their specificity for any particular cyclin–CDK complex. In recent years, small interfering RNA (siRNA) has become a specific and powerful tool to turn off the expression of target genes, and has become a promising tool in molecular medicine. Thus, it may be possible to use RNAi to specifically suppress the overexpression of cyclin A_2 and inhibit cyclin A_2–CDK complex activity.

Carbon nanotubes are formed by rolling sheets of graphite-like carbon thus creating hollow tubes and these tubes possess the unique feature of being able to enter a living cell without causing its death or without inflicting other damage.[20–34] They can shuttle biological molecules such as DNA, protein, oligodeoxynucleotides, and siRNA into mammalian cells, indicating their potential application as a vector for the delivery of therapeutic molecules. Among the molecules that can noncovalently bind to the surface of SWNTs, RNA/DNA has been the focus of research, which adsorbs as a single-strand or double strand complexes. Previous reports have shown that a particular sequence of single stranded RNA/DNA self-assembles into a helical structure around individual carbon nanotube and can be used to separate carbon nanotubes based on diameter and electronic properties.[35–36] Recently we have reported that SWNTs can induce a sequence-dependent B-A DNA transition and selectively induce human telomeric i-motif DNA formation and accelerate S1 nuclease cleavage rate.[37–38] Herein, we report our preliminary attempt for using RNAi against cyclin A_2 overexpression delivered by

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functionalized single wall carbon nanotubes (f-SWNTs) as a therapeutic strategy in chronic myelogenous leukemia cells.

Results

Ammonium functionalization of SWNTs was characterized by FT-IR and X-ray photoelectron spectra (XPS). The peak of C=O stretching shifted to 1652 cm^{-1} as a result of the formation of an amide bond which was supported by the appearance of the bending mode of N-H at 1550 cm^{-1} and the stretching of C-N at 1212 cm^{-1} (Figure S1). X-ray photoelectron spectroscopy (XPS) spectra (Figure S2) were obtained with an ESCALAB Thermal 250 instrument and monochromatic $Mg_{K\alpha}$ (E = 1253.6 eV) was used for photoexcitation. The disappearance of the peak at 289 eV[39] associated with carboxylic acid in C(1s) spectra and the appearance of the peak at 399.9 eV[40] in N(1s) spectra further indicate the formation of amino-modified SWNTs.

Ammonium functionalized carbon nanotubes have been demonstrated to effectively condense nuclear acid and deliver them into mammalian cells.[22,29–30] To characterize whether SWNT-NH_2 interacts with cyclin A_2 siRNA, we studied siRNA–f-SWNTs complexes by native polyacrylamide gel electrophoresis. The intensity of the free siRNA band decreased with increasing f-SWNTs–siRNA ratio (Figure 1). When the ratio

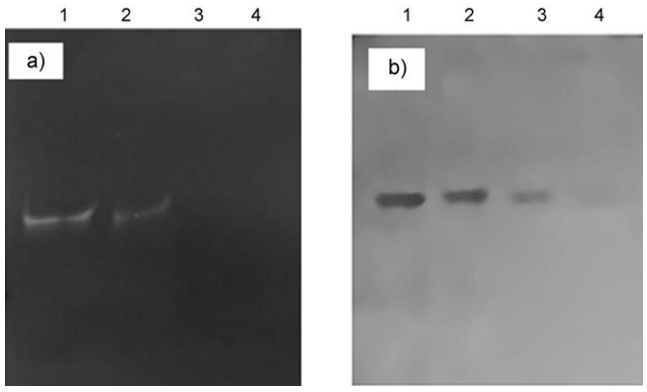

Figure 1. Electrophoretic mobility of siRNA–f-SWNTs complexes. The gel was stained by a) EB or b) silver. Lane 1 represented 0.3 μg of free siRNA. All other lanes contain f-SWNTs complexed to 0.3 μg of siRNA at various ratios (*w*/*w*): lane 2: 10; lane 3: 20; lane 4: 40.

reached 40, no band was observed. The result suggest the absorption and condensation of siRNA by f-SWNTs, which exclude EtBr intercalation or silver impregnation. Thus, positive charge functionalized SWNTs can effectively assist conjugation of cyclin A_2 siRNA. The coupling of siRNA and f-SWNT could also be observed using AFM (Figure S3b).

As shown in Figure 2, treatment of cyclin A_2 siRNA–f-SWNTs reduced cellular levels of both cyclin A_2 mRNA and cyclin A_2 protein in K562, whereas addition of siRNA alone, f-SWNTs, or control siRNA–f-SWNT did not significantly alter the level of either cyclin A_2 mRNA or cyclin A_2 protein. The result showed that cyclin A_2–f-SWNTs could enter into cells and that the inhibitory effect of the cyclin A_2 siRNA was specific.

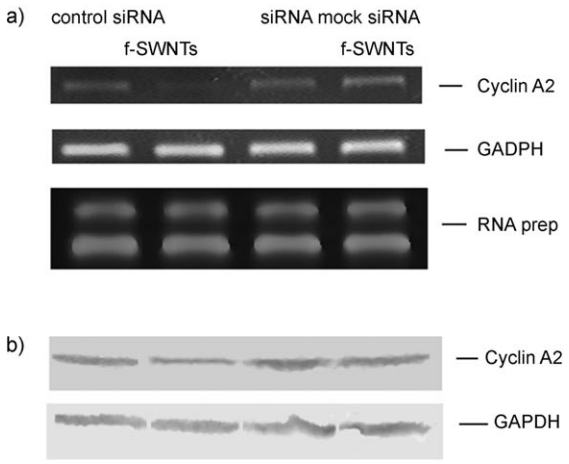

Figure 2. Specific knockdown of gene expression by cyclin A_2 siRNA–f-SWNT. a) Levels of cyclin A_2 transcripts after treatment of K562 cells for 32 h with cyclin A_2 siRNA–f-SWNTs, cyclin A_2 siRNA alone, and mock siRNA–f-SWNTs versus untreated control cells. Both GADPH and total RNA were used as internal control. b) Protein levels of cyclin A_2 and GAPDH in K562 cells treated as in (a).

We found that cyclin A_2 siRNA–f-SWNTs clearly suppressed the growth of K562 and reduced cell number, whereas addition of cyclin A_2 siRNA alone, f-SWNTs, or mock siRNA–f-SWNTs did not significantly affect cell proliferation (Figure 3a). The inhibitory effect on K562 cell growth was confirmed by soft agar assay as shown in Figure 3b. Functionalized single wall carbon nanotubes did not exhibit obvious toxicity in K562 cell.

To determine whether depletion of cyclin A_2 promotes tumor cell death, flow cytometry[42] was performed after transfection of cyclin A_2–f-SWNTs into K562. The cells were analyzed six days after two transfection and sub-G1 (apoptosis) populations were observed (Figure 4). As a control, we transfected mock siRNA–f-SWNTs complexes into cyclin A_2 overexpressing K562, and no significant apoptosis were observed. A typical apoptosis DNA ladder was also obtained for the sample treated with cyclin A_2 siRNA: f-SWNTs but not for the control sample (Figure 5). Cell cycle analysis showed a decreased cell population in S phase after cyclin A_2 siRNA–f-SWNTs treatment (31%) in K562 compared with control samples (45%).

Taken together, our results indicate that the cyclin A_2 siRNA–f-SWNTs exhibited a specific inhibitory effect on cyclin A_2 overexpressing K562 cells through blockage of cell proliferation as well as promotion of apoptosis.

Discussion

Cyclin and cyclin-dependent kinase complexes play an important role in cell cycle regulation.[2] The overexpression of cyclin A_2 in malignant tumors contributes to cancer progression and poor patient survival rates,[8–16] suggesting that the down-regulation of cyclin A_2 could be an attractive strategy to prevent tumor progression. The progress of RNAi techniques has demonstrated the potential to specifically suppress the expression of target genes. However, efficiency of RNAi is often compromised by the instability of siRNA and low uptake efficiency.

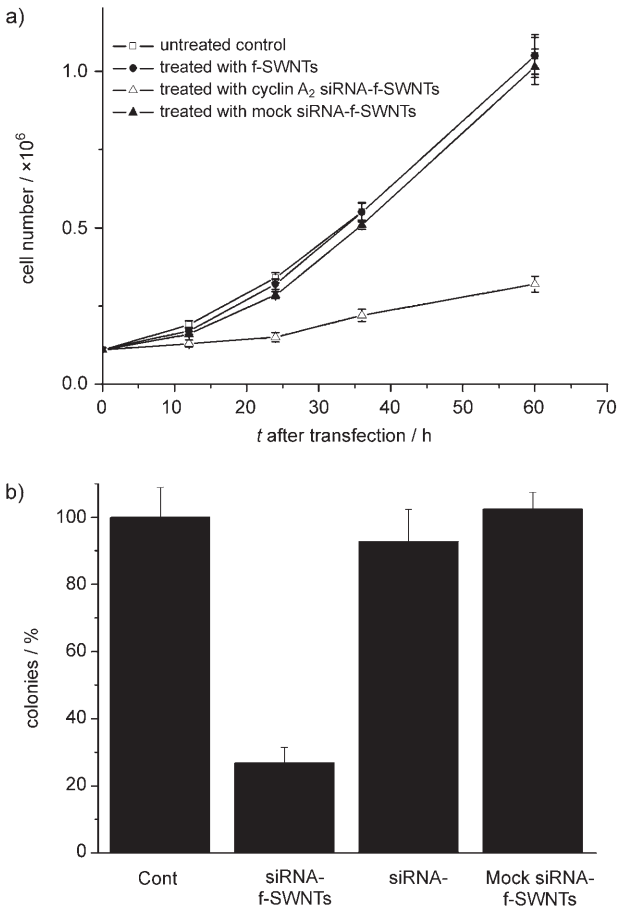

Figure 3. Inhibition of cell growth by cyclin A_2 siRNA in vitro. a) Growth curves of K562 in response to siRNA. The viable cells were counted at the indicated time points. The data shown here represent the average of two independent experiments. b) Suppression of colony formation in soft agar. K562 cells were transfected with siRNA targeting on cyclin A_2 and then seeded in 0.35% agarose containing IMDM with 10% fetal calf serum. The cells without transfection (mock) were used as control. The colony numbers were counted three weeks later. The numbers of colonies were then standardized against the control cells (set as 100%).

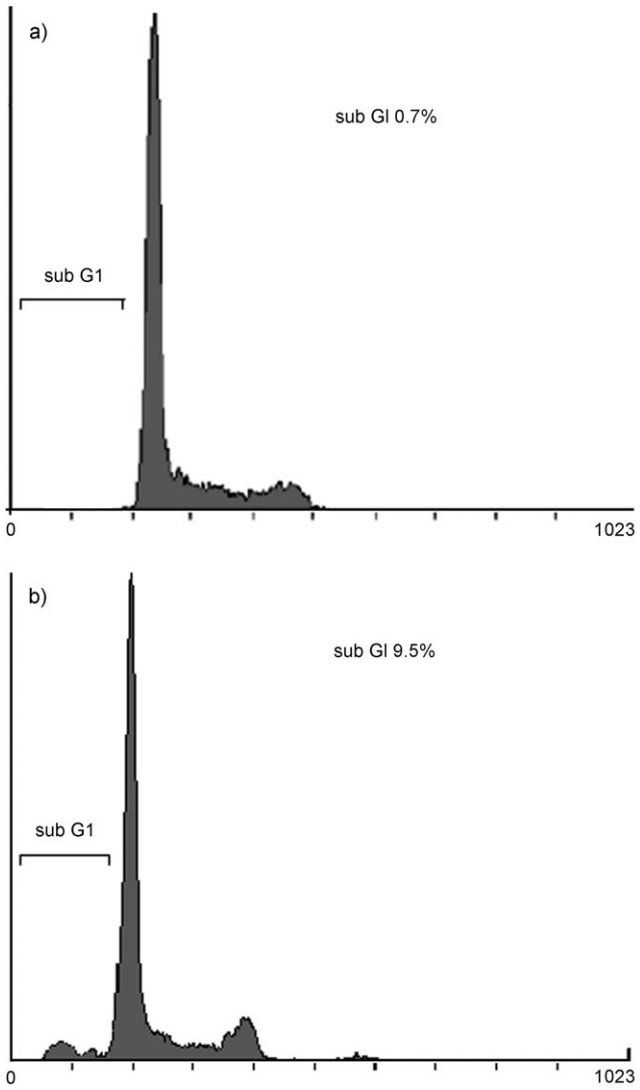

Figure 4. Down regulation of cyclin A_2 in K562 cells by RNAi mediated by functionalized single nanotubes promoted apoptosis. a) Mock siRNA–f-SWNTs treated; b) cyclin A_2 siRNA–f-SWNTs treated. The experimental details are described in the materials and methods section.

In the present studies, we used ammonium functionalized SWNTs to deliver cyclin A_2 siRNA into K562 cells and examined whether depletion of cyclin A_2 in this manner would inhibit the proliferation and induce cell apoptosis.

Compared to classical delivery systems, such as liposomes, single wall carbon nanotubes possess an enormous aspect ratio. This feature allows for more siRNA load and more efficient permeation through cell membranes. Dai and Kateb have demonstrated that carbon-nanotube transporters can efficiently deliver siRNA into human T cells and primary cells and microglia, whereas conventional transfection vectors such as lipofectamine show little effect in the internalization of siRNA.[30,34] Yang et al. have shown that carbon-nanotube vectors can successfully mediate target gene interference even when siRNA concentration is 2 N mol L^{-1}.[29] Moreover, carbon-nanotube vectors have no apparent cell toxicity,[20–21,29–30,32–34] which is also confirmed in this work (Figure S5a), whereas lipofectamine 2000 causes apoptosis and necrosis in some cells (Figure S5b).

Our results demonstrate that siRNA delivered by functionalized SWNTs can effectively downregulate oncogene cyclin A_2 overexpression with specificity, as shown in Figure 2, indicating the potency of RNAi against cyclin A_2 as a new strategy for chronic myelogenous leukemia therapy. Also, the blockage of proliferation and induction of apoptosis in cyclin A_2 siRNA–f-SWNTs treated cells further support the effectiveness of this treatment. It has been reported that normal cells are less sensitive than transformed cells to siRNA, and these differences may be due to a difference in cell membrane.[43] Hence, this specificity would increase the therapeutic index of this RNAi based therapy strategy.

Cyclin A_2 has been known to play an important role at G1-S and G2-M transitions, as well as DNA replication.[2–5] The S-phase fraction decreased in the cyclin A_2 siRNA–f-SWNTs group versus the control group, which was probably associated with the depletion of cyclin A_2 and hence reduced CDK2 activity.

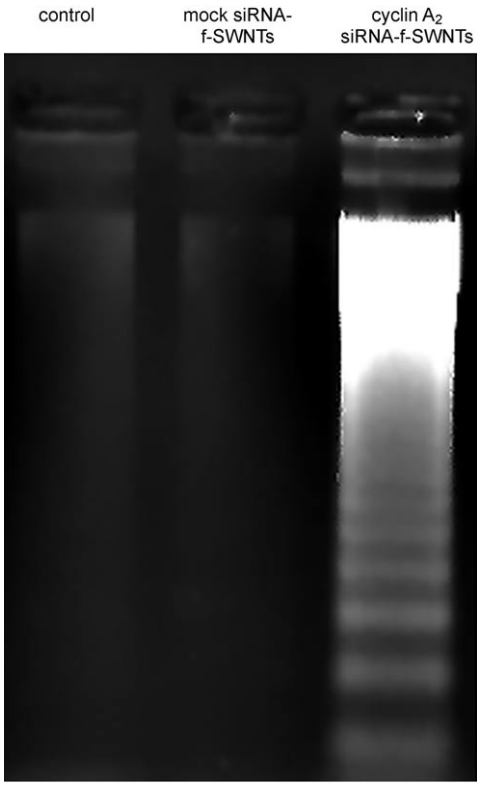

Figure 5. Agarose gel electrophoresis patterns of DNA obtained from K562 cells treated with cyclin A_2 siRNA–f-SWNTs or mock siRNA–f-SWNTs.

Therefore, the inhibition of cell proliferation may result from both the blockage of S-phase entry and DNA replication.

Herein, we showed that when overexpressed cyclin A_2 was depleted in K562 cells, it resulted in blockage of cell proliferation and induction of apoptosis. Our primary results suggest that cyclin A_2 overexpression may be essential for maintaining cell proliferation and cell survival in cyclin A_2-overexpressing K562 cells. This is the first study, to our knowledge, showing that targeting cyclin A_2 overexpression is a potential effective approach to treating cyclin A_2-overexpressing chronic myelogenous leukemia cells.

In summary, cyclin A_2 is a promising anticancer target molecule[2] as the depletion of cyclin A_2 results in reduced cell proliferation and cell apoptosis, and our primary studies suggest siRNA against cyclin A_2 mediated by functionalized single wall carbon nanotubes might be a useful therapeutic strategy for chronic myelogenous leukemia cells. The results would be of importance for providing an additional therapeutic option for chronic myelogenous leukemia beyond chemotherapy in light of increasing multidrug resistances. Different types of cancer cell lines need to be studied. The highly specific function of siRNA to their target genes and functionalized single wall carbon nanotubes may offer the possibility to inhibit different cancer-promoting genes such as cyclin A_2.

Experimental Section

Cell culture

The human erythroleukemic cell line K562 was grown in Iscove's modified Dulbecco's medium (Gibco BRL) supplemented with 10% fetal calf serum and 4 mM L-glutamine in a humidified 37 °C incubator with 5% CO_2.

Preparation of cyclin A_2 siRNA–f-SWNT complexes

SWNTs ($\phi = 1.1$ nm, purity > 90%) were purchased from Aldrich (St. Louis, MO,USA), purified as described previously by sonicating SWNTs in a 3:1 *v/v* solution of concentrated sulfuric acid (98%) and concentrated nitric acid (70%) for 24 h at 35–40 °C, and washed with water, leaving an open hole in the tube side, and functionalizing the open end of SWNTs with a carboxyl group to increase their solubility in aqueous solution.[37–38,41] The shortened length of SWNTs was in the range of 50–300 nm (Figure S3a). SWNTs-NH_2 was prepared using the method described by Chen et al.[41] Briefly, shortened SWNTs (100 mg) were stirred in $SOCl_2$ (20 mL, containing 1 mL of dimethylformamide) at 70 °C for 24 h. After centrifugation, the supernatant was decanted and the remaining solid was washed with anhydrous tetrahydrofuran. After centrifugation, the remaining solid was dried at room temperature under vacuum. A mixture of the resulting SWNTs-COCl (20 mg) and 1, 6 hexanediamine (5 mL) was reacted in dimethylformamide at 120 °C for 96 h under nitrogen. After cooling to room temperature, the excess 1, 6 hexanediamine was removed by washing with ethanol four times (10 min sonication at 40 KHz). The remaining solid was dissolved in dioxane. Finally, HCl solution (about 6 M) was added into the aqueous suspension of SWNTs-NH_2, and the positively charged functionalized SWNTs were collected by standard centrifugation and membrane filtration. Functionalized SWNTs solution is stable for at least four weeks at 4 °C (Figure S4). To prepare cyclin A_2 siRNA–f-SWNTs complex, the SWNT-NH_2 was incubated with siRNA at various ratios for 1 h at room temperature.

Electrophoretic mobility shift assay

A 0.3 μg amount of siRNA (targeting for cyclin A_2) complexed to the ammonium-functionalized single wall carbon nanotubes at different ratio (*w/w*), or 0.3 μg of free siRNA as a control, was added to 20% native PAGE gel in 1×TBE buffer. The gel was run for 2 h at 100 V, and stained by EB or silver, then photographed using a UVP gel documentation system (Ultraviolet Products, Upland, CA, USA).

Transfection of cyclin A_2 siRNA–f-SWNT complex

The siRNA oligos were synthesized by Genepharma Corporation (Shanghai, China). The siRNA corresponded to nts 524–544 of the human cyclin A_2 coding region (GeneBank accession no. NM_001237).[17] The nonsilencing control siRNA is an irrelevant siRNA with random nucleotides UUCUCCGAACGUGUCACGUTT. cyclin A_2 siRNA–f-SWNTs complexes ($w_{\text{f-SWNTs}}/w_{\text{siRNA}} = 40$) were added at 25 nmol/L(siRNA concentration) to culture of cell.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and then treated with RNase-free DNase (1 U μL^{-1} Takara; Dalian, China), for 30 min at 37 °C fol-

lowed by inactivation of DNase at 75 °C for 5 min. To control for genomic DNA contamination, a RT-PCR reaction was performed without the addition of the transcriptase enzyme. The primers and conditions for cyclin A_2 were TCCATGTCAGTGCTGAGAGGA (5′), GAAGGTCCATGAGACAAGGC (3′); 94 °C for 30 seconds , 60 °C for 30 seconds, 72 °C for 1 min for 25 cycles. Three introns are present in this pair of primer so that any contaminating genomic DNA would not be amplified. Primers used for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were ACCTGACCTGCCGTCTAGAA (5′), TCCACCACCCTGTTGCTGTA (3′). Two sets of primers were used for each sample, including primers specific for the gene of cyclin A_2 and primers for GAPDH as an internal control. All PCR products were visualized on 1.5% agarose gel with added ethidium bromide.

Western blotting

For Western blot analysis, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mmol L^{-1} NaCl, 50 mmol L^{-1} Tris-HCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, pH 7.6) containing protease inhibitors for protein extraction. Protein concentrations were determined using the Bradford assay as described by the manufacturer.

Cell lysate samples (20 μg) were denatured by addition of 2× reducing sample buffer (100 mmol L^{-1} Tris, 4% SDS, 25% glycerol, 10% β-mercaptoethanol, 0.01% bromphenol blue, pH 6.8), incubated for 10 min at 95 °C, and separated on a 12% SDS-PAGE. The proteins were electroblotted to PVDF membrane as described by the manufacturer. After blocking with 2% BSA, the membrane was incubated with rabbit polyclonal anticyclin A_2 primary antibody (Lab Vision Corporation, CA, USA). The protein–antibody complexes were detected using peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, Stratech, UK) and visualized by CN/DAB substrate according to reference [44]. Complete transfer of proteins was checked by staining gels with Coomassie blue. Immunoblotting for GAPDH was performed to verify equivalent protein loading. Images were photographed using a UVP gel documentation system (Ultraviolet Products, Upland, CA, USA).

Cell cycle analysis

Standard fluorescence-activated cell sorter analysis was used to determine apoptosis of the cells or the distribution of cells in cell cycle.[42,45] The apoptotic cells were assessed by flow cytometric detection of sub-G1 DNA content after being stained with propidium iodide.

Apoptotic DNA ladder assays

We isolated and analyzed cellular DNA of K562 cells in 1% agarose gel according to the method described in reference [46].

Colony Formation Assay in Soft Agarose

Briefly, K562 was transfected with cyclin A_2 siRNA–f-SWNTs or negative control. Forty hours after the transfection, the cells were plated in 24-well plates in culture medium containing 0.35% agarose overlying a 0.7% agarose bottom layer and cultured at 37 °C with 5% CO_2. Three weeks later, colonies were counted using a microscope.

Statistical analysis

Data are expressed as mean ± s.d. and analysis of variance was carried out using Student's t test with Origin 7.5 (OriginLab Corporation, Northampton, MA, USA), where $P < 0.05$ was considered significant.

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Keywords: apoptosis · cyclin A_2 · leukemia · RNA interference (RNAi) · single-walled carbon nanotube

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