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Toxicology Letters 155 (2005) 73–85
Toxicology Letters
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Effect of single wall carbon nanotubes on human HEK293 cells

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Received 19 April 2004; received in revised form 19 August 2004; accepted 31 August 2004
Available online 19 October 2004

Abstract

The influence of single-walled carbon nanotubes (SWCNTs) on human HEK293 cells is investigated with the aim of exploring SWCNTs biocompatibility. Results showed that SWCNTs can inhibit HEK293 cell proliferation, decrease cell adhesive ability in a dose- and time-dependent manner. HEK293 cells exhibit active responses to SWCNTs such as secretion of some 20–30 kd proteins to wrap SWCNTs, aggregation of cells attached by SWCNTs and formation of nodular structures. Cell cycle analysis showed that 25 μg/ml SWCNTs in medium induced G_1 arrest and cell apoptosis in HEK293 cells. Biochip analysis showed that SWCNTs can induce up-regulation expression of cell cycle-associated genes such as *p16*, *bax*, *p57*, *hrk*, *cdc42* and *cdc37*, down-regulation expression of cell cycle genes such as *cdk2*, *cdk4*, *cdk6* and *cyclin D3*, and down-regulation expression of signal transduction-associated genes such as *mad2*, *jak1*, *ttk*, *pcdha9* and *erk*. Western blot analysis showed that SWCNTs can induce down-regulation expression of adhesion-associated proteins such as laminin, fibronectin, cadherin, FAK and collagen IV. These results suggest that down-regulation of G_1-assoicated *cdks* and *cyclins* and upregulation of apoptosis-associated genes may contribute to SWCNTs induced G_1 phase arrest and cell apoptosis. In conclusion, SWCNTs can inhibit HEK293 cells growth by inducing cell apoptosis and decreasing cellular adhesion ability.

Keywords: HEK293 cell; Single-walled carbon nanotube; Apoptosis; Biochip; Flow cytometry analysis; Western blot; Immunofluorescent analysis

1. Introduction

Carbon nanotubes, as a class of stiff, stable and hollow nanomaterials with many unique properties such as mechanical, physical and chemical properties, have been being explored application in biomedical engineering and medical chemistry (Baughman et al., 2002;

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doi:10.1016/j.toxlet.2004.08.015

Bianco and Prato, 2003; Pantarotto et al., 2004). For example, carbon nanotubes have been used as AFM tip to obtain atomic-resolution imaging of biological molecules such as DNA and proteins (Nagao et al., 2000; Hafner et al., 2001). Our research also show that carbon nanotubes can be filled with DNA or peptide molecules and have highly potential in gene or peptide storage and delivery system in molecular therapy of diseases (Gao et al., 2003; Cui and Gao, 2003; Cui et al., 2004a). Carbon nanotubes can be used to fabricate nanomotors, which likely enter inside cells to treat diseases. So far, it has becoming a focus to investigate the influence of carbon nanotubes and associated nanomaterials or nanodevices on human cells and environment. Carbon nanotubes can be functionalized to achieve improved properties and functions such as biocompatibility and biomolecular recognition capabilities (Shim et al., 2002; Bahr and Tour, 2002). The potential with which carbon nanotubes can be applied in biomedical engineering and medicinal chemistry is highly dependent upon their biocompatibility. Carbon nanotubes exhibit cytotoxicity to human keratinocyte cells (Robert, 2003; Shvedova et al., 2003), can inhibit the growth of embryonic rat-brain neuron cells (Mattson et al., 2001) and induce the formation of mouse-lung granulomas (Chan et al., 2003; Warheit et al., 2004; Maynard et al., 2004; Lam et al., 2004). However, so far few reports are closely associated with mechanism of effect of carbon nanotubes on human normal or tumor or embryonic tissue cells. Therefore, investigating effect of carbon nanotubes on human cells and their interaction mechanism is of very importance.

Here, we selected human HEK293 cells as research target, investigated the interaction between SWCNTs and HEK293 cells by morphological observation, Western blot, flow cytometry, immunofluorescent analysis and biochip analysis. Result shows that SWCNTs can inhibit the proliferation of human HEK293 cells, induce cell apoptosis, decrease cell adhesive ability, and cause the cells to secrete some 20–30 kd proteins, which wrap SWCNTs into nodular structures and isolate SWCNTs-attached cells from the main cell populations. A model of signal transduction and cellular pathways of SWCNTs-cell interaction was proposed. The main purpose of current study was to explore the effect of SWCNTs on human HEK293 cells and potential biochemical mechanism, laying foundation for further exploring carbon nanotubes application on molecular therapy of diseases in near future.

2. Materials and methods

2.1. Single-walled carbon nanotubes and antibodies

Single-walled carbon nanotubes (SWCNTs) were purchased from Carbon Nanotechnologies, Inc. (CAS no. 7782-42-5). Monoclonal anti-human fibronectin antibody (product no. F7387), anti-focal adhesion kinase ($pp125^{FAK}$) antibody (product no. F2918), anti-pan cadherin antibody (product no. C3678), monoclonal anti-collagen type IV clone COL-94 (product no. 1926), monoclonal anti-laminin clone lam-89 (product no. L8271), anti-cyclin D3 antibody (product no. C7214), monoclonal anti-β-actin clone AC-15 (product no. A5441), anti-rabbit IgG FITC (product no. F9037) and anti-mouse IgG Cy3 conjugate antibody (product no. C2181) were purchased from Sigma Inc.

2.2. Cell viability and proliferation assay

HEK293 cells (human embryo kidney cells) were obtained from American ATCC Cell Line Center. The cells were cultured in essential medium with Earle's salt supplemented with 10% fetal calf serum, 1% kanamycin and 2 mM glutamine (GIBCO-BRL Life Technologies, Gaithersburg, MD), at 37 °C in 5% CO_2 humidified incubator. The medium was exchanged once per two days. MTT (tetrazolium salt) assay was applied to evaluate the effect of SWCNTs on HEK293 cells viability by measuring the uptake and reduction of tetrazolium salt to an insoluble formazan dye by cellular microsomal enzymes (Sasada et al., 1996). SWCNTs with different concentrations of 0.78125 μg/ml, 1.5625 μg/ml, 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml were added inside the 24-well plates. HEK293 cells without SWCNTs were used as the control. The cell viability was calculated by the follow formula: cell viability (%) = optical density (OD) of the treated cells/OD of the non-treated cells. The cell number was counted daily by using the Trypan blue dye exclusion method and the percentage of cell growth was calculated as a ratio of numbers of

SWCNTs-treated cells and control cells treated with 0.5% DMSO vehicle (Alley et al., 1988).

2.3. Detection of adhesion ability

The cell attachment assay was performed as previously described (Akiko et al., 2000). Essentially, 6-well plates were coated with fibrinogen (5 μg/ml) and vitronectin (1.5 μg/ml) in DPBS. Cells were harvested, washed three times with serum-free minimal essential medium with Eargle's salt and resuspended in attachment solution (calcium- and magnesium-free Hanks' balanced salt solution, 20 mM HEPES, 1 mg/ml heat-inactivated BSA, 1 mM $CaCl_2$ and 1mM $MgCl_2$). Cells (1×10^4) were added to each well and allowed to culture for 1–5 days at 37 °C in a humidified 5% CO_2 incubator. These plates of respective 25 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml SWCNTs-treated cells were cultured for 1–5 days and 1 control plate (1×10^4 cells were added to each well-treated with 0.5% DMSO vehicle and allowed to culture for 1–5 days at 37 °C in a humidified 5% CO_2 incubator) was centrifuged for 10 min at the speed of 4000 rpm. Unattached cells were washed with Hanks' balanced salt solution. The number of remaining attached cells after centrifugation was quantified spectrophotometrically at 405 nm in triplicate (Lotz et al., 1989). Cell adhesion ability (%) = the number of SWCNTs-treated adhesive cells/the number of control adhesive cells.

2.4. DNA fragmentation

HEK293 cells were cultured with 25 μg/ml of or without SWCNTs for 1–5 days, and resuspended in lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA and 0.3% Triton X-100) for 30 min on ice. Cell lysates were treated with RNase (100 μg/ml) for 30 min at 55 °C and then with proteinase K (400 μg/ml) for another 1 h at 55 °C. The supernatant was extracted with phenol/chloroform. The DNA was precipitated and electrophoresed on 2% agarose gels (Wang et al., 2001).

2.5. Flow cytometry analysis

HEK293 cells were treated without or with 25 μg/ml of SWCNTs for 1–5 days, and harvested at respective days. After washing with PBS, the cells were fixed in 70% ethanol/PBS for 30 min on ice. Approximately 4×10^5 cells were centrifuged. The cell pellets were resuspended with PBS, and further treated with RNase (DNase free, 100 μg/ml, final concentration in PBS) and propidium iodide (40 μg/ml, final concentration in PBS) for 30 min at 37 °C. The treated cells were centrifuged. The cell pellets were resuspended with PBS. The cell suspension was passed through a 19-gauge needle and kept on ice until analysis. The number of cells in different phases of the cell cycle was analyzed using a FACScan Flow cytometer with Cell-FIT software (Becton Dickinson Instruments).

2.6. SDS-PAGE analysis and Western blot analysis

HEK293 cells were treated with 25 μg/ml of SWCNTs for 1–5 days. After incubation, cells were lysed in protein lysis buffer (50mM Tris pH 7.4,150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% 2-mercaptoethanol, 1% NP-40, 0.25% sodium deoxycholate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 0.2 mM phenylmethyl sulfonylfluoride); protein concentrations were determined using the Bradford method. Equal amounts of sample lysate were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was blocked with 0.1% BSA in TBST buffer (20 mM Tris, pH7.4, 150 mM NaCl and 0.1% Tween-20), and incubated overnight at 4 °C with specific primary antibodies. Subsequently, the membrane was washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence kits were used (Amersham, ECL kits). In order to confirm whether SWCNTs can stimulate HEK293 cells secrete small molecular proteins, HEK293 cells were cultured for 1–5 days in essential medium without 10% fetal calf serum with the aim of excluding mistaking fetal calf serum proteins as secreted small molecular proteins.

2.7. Immunofluorescent staining analysis

HEK293 cells with 25 μg/ml of SWCNTs were cultured on sterile coverslips at 37 °C in a humidified atmosphere of 5% CO_2 in air and cultured for 1–5 days. The cells were then washed briefly in PBS, fixed with

−20 °C methanol for 10 min and with −20 °C acetone for 1 min. The coverslips were washed twice in PBS, and blocked with PBS containing 0.1% BSA for 10 min at room temperature followed by draining. The cell-side-up of coverslips was incubated with antibody (1:2000) in PBS containing 1% BSA for 60 min, and was washed for three times in PBS. The coverslips cell-side-up was incubated with anti-mouse FITC conjugate as the secondary antibody, at the recommended dilution, in PBS containing 1% BSA, for 30 min, and then was washed for three times in PBS. One drop of aqueous mounting medium was added on the coverslip and inverted carefully on a glass slide. The cells were observed by a fluorescence microscope with appropriate filters and taken photography.

2.8. Microarray analysis

2.8.1. Fabrication of microarrays

Hundred pairs of oligonucleotides probes associated with cell cycle, cell apoptosis and signal transduction were designed and synthesized in Germany MWG company, and were fabricated into microarrays according to standard method (Schena, 1999). As a quality control, Spot Report™ Oligo™ Array Validation System (Cat # 252170-7) was purchased from Stratagene® company.

2.8.2. Extraction of total RNAs and probe preparation

HEK293 cells with 25 μg/ml of or without SWCNTs were cultured in 200 ml culture bottles at 37 °C in a humidified atmosphere of 5% CO_2 in air for two days. Then the cells were collected and washed repeatedly with PBS (pH 7.4) until the SWCNTs were completely removed away. Total RNAs from 1×10^6 HEK293 cells, cultured with 25 μg/ml of or without SWCNTs for two days, were extracted by using total RNA extraction kit from Promega Inc. Final total RNAs were dissolved in RNase-free H_2O and diluted into the concentration of 0.5 μg/μl. Fluorescent-labeled cRNA probes were prepared through reverse transcription and purified according to the protocol of Schena (1999). The probes from HEK293 cells with 25 μg/ml of SWCNTs were labeled with Cy5-dUTP, and the probes from HEK293 cells without SWCNTs were labeled with Cy3-dUTP. These probes were mixed and precipitated by ethanol, and finally dissolved in 20 μl hybridization solution (5 × SSC + 0.2% SDS) (DeRisi et al., 1997).

2.8.3. Hybridization and washing

After denaturing at 95 °C for 5 min, the probes were added onto slides, covered with a cover and incubated at 42 °C for 17 h. The slides were subsequently washed in solutions of 2 × SSC + 0.2%SDS, 0.1 × SSC + 0.2% SDS and 0.1 × SSC, 10 min each time, and dried at room temperature.

2.8.4. Detection and analysis

Affymetrix®428™ Array Scanner was used to collect the image of post-hybridization chip. ImageGene 3.0 software (BioDiscovery Inc.) was used to quantify, correct for background noise and normalize signals from hybridization chip.

2.8.5. Data analysis

All data are presented in this paper as means result ± S.D. Statistical differences were evaluated using the *t*-test and considered significance at $P < 0.01$ level. All figures shown in this article were obtained from three independent experiments with similar results.

3. Results

3.1. Effect of SWCNTs on the viability and proliferation of HEK293 cells

Since cell viability is positively correlated with the degree of MTT reduction, the cell viability of SWCNTs-treated HEK293 cells were evaluated by using MTT reduction assays. As indicated in Fig. 1A, treatment of HEK293 cells with various concentrations (0.78125 μg/ml, 1.5625 μg/ml, 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml) of SWCNTs caused a time- and dose-dependent decrease in cell viability relative to the control culture.

As indicated in Fig. 1B, treatment of HEK293 cells with various concentrations of SWCNTs caused a time- and dose-dependent decrease in cell number relative to control cultures. This result showed that SWCNTs can inhibit the proliferation of HEK293 cells.

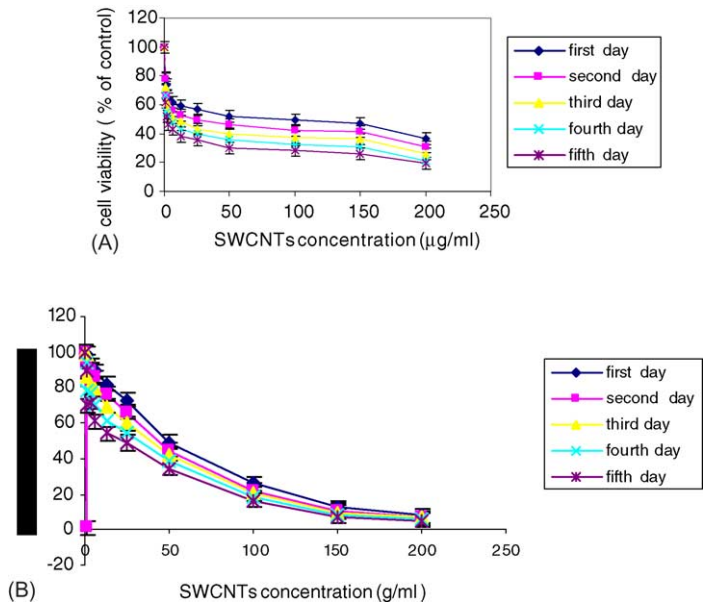

Fig. 1. (A) HEK293 cells viability curve measured by MTT assay and the percentage of cell viability was calculated as a ratio of OD of SWCNTs-treated cells and control cells. (B) Antiproliferation effect of SWCNTs in HEK293 cells. Cell number was measured by Trypan blue dye exclusion method and the percentage of cell growth was calculated as a ratio of numbers of SWCNTs-treated cells and control cells (treated with 0.5% DMSO vehicle).

3.2. Effect of SWCNTs on cell adhesion

The adhesive ability of SWCNTs-treated HEK293 cells can be evaluated with the ratio of SWCNTs-treated adhesive cell number to the control adhesive cell number after centrifuge. As shown in Fig. 2, the cell adhesive ability decreased markedly with the increase in SWCNT concentration and culture time.

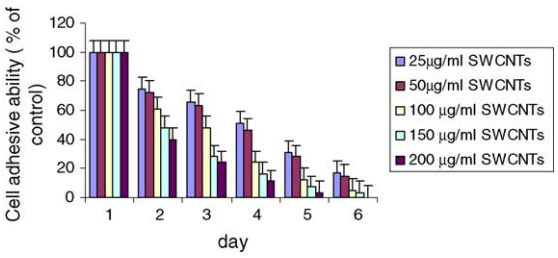

Fig. 2. SWCNT-treated HEK293 cell adhesion ability measured by centrifugation method. The percentage of adhesive cells decreased markedly with the increase in SWCNT concentration and culture time.

3.3. Induction of apoptosis of HEK293 cells by SWCNTs

Microscopic observation of SWCNTs-treated HEK293 cells showed that some HEK293 cells rounded up and detached from the culture plates after 24 h of incubation. As the dose of SWCNTs in the medium reached 25 μg/ml and cultured for over 24 h, the cultured cells displayed morphological changes characteristic of apoptosis. As shown in Fig. 3A, HEK293 cells cultured with 25 μg/ml SWCNTs for 72 h exhibited features characteristic of apoptosis. Cells became round, small and floated as shown in A_0 compared with control cells; apoptotic cells formed nodular structure encapsulating SWCNTs as shown in A_1; black SWCNTs attached to apoptotic cells as shown in A_2. These apoptotic cells were further observed to exhibit typical apoptosis features such as membrane vesicles, nucleus condensation, fragmentation and apoptotic bodies. DNA ladder electrophoresis in Fig. 3B showed that HEK293 cells cultured with 25 μg/ml of SWCNTs for 24 h exhibited typical apoptosis ladder, which became more and more

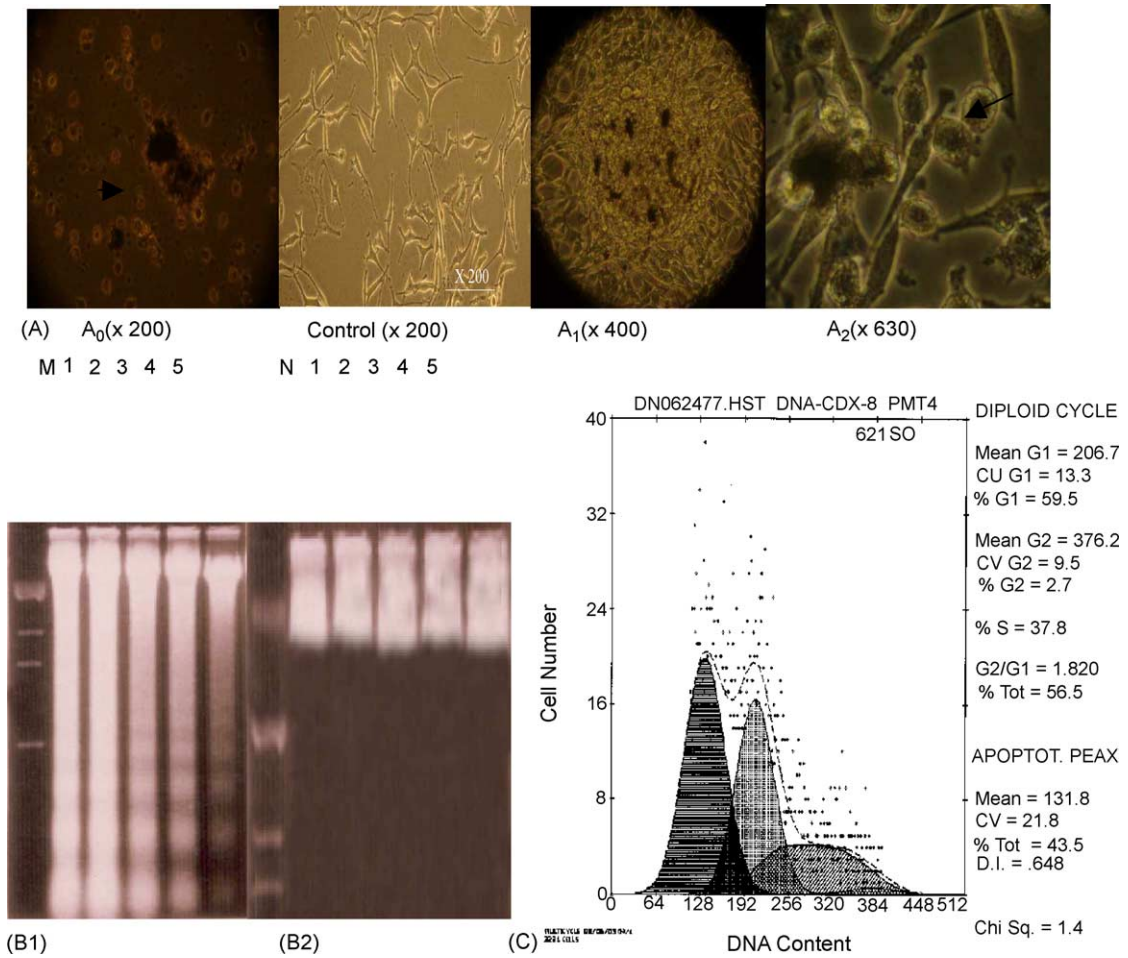

Fig. 3. Apoptosis of HEK293 cells induced by SWCNTs. A: morphological changes of HEK293 cells cultured with 25 μg/ml SWCNTs for three days; A_0: showing cells become round and floating with apoptotic characteristics; control: showing normal morphological cells; A_1: showing nodular structure composed of SWCNTs and apoptotic cells; A_2 showing apoptotic cells attached by SWCNTs. B1: DNA electrophoresis of cells cultured with 25 μg/ml SWCNTs for 1–5 days, *M* molecular marker, no. 1–5 denote the results of cells cultured for day 1–5, respectively; B2: DNA electrophoresis results of control cells cultured for day 1–5; C: the cell cycle distribution of HEK293 cells cultured with 25 μg/ml SWCNTs for four days, the percentage of sub-G_1 cells (apoptosis cells) was 43.5%.

marked as the cell culture days increased, however, the control cells exhibited no DNA ladder.

Cell cycle analysis of HEK293 cells with 25 μg/ml, 50 μg/ml, 100 μg/ml and 150 μg/ml SWCNTs is shown in Table 1. When the concentration of SWCNTs reach 25 μg/ml and cultured for 24 h, the cell cycle was arrested in G_1, 5.3% cells exhibited apoptotic feature. Similar results were also observed respectively at 50 μg/ml, 100 μg/ml, 150 μg/ml SWCNTs cultured for 24 h. As cell culture days increased, the number of cells following the normal cell cycle of G_1, G_2 and M phases continued to drop, while that of apoptotic and dead cells continued to rise. Fig. 3C depicts the cell cycle distribution of HEK293 cells with 25 μg/ml SWCNTs cultured for four days, indicating that the percentage of apoptotic cells reach 43.5%, a high sub-G_1 peak (apoptosis peak) appeared before the G_1 phase peak. These results confirmed that SWCNTs could cause cell cycle arrest in G_1 and induce HEK293 cells apoptosis in a dose- and time- dependent manner.

Table 1
Cell cycle analysis of control 293 cells and SWCNTs-treated 293 cells

SWCNT concentration (μg/ml)	Time (h)	Distribution ratio (%)			
		G_1	S	G_2/M	Apoptotic cells[a]
0	24	35.0 ± 1.7	51.0 ± 2.6	11.0 ± 0.6	0.0
0	48	37.9 ± 1.8	50.0 ± 2.5	12.1 ± 0.7	0.0
0	72	37.1 ± 1.8	49.8 ± 2.4	13.1 ± 0.6	0.0
0	96	32.2 ± 1.9	50.9 ± 2.5	12.9 ± 0.5	0.0
25	24	70.0 ± 3.5	26.0 ± 1.3	4.0 ± 0.2	5.3 ± 0.3
25	48	64.5 ± 3.2	32.7 ± 1.6	2.7 ± 0.1	16.4 ± 0.8
25	72	61.0 ± 3.1	28.9 ± 1.4	10.1 ± 0.5	25.4 ± 1.3
25	96	59.5 ± 2.9	37.8 ± 1.8	2.7 ± 0.1	43.5 ± 2.2
50	24	45.8 ± 2.3	38.2 ± 1.9	16.0 ± 0.8	32.2 ± 1.6
50	48	62.4 ± 3.1	33.3 ± 1.7	4.3 ± 0.2	51.4 ± 2.6
50	72	38.2 ± 1.9	33.1 ± 1.6	29.7 ± 1.5	60.5 ± 3.0
100	24	88.2 ± 4.4	9.6 ± 0.4	2.2 ± 0.1	48.8 ± 2.4
100	48	12.7 ± 0.6	36.7 ± 1.8	50.6 ± 2.5	57.9 ± 2.8
100	72	0.0 ± 0.2	33.1 ± 1.6	66.9 ± 3.3	64.8 ± 3.2
150	24	80.1 ± 4.0	5.0 ± 0.2	9.0 ± 0.4	52.1 ± 2.6
150	48	58.0 ± 2.9	31.0 ± 1.6	4.0 ± 0.2	61.9 ± 3.1
150	72	32.4 ± 1.6	9.6 ± 0.4	1.9 ± 0.1	78.5 ± 3.9

Normal control: HEK293 cells cultured without SWCNTs.
[a] Means main sub-G_1 cells, also includes second necrosis death cells of post-apoptotic cells.

3.4. Effect of SWCNTs on adhesive proteins and cyclin D3 in HEK293 cells

As shown in Fig. 4, Western blot analysis showed that the expression of adhesive proteins such as laminin, fibronectin, FAK, cadherin and cell cycle protein cyclin D3 in HEK293 cells decreased gradually as the culture days and the amount of SWCNTs increased. The expression of these adhesive proteins in the control cells exhibited no significant difference ($P > 0.05$). Indirect immunofluorescent staining analysis demonstrated, the expression levels of cadherin and collagen IV in the control cells cultured for day 1–5 didn't exhibit significant difference ($P > 0.05$), however, their expression levels in HEK293 cells cultured with SWCNTs for day 1–5 decreased grad-

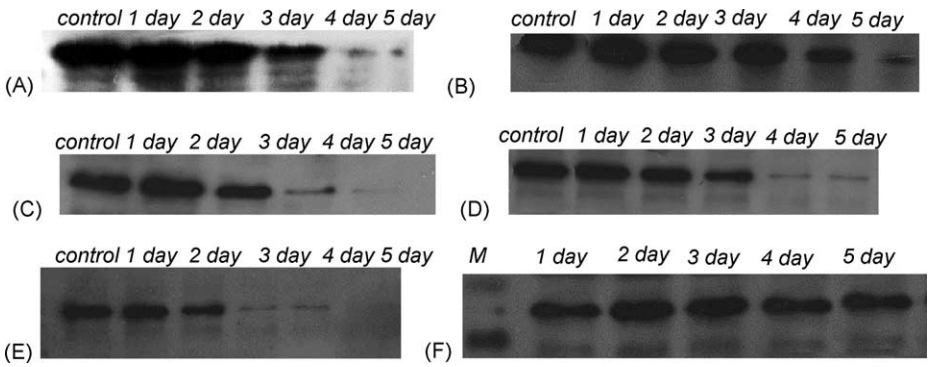

Fig. 4. Western blot analysis of adhesion proteins in HEK293 cells cultured with 25 μg/ml of SWCNTs for 1–5 days. A–E: expression results of laminin, fibronectin, FAK, cadherin, cyclin D3, respectively; controls in A–E: expression results of matched adhesion proteins in normal cells culture; F: expression of β-actin in these samples to normalize each lane for protein content; *M*: protein marker, F1–F5: expression of β-actin in these samples cultured for day 1–5.

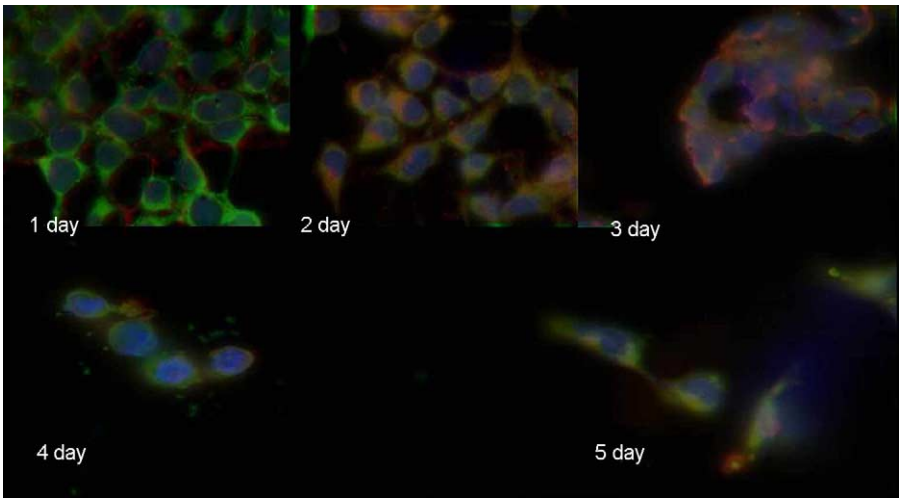

Fig. 5. Indirect immunofluorescent staining of SWCNTs-treated HEK293 cells for day 1–5 by fluorescent microscopy (×630). Green: cadherin; red: collagen; blue: cellular nucleus staining with DAPI. The expression levels of cadherin and collagen IV in cells decreased gradually as the culture days increased; HEK293 cells gradually detached from the cell populations as the culture days increased.

ually as the culture days increased as shown in Fig. 5 ($P < 0.01$).

3.5. Active responses of HEK293 cells to SWCNTs

When added in the culture bottle of HEK293 cells, SWCNTs were initially uniformly distributed but began to attach to cell surfaces within a few hours. When the concentration of SWCNTs reached 5 μg/ml, the HEK293 cells produced a series of interesting reactions. On the 2nd day, some SWCNTs were aggregated together and surrounded by HEK293 cells, as shown in Fig. 6A. The aggregated SWCNTs were wrapped by some proteins secreted by HEK293 cells (Fig. 6B). As indicated in Fig. 6C, the cells began to secrete several 20–30 kd proteins on the 2nd day after SWCNTs were added into the culture, these proteins were still secreted on the 3rd day, but can't be secreted on the 4th and 5th days. These proteins were also not detected in HEK293 cells cultured in the absence of SWCNTs. In order to

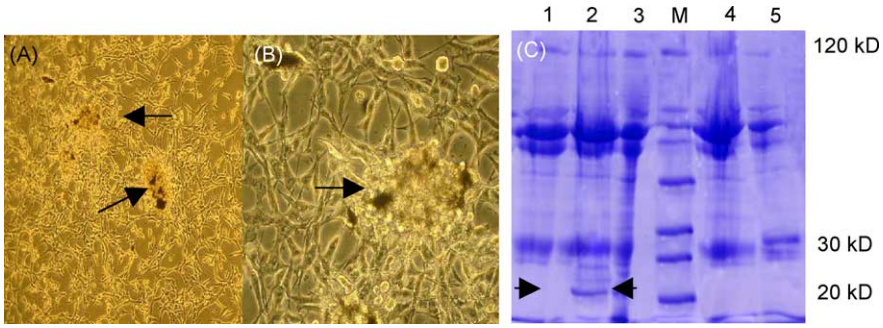

Fig. 6. Responses of HEK293 to single-walled carbon nanotubes. (A) SWCNTs were aggregated together and surrounded by HEK293 cells (×200). (B) The aggregated SWCNTs were surrounded by secretion from HEK293 cells (×400). (C) SDS-PAGE result showed that some 20–30 kd proteins existed in the supernatants of HEK293 cells with 25 μg/ml SWCNTs. *M* is protein marker; the no. 1–5 denote the results for HEK293 cells cultured with SWCNTs for 1–5 days, respectively, showing some small secreted proteins were only detected on the day 2 and day 3 after SWCNTs were added inside the cell culture.

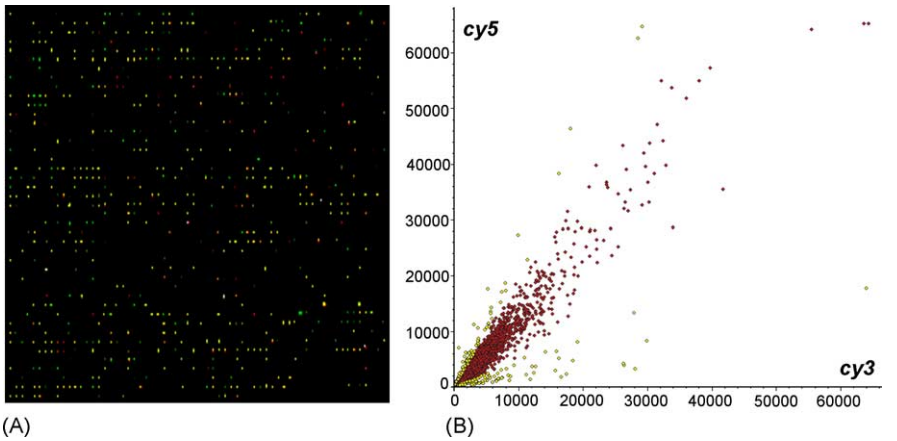

Fig. 7. Result of oligonucleotide microarrays analysis. A: the partial scanning image of hybridization of microarrays with samples, the red colour means higher expression of genes, blue colour means lower expression of genes; B: scatter plot of expression levels of genes in samples.

further confirm that HEK293 cells secret small molecular proteins, the free-serum culture reagents were respectively used to culture HEK293 cells with 25 μg/ml, 50 μg/ml and 100 μg/ml SWCNTs for day 1–5. The collected cell supernatant fluids were confirmed to exist 20–30 kd proteins on the 2nd day after SWCNTs were added into the culture reagents by 15% SDS-PAGE analysis. The structure and functions as well as secreted mechanism of secreted small proteins are under way of investigation.

3.6. Gene expression profile between HEK293 cells with or without SWCNTs by oligonucleotide microarrays

The expression levels of genes associated with cell cycle, cell apoptosis and signal transduction in HEK293 cells cultured with 25 μg/ml of or without SWCNTs for two days were analyzed by oligonucleotide microarrays as shown in Fig. 7 and Table 2 . The analysis showed that HEK293 cells were arrested in the G_1 phase, with Rb/P53 as the main apoptosis pathway induced by SWCNTs as shown in Table 1. Apoptosis-associated genes such as *p16*, *bax*, *hrk*, *bak1*, *p53*, *p57*, *FGFR2*, *TGF beta receptor 1* (*TGF-betaR1*) and *TNFAIP2* exhibited up-regulation expression, further supporting that SWCNTs may induce apoptosis of HEK293 cells.

G_1/S phase associated genes (*cyclin D1*, *cdk4*, *cdk6*, *cyclin E2*, *cyclin-E binding protein 1*, *cdk2*, *cdk3* and *cdc25*), S phase associated genes (*cyclin A1*, *cdc25a* and *cdk2*), G_2 phase associated genes (*cyclin A2*, *cdk8*, *cdk9*, *cyclin C*, *cdc25c* and *cyclin D3*), M phase associated genes (*cyclin M3*, *cyclin b2*, *cdc14a* and *cyclin M2*) all showed down-regulation expression, cell cycle genes such as *PP2A*, *cyclin G1*, *cyclin G2*, *cyclin I*, *cdc37*, *cdc42* and *cdc46* exhibited up-regulation expression, these results fully confirmed that the HEK293 cells were arrested in the G_1/S phase, and SWCNTs prevented the cells from continuation into the S, G_2 and M phases.

The biochip analysis also showed that genes associated with signal transduction such as *mad2*, *jak1*, *ttk*, *tyk2*, *early growth response 1*, *Pa2g4*, *cadherin-5*, *CNGA1*, *PCDHA9* and *CACNA2D3* exhibited down-regulation expression, those genes such as *bmp2*/*bmp4*, *mmp9*, *GABA*, *FGFR2*, *TGF beta receptor 1* and *TNFAIP2* exhibited up-regulation expression.

We also observed that SWCNTs can decrease tyrosine kinase activities, induce down-regulation expression of *mad2*, *cyclin D1*, *cyclin A*, *cyclin E* and up-regulation expression of *TGF beta receptor 1*, *cdc37*, *cdc42* and down-regulation expression of special complexes such as *cyclin–cdk*, *GTP–cdc42* complex.

4. Discussion

Carbon nanotubes, because of their unique properties, own great potential applications on biomedical

Table 2
Differential genes between HEK293 cells with or without CNTs by biochip

GenBank no.	Gene	Ratio	*P* value
L49240_1	*FGFR2*	3.9	1.92E-05
NM_004994_1	*Mmp9*	4.3	4.56E-05
XM_008531_1	*Rabaptin-5*	4.5	1.34E-05
U064137_2	*P57 kip2*	3.4	3. 66E-05
NM_004612_1	*TGFbetaR1*	2.9	5. 65E-06
NM_033312_1	*Cdc14a*	−3.5	3. 63E-05
M22490_1	*Bmp2/bmp4*	3.7	1. 37E-03
XM_007258_1	*TNFAIP2*	2.6	6. 41E-06
NM_000807_1	*GABA*	3. 2	2. 88E-05
NM_007065_1	*Cdc37*	3.3	7.13E-04
X74795_1	*Cdc46*	3.7	1.36E-03
NM_006835_1	*Cyclin I*	3.6	1.19E-05
XM_003628_1	*Cyclin G2*	3.4	3.66E-05
NM_003806_1	*Hrk*	3.3	7.13E-04
BC007199_1	*Cyclin M3*	−3.9	2.60E-04
AF255306_1	*Cdk9*	−2.5	3.48E-04
NM_001260_1	*Cdk8*	−2.7	2.65E-05
XM_057942_1	*Bak1*	4.5	1.34E-05
L10844_1	*Cdc42*	4.1	2.82E-05
NM_052987_1	*Cdk2*	−3.2	3.42E-05
NM_001259_1	*Cdk6*	−2.5	7.89E-07
AF045161_1	*Cyclin t*	3.1	6.03E-03
D25418_1	*Human prostacyclin receptor*	3.6	4.37E-06
AF216962_1	*Cyclin M2*	4.1	4.96E-05
NM_006542_1	*SPHAR*	2.8	1.24E-06
NM_001258_1	*Cdk3*	−3.4	6.03E-03
NM_001760_1	*Cyclin d3*	−4.5	1.34E-05
AF275603_1	*Pnas-107*	3.7	1.37E-03
NM_005190_1	*Cyclin C*	−2.5	5.01E-05
Y13120_1	*Cdk4*	−3.1	4.37E-06
AJ243010_1	*Apoptotic protease activating factor 1*	4.6	2.74E-04
NM_004324_1	*Bax*	3.5	3.66E-05
U46917_1	*BAG1*	2.9	3.65E-05
M13077_1	*PP2A*	3.5	3.63E-05
U12820-1	*P16*	4.4	2.92E-06
NM_002134_1	*Hmox2*	−2.7	1.34E-05
NM_004701_1	*Cyclin b2*	−4.4	1.89E-05
U97680_1	*Cyclin A1*	−3.0	2.92E-06
NM_001237_1	*Cyclin A2*	−3.3	3.67E-05
XM_003492_1	*Cyclin-E binding protein1*	−3.0	2.92E-06
AF060515_1	*Cyclin K*	−3.4	3.67E-05
NM_001758_1	*Cyclin D1*	−3.9	7.93E-07
NM_004702	*Cyclin E2*	−5.2	1.88E-05
AF213046_1	*CDKN3*	−3.3	7.13E-04
NM_004748_1	*Cpr8*	−4.0	2.92E-06
NM_021872	*Cdc25b*	−3.1	9.35E-06
NM_018398_1	*CACNA2D3*	−3.6	7.92E-07
NM_031857_1	*PCDHA9*	−2.5	1.59E-04
S42457_1	*CNGA1*	−4.1	9.45E-03
NM_003331_1	*Tyk2*	−2.9	9.35E-06
XM_041406_1	*Ttk*	−3.5	3.37E-05
U65410	*Mad2*	−3.4	1.89E-05
NM_002227_1	*Jak1*	−4.3	1.32E-05
NM_001789	*Cdc25a*	−3.3	3.67E-05
AB035304_1	*Cadherin-5*	−3.9	7.93E-07
AF266723_1	*Bmx*	−2.8	3.62E-05
NM_001964_1	*Early growth response 1*	−4.6	2.64E-05
X74795_1	*Cdc46*	−4.4	1.89E-05
NM_006191_1	*Pa2g4*	−2.9	2.92E-06
NM-000546	*P53*	−3.1	4.89E-04
Z47993_1	*Fas soluble protein*	−3.3	5.66E-04

Specification: cut off value of ratio associated with differential expressed genes is set as >2.5, + denotes up-regulation expression, − denotes down-regulation expression.

engineering and medical chemistry. For example, carbon nanotubes own catalytic function, which possibly affect cellular metabolism (Cui et al., 2004b). Carbon nanotubes can be filled with target DNA molecules or peptides, which has high potential in delivering target DNA molecules or peptides into special tissue region to treat the diseases (Guo et al., 1998; Gao et al., 2003; Cui et al., 2004a,b). Carbon nanotubes were previously shown to inhibit the growth of rat nerve cells (Mattson et al., 2001) and have cytotoxicity to human keratinocyte cells (Shvedova et al., 2003). Our present study mainly investigates the effect of SWCNTs on human embryo kidney cell line HEK293 and potential biochemistry mechanism with the aiming at exploring biocompatibility and potential therapy value. Our observation shows that SWCNTs can inhibit the proliferation of HEK293 cells, induce cell apoptosis and decrease cellular adhesive ability in a time- and dose-dependent manner. While a high SWCNT concentration of 250 μg/ml induces death of HEK293 cells within 24 h, less than 1 μg/ml SWCNTs in the medium appear to have only slight influence (data not shown here). HEK293 cells are attached by SWCNTs and secrete some 20–30 kd proteins to wrap and aggregate SWCNTs to form nodular structures. These appear to be a series of cell active responses aimed at isolating SWCNTs from the remaining cell mass.

In order to explore the molecular mechanism of effect of SWCNTs on HEK293 cells, we analyzed

changes in cell cycle induced by SWCNTs. The analysis revealed G_1 arrest of HEK293 cells after exposure to 25 μg/ml of SWCNTs in the medium, and this arrest was accompanied by a dramatic decrease in the number of cells in the S phase. We also observed a significant up-regulation expression of p16, which is known to regulate the activity of cyclin-dependent kinases (cdks), the heart of the eukaryotic cell cycle engine (Sherr, 1993; Sherr and Roberts, 1995). In SWCNTs-treated HEK293 cells, accumulated p16 protein may bind to and inhibit the kinase activity of cdk2, cdk4 and cdk6, hence prevent the cells from entering into the S phase and subsequently arrest the cell cycle in the G_1 phase. The SWCNTs-treated HEK293 cells showed marked down-regulation expression of *cdk2*, *cdk4*, *cyclin A*, *cyclin D3* and *cyclin E* genes. Western blot indicated that the expression of cyclin D3 in SWCNTs-treated HEK293 cells decreased gradually as the culture time and dose of SWCNTs increased. It has been documented that cdk4 and cdk6 are activated in association with D-type cyclin in the mid G_1 phase (Agami and Bernards, 2002). Cyclin D, a G_1/S cyclin, promotes S phase by inhibiting Rb. A second G_1/S cyclin, cyclin E, is a principal regulator of the S phase during cell development. Although cyclin E is an inhibitor of Rb, it also has additional Rb/E2F-independent cell-cycle roles (Baldin et al., 1993; Liu and Greene, 2001). Cdk2 is associated with cyclin E in the late G_1 phase and its activity is rate-limiting for progression from the G_1 to the S phase, regulating both the passage from G_1 into S and the S phase progression. Cdk2 down-regulation expression may result in cell arrest in the G_1 phase (Baldin et al., 1993; Tsai et al., 1993). Thus, reduced levels of these G_1-associated cdks and cyclins may also facilitate blockade of the cell cycle in mid G_1 and G_1/S in SWCNTs-treated cells. These results provided solid evidences that SWCNTs suppress the proliferation of HEK293 cells by the p16–cyclin D–Rb pathway.

Our observations show that SWCNTs can induce HEK293 cell apoptosis, which were characterized by morphological changes, chromatin condensation and internucleosomal DNA fragmentation, accompanied by up-regulation expression of apoptosis-associated genes such as *p16*, *bax*, *hrk*, *bak1*, *p57*, *FGFR2*, *TGF beta receptor 1* and *TNFAIP2* genes and down-regulation expression of cell cycle-associated genes such as *cyclin D1*, *cdk2*, *cdk4* and *cdk6* compared to normal HEK293 cells. The family of bcl-2 related proteins regulates susceptibility to apoptosis. Anti-apoptotic members of the bcl-2 family, including bcl-2 and bcl-X_L, which act to prevent or delay cell death, while pro-apoptotic members, including bax and bcl-Xs, which promote apoptosis (Reed, 1997). Our data showed that the expression of bax and bcl-Xs were up-regulated in SWCNTs-treated HEK293 cells. It has been reported that Bax is up-regulated by p53 protein. Therefore we consider that the bcl-2 family is involved in the cell apoptosis induced by SWCNTs.

Our data showed that the adhesion ability of HEK293 cells decreased gradually as the amount of SWCNTs and number of culture days increased. Cell adhesion to a substrate controls the behavior of cells such as cell morphology, migration, growth, apoptosis and differentiation (Hayashi, 1995; Kleinman et al., 1981; Hynes, 1992). Extracellular matrix (ECM) is the substrate for cell adhesion, growth, and differentiation, and it provides mechanical support to tissues. Local disruption of ECM results in selective programmed cell death within adjacent cells (Boudreau et al., 1995). Laminin, FAK, cadherin, fibronectin and collagen IV are important components of ECM. Biochip analyses showed that adhesion-associated genes such as *laminin*, *fibronectin*, *FAK*, *cadherin*, *collagen IV* and *padh9* exhibited down-regulation expression. Western blot analysis showed that the adhesive proteins such as laminin, fibronectin, FAK, cadherin and collagen IV exhibited gradual down-regulation expression as cell culture days increased. Our data also showed that cdc42 and cdc37 exhibited up-regulation expression, which can lead to cytoskeletal reorganization and cell shape alteration. These results showed that SWCNTs can induce adhesion-associated genes and proteins exhibit down-regulation expression, leading to thinning of cell basement membrane and decrease of the adhesion ability, finally resulting in cellular apoptosis or death.

However, we also observed HEK293 cells actively respond to SWCNTs such as secreting proteins to aggregate and wrap SWCNTs. SDS-PAGE analysis confirmed the existence and size (20–30 kd) of these secreted proteins in the medium. Our observation showed that HEK293 cells cultured with SWCNTs began to secrete some small proteins on the 2nd day. The structure, function and secretion mechanism of these small proteins remain to be clarified. We also observed, no matter how uniformly distributed the

SWCNTs were at the initial stage, after the cells with SWCNTs were cultured for several hours, some SWCNTs aggregated together and formed bundles in the medium, while some SWCNTs attached to HEK293 cells and resulted in cell aggregation. These cells attached by SWCNTs gradually appeared apoptosis. The cells far from SWCNTs still grew very well. These observations demonstrate that HEK293 cells can make active responses of self-protection to SWCNTs. The detailed mechanism of active response is under way study.

According to the above-mentioned results, we suggest a possible model of interaction between single-walled carbon nanotubes and HEK293 cells. The SWCNTs attach to the surface of HEK293 cells, providing a stimuli signal to the cells. The signal is transduced inside the cells and the nucleus, leading to down-regulation of adhesion-associated genes and corresponding adhesive proteins, resulting in decrease of cell adhesion and causing cells to detach, float and shrink in size. At the same time, SWCNTs induce up-regulation of apoptosis-associated genes such as *p16*, *Rb*, *p53* and causes HEK293 cells arrest in the G_1 phase, finally resulting in apoptosis. During this period, HEK293 cells make active responses of self-protection to SWCNTs, secrete some small proteins into the medium to wrap SWCNTs into nodular structures, which isolate the cells attached by SWCNTs from the remaining cell mass.

In conclusion, SWCNTs can inhibit the proliferation of HEK293 cells by inducing cell apoptosis and decreasing cellular adhesive ability. In return, HEK293 cells can also mobilize active responses including secretion of small "isolation" proteins to isolate SWCNTs-attached cells from the remaining cell mass. This phenomena has potential application in medical chemistry and disease therapy. The secreted proteins possibly are valuable target molecules. The detailed network pathways responsible for the transduction of these signals are complex and still need further clarification.

Acknowledgment

We thank Dr. Heinz Schwarz of Max Planck Institute for Development Research for help on immunostaining experiments.

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