Aligned Carbon Nanotubes Reduce Hypertrophic Scar via Regulating Cell Behavior

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Supporting Information

ABSTRACT: Hypertrophic scars, characterized by excessive cell proliferation, disordered cell growth, and aberrant deposition of collagens, could cause significant clinical problems. Herein, aligned carbon nanotubes (ACNTs) were synthesized via chemical vapor deposition, and bulk ACNTs were pulled out from the arrays. The capacity of the ACNTs to reduce hypertrophic scar formation was evaluated both in vitro and in vivo. The results demonstrated that the ACNTs suppressed the over-proliferation of fibroblast cells, directed their growth, and inhibited collagen expression in vitro without cell cytotoxicity. Moreover, in vivo evaluation in a rabbit ear model indicated relieved scar hypertrophy after the ACNTs treatment. The gene expression microarray was further used to understand the mechanism, which showed that ACNTs could inhibit the TGFβ pathway to alter the components in the extracellular matrix, cell proliferation, cell cytoskeleton, and cell motility. These findings may provide a potent strategy of using carbon nanotubes in the bioengineering field.

KEYWORDS: carbon nanotubes, alignment, reduce, hypertrophic scar, mechanism

As common complications after surgery, trauma, and burn injuries, hypertrophic scars are intractable challenges for surgeons.1–3 People with excessive scarring usually face physical and psychosocial consequences, for example, pruritus, stiffness, pain, diminished self-esteem, and mental suffering.4,5 Scar formation typically includes inflammatory, proliferative, and remodelling phases.6 During the inflammatory phase, the extrinsic clotting pathway is activated to form a fibrin hemostatic plug, while the inflammatory cells and macrophages enter the injury zone; this phase usually lasts for 3 days. The proliferative phase begins with the formation of granulation tissue, which is composed of macrophages, fibroblasts, and endothelial cells, and usually lasts for 2–3 weeks. Growth factors secreted from macrophages can induce fibroblast cells to proliferate, and these cells can lay down collagens for the growth of endothelial cells. The remodelling phase lasts for 1 year, during which Type-III collagen is degraded and replaced by Type-I collagen.6,7 The vital factors of reducing scar formation include the sufficient inhibition of fibroblast overproliferation, the excessive deposition of collagen, and the promotion of well-structured fibrous tissue formation in the wound site. However, treatments aimed at reducing hypertrophic scar formation, including surgical procedures, dressings, and drugs, are far from satisfactory.8–11 Recently, aligned structure materials have shown great potential in tissue engineering. Aligned collagen hydrogels, collagen fibers, nanotopography scaffolds, and natural–synthetic polyblend nanofibers are reported to direct multiple cell growth and improve nerve regeneration.12–15 Aligned polymers and collagen fibers could affect extracellular matrix texture and promote the quiescent keratocyte phenotype,6 which hold the promise for scar prevention. Fiber aligned poly-l-lactic acid scaffolds have also been proven potent in cardiac regeneration and scar prevention.16 These studies suggest that aligned structure materials with proper fiber diameter and interval are capable of inducing oriented cell growth. However, the synthesis procedures of mentioned materials might be...
relatively complicated and costly, which may hinder their efforts toward mass production. Furthermore, due to their nanoscale function monomers, solid substrate materials are usually required to be attached on to extend their properties to the macroscopic scale, which make it hard for the fabrication of dressings with various shapes.

Due to their characteristic one-dimensional structure and availability for various modifications on the outer surface, carbon nanotubes (CNTs) have been widely investigated for biomedical applications such as drug delivery, tissue engineering, and biosensing. Previously, aligned arrangements of CNTs were found to direct human mesenchymal stem cells, Chinese hamster ovary cells, muscle cells, and neurite growth as well, suggesting the promising ability of aligned carbon nanotubes (ACNTs) to regulate the location and direction of cell growth in tissue engineering scaffolds. However, arrangements of individual CNTs are limited by the inconvenience of fabricating CNTs into a bulk material. Therefore, they are often applied only as a coating or incorporated along with other materials. Recently, ACNT arrays were successfully synthesized into freestanding bulk materials, which can be produced in commercial scale and placed on various kinds of substrate materials. These bulk ACNT arrays with various shapes in energy applications have been well demonstrated. The prospects of such ACNT bulk materials in bioengineering, however, have not been fully explored.

Herein, ACNTs were synthesized in a highly aligned, arranged structure, and their potential for scar prevention was investigated. After being cultured on ACNT films, fibroblast cell proliferation was found to be significantly inhibited without obvious toxicity. Additionally, cells were found to be generally growing in alignment, and filopodia were also observed to be attached to the arrays and stretched in an orientation parallel to the ACNTs. Gene expression microarray assays suggested the ACNTs’ influence on cell behavior, and the extracellular matrix (ECM) mainly suppressed cell proliferation, regulated the cell cytoskeleton and cell motility, and inhibited collagen deposition. In vivo evaluation of the rabbit hypertrophic scar model indicated that ACNTs efficiently reduced hypertrophic scar formation.
**RESULTS AND DISCUSSION**

**Preparation and Characterization of ACNTs.** Primarily, the ACNT films (Figure 2a, b) were dry-drawn from spinnable CNT arrays prepared via chemical vapor deposition (CVD) procedure. The CNTs were multiwalled with diameters of ~8 nm, and the average distance among neighboring CNTs was approximately 300 nm (Figure 2c). The high alignment of the CNTs was verified by small-angle X-ray scattering (Figure 2d).

**ACNTs Suppressed Fibroblasts Growth in Vitro.** The excessive proliferation of fibroblasts is one of the main reasons for the formation of hypertrophic scars. Human dermal fibroblasts (HDF) and mouse embryonic fibroblasts (NIH3T3) are commonly used cells for in vitro scar formation investigations. In the attempt to investigate the effect of ACNTs on cell behavior, fibroblasts (HDF, NIH3T3) and other cell types such as human umbilical vein endothelial cells (HUVECs), human smooth muscle cells (HSMCs), and preosteoblastic MC3T3 cells were seeded on the ACNT films for 48 h, and the cell number was determined by observation and counting under a microscope (IX81, Olympus, Tokyo, Japan) with 200X magnification. These other three types of cells were selected because they are involved in the healing process in different tissues. The cell proliferation of the fibroblasts and the other three major types of cells was inhibited by 11.3% and 11.5%, respectively (Figure 3). These data suggested that ACNTs could suppress multiple cells growth in vitro without toxicity.

**ACNTs Induced Oriented Movement of Fibroblasts.** It is worth noting that the ideal tissue reconstruction of a wound requires the oriented growth of cells in dermis tissue, while the irregular distribution and stretching of fibroblast cells will contribute to hypertrophic scar formation. As a result, regulating the cell location, direction, and migration in an ordered state is crucial to preventing chaotic cell growth and further reducing hypertrophic scar formation. Here, the growth and stretch orientation of various cells cultured on the ACNT films was traced via immunofluorescence (phalloidin, α-tubulin, and 4′,6-diamidino-2-phenylindole). Both HDF and NIH3T3 cells were observed growing along the alignment direction of the ACNT films (Figure 4a, b), and a similar phenomenon was also observed with the other three types of cells (Figure S5). Meanwhile, time-lapse imaging was applied to monitor the movement of fibroblasts on ACNTs and found that the cells migrated alongside the nanotubes (Figure 4c, d, Videos S1–S4). Quantitative measurements further confirmed the oriented cell growth on ACNTs (Figure 4e). These data suggested that the ACNTs could induce oriented movement of multiple cells efficiently.

**ACNTs Change Fibroblasts Cytoskeleton Assembly.** Furthermore, the cytoskeleton contributes to cell structure, division, motility, intracellular transport, and interaction with the ECM. The interaction between the cell and ECM may trigger numerous responses that are essential for the cellular behavior and fate, and two actin-based structures, lamellipodia and filopodia, play key roles in this interaction. Specifically, lamellipodia are sheet-like protrusions consisting of short, lamellipodia, play key roles in this interaction. Specifically, lamellipodia are sheet-like protrusions consisting of short, thin filaments (Figure S3). Cell dead/live staining showed that ACNTs did not induce obvious cell death.
New Zealand rabbits were divided randomly into ACNTs and control groups \((n = 15)\). After establishing the hypertrophic scar model according to a previous report,\(^{5,50}\) the rabbits of the ACNTs group were covered with ACNTs films over the wound and followed by a sterilized bandage, while the control group was covered only with bandages. Afterward, the wound healing was monitored and evaluated via gross observation and histological examinations. In particular, the gross appearances of the wounds were observed, and digital photographs were acquired at different time points. At day 35, stiff and visibly elevated scars were formed in both the control and ACNTs groups. However, the color of the scars formed in ACNTs group was relatively pale, and the appearance was platter, while the scars of the control group were dark red, and a significantly elevated tubercular scar was observed (Figure 6a). Interestingly, we also found a disparity between the groups in wound healing time, wherein the healing time of the ACNTs group was recorded to be 19.20 ± 2.62 days \textit{versus} 17.73 ± 3.53 days in the control group, but no statistical significance was detected \((p > 0.05, \text{Figure } 6b)\). These results might correspond with the lower \textit{in vitro} proliferation of cells cultured on the ACNTs but without apoptosis induction (Figure S2).

Histological evaluation was further performed for details. The hematoxylin–eosin (H&E) stained sections indicated that the dermal layers of the scars were relatively flatter in the ACNTs group compared with the control group (Figure 6c-1, c-2). Modified Masson’s trichrome was applied to specifically identify the collagen fibers, which can be stained dark blue.\(^{51}\) Significantly, the coarse collagen fibers of the control group were irregularly arranged and densely distributed (Figure 6c-3). The magnified images further revealed the obviously tangled and disordered arrangement of the collagen fibers in the control group (Figure 6c-3, inset). In sharp contrast, the ACNTs group did not exhibit a significantly thickened dermal layer, and the collagen fibers were arranged in a relatively regular manner (Figure 6c-4).

For a detailed analysis, the scar elevation index (SEI) was further applied in an attempt to quantify the H&E evaluation, as previously reported (Figure 6d).\(^{52,53}\) The SEI of the ACNTs group was 1.694 ± 0.182 \textit{versus} 2.046 ± 0.281 in the control group, which was significantly reduced \((p < 0.05)\) (Figure 6d). Normal dermis sections were set as references (Figure S7).

Additionally, Sirius red staining was used to discriminate between different types of collagen (Figure 6e-1). \textit{Specifically}, there was a dense distributed area of the Type-I collagen stained red-orange in the control group (Figure 6e, h). In contrast, images of the ACNTs group showed considerable amounts of Type-III collagen fibers stained fluorescent green, and orange-stained Type-I collagen was observed to be less densely distributed in the vicinity (Figure 6f, i). Normal dermal tissue was set as the reference, in which most of the fibers were Type-III collagen stained with yellow and green (Figure 6g, j).

As Type-I collagen is stronger than the other types and usually the most abundant in hypertrophy scar tissue, the inhibition of Type-I collagen will definitely contribute to its remission.\(^{6,7,54}\) Overall, in the two groups stained with H&E, Masson’s, and Sirius red indicated that the ACNT-treated scars resulted in thinner and more organized layers of fibrous tissue and lower Type-I collagen deposition compared with the normal group.

Silicone gel sheet (SGS) has been approved by U.S. Food and Drug Administration (FDA) for scar treatment. The gel sheet is usually applied when the wound is completely healed, and it functions mainly through covering the scars and producing a

dendritically branched actin filaments. Filopodia are finger-like membrane protrusions consisting of long, unbranched filaments extending out from the cell edge.\(^ {42}\) They are both involved in substrate tethering and environment sensing.\(^ {43,44}\) Here, for observation under the confocal microscope, cells were seeded on ACNTs films and cultured for 3 days. F-actin and α-tubulin, two major components of the cytoskeleton, were stained by phalloidin and α-tubulin, respectively.

After co-culture, cells on ACNTs were observed elongated into spindle shapes, extending less randomly stretched filopodia that assembled into a linear structure (Figure 5a, c). F-actin and α-tubulin were also observed rearranged in parallel to the alignment of the CNT arrays (Figure 5a, c). The role of filopodia in the rearrangement of the cytoskeleton was further studied by scanning electron microscopy (SEM) (Figure 5b, d). The cells cultured on glass extended abundant filopodia, which spread randomly in the vicinity. In sharp contrast, cells cultured on the ACNTs extended fewer filopodia, which attached to the ACNTs and spread mostly along the radial direction thereof. Many investigations have proven that the filopodia directly affect the cell cytoskeleton and migration.\(^ {45,46}\) Bundles of long and parallel filopodia are considered to induct directional migration.\(^ {47}\) Therefore, the extended filopodia inducted by ACNTs might account for the directional growth of multiple cells.

**ACNTs Reduce Scar Formation \textit{in vivo}.** The feasibility of the ACNTs in reducing the formation of hypertrophic scar was further assessed in a rabbit ear model.\(^ {48,49}\) The bulk ACNTs fabricated into freestanding films facilitated the ease of application as a wound dressing material. Briefly, 30 adult

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Figure 3. ACNTs inhibited the proliferation of fibroblast cells. Fluorescent images of EdU-labeled proliferating HDF cells and NIH3T3 cells cultured on ACNTs for 3 days indicated relatively more sporadic distribution than on the control. Note that proliferating cells are sensitive to EdU labeling. Red, proliferating cells; Blue, total cells (a, c). Quantitative measurement of the EdU-proliferating cells are sensitive to EdU labeling. Red, proliferating

Figure 4. ACNTs induced the alignment of the CNT arrays (Figure 5a, c). The role of ACNTs extended fewer filopodia, which attached to the ACNTs and spread mostly along the radial direction thereof. Many investigations have proven that the filopodia directly affect the cell cytoskeleton and migration.\(^ {45,46}\) Bundles of long and parallel filopodia are considered to induct directional migration.\(^ {47}\) Therefore, the extended filopodia inducted by ACNTs might account for the directional growth of multiple cells.

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moisturizing effect. Specifically, the evaporation of water from the skin was decreased, while hydration of the stratum corneum was increased after application of SGS on the scar, which led to relieved symptoms. Besides, studies concluded that the primary mechanism of SGS products in hypertrophic scar treatment is also partly attributed to the occlusion effect.

Either hydration or occlusion is not a direct contact or interaction with fibroblasts and ECM, so it usually takes a long course to reduce the scar hypertrophy.

On the other hand, the mechanism of ACNTs prevented scar formation is largely different from that of a silicone gel sheet. ACNTs were used during the scar formation process and could inhibit fibroblasts proliferation, direct cell growth, and alter extracellular texture composition. Here, we used a rabbit ear model to compare the therapeutic efficiency of ACNTs and SGS. The data showed that at day 35, more ideal results were observed in the ACNTs group including the color and the thickness of scar compared with those of the SGS group (Figure S8). The SEI of the ACNTs group was decreased compared with the SGS group. Meanwhile, there is no apparent difference between the control group and SGS group in the color, thickness, and SEI of scar (Figure S8). The reason might be that SGS did not exert function during a short course of 10 days’ treatment. Despite the different function mechanism and action phase of ACNTs compared with SGS, this result can be prudently interpreted that ACNTs may be a potential option for scar hypertrophy treatment in addition to SGS.

**Mechanism Investigation.** Scar formation is a complex process based on a phasic and well-structured wound healing process that involves various cells, mediators, cytokines, and matrix molecules. The pivotal method to regulate scar formation is to balance the proliferative and remodelling processes. Additionally, suppressed collagen deposition is necessary to relieve pathological scarring. Here, a gene expression microarray was applied to further understand the mechanism of ACNTs in inhibiting excessive cell proliferation and guiding cell growth. More importantly, the role of ACNTs in suppressing the deposition of collagen was investigated.

For the following experiments, HDF cells were applied as a dominant cell type in dermal tissue. Briefly, after being cultured on ACNTs for 3 days, total RNA (approximately 40 μg) from HDF cells cultured on glass and ACNTs was extracted using Trizol reagent. Then, a microarray expression analysis was performed and analyzed according to gene ontology (GO) enrichment. Specifically, the fold-change analysis showed that...
The directed cytoskeleton stained with phalloidin; green, cytoskeleton stained over a larger cell area and exhibited more the ACNTs (Figure 7a). GO enrichment analysis was conducted elevated and 173 decreased in HDF cells after being cultured on matrix components, cell Notably, the GO categories for collagen binding, extracellular randomly stretched cultured on the ACNTs showed spindle shapes and apparently less cytoskeleton. The cytoskeleton of HDF cells and NIH3T3 cells more than 250 genes were di
...belong to the "negative regulation of cell motility" and "cytoskeleton organization" categories. These genes were all found to be down-regulated after the cells were cultured on the ACNTs. They are vital for cell migration and cytoskeleton organization, which may explain the oriented growth and stretch of cells along the ACNT arrays. Additionally, their expression was examined using real-time PCR (RT-PCR) (Figure 7c, d) and Western blot (Figure 7e). These six genes were significantly down-regulated in both HDF and NIH3T3 cells. Together with the finding that the filopodia sensed and spread along the ACNTs, it was reasonable to deduce that the filopodia caused the expression change of cellular genes, which finally led to directed cell growth. Scar formation also largely results from the progressive and uncontrolled deposition of ECM, especially collagens. The gene expression profiles indicated that COL1A1, COL3A1, COL5A1, and COL15A1 were down-regulated in HDF cells cultured on the ACNTs. We then investigated their expressions in both HDF and NIH3T3 cells and found that the mRNA and protein expressions of these genes were apparently inhibited, which further strengthened the inhibitory effects of the ACNTs on hypertrophic scar formation (Figure 7c–e).

Furthermore, we investigated the possible molecular mechanism. The result of KEGG pathway showed that focal adhesion pathway was one of the significantly altered pathways (Figure S10). Focal adhesions are sites where proteoglycan and integrin mediated adhesion links to the actin cytoskeleton. They are large macromolecular assemblies through which regulatory signals and mechanical force are transmitted between the interacting cells and ECM. Our data showed that ACNTs could suppress the expression of Myosin light chain 2 (MLC2), myosin light chain kinase (MLCK), Rho-associated coiled-coil containing protein kinase 2 (ROCK2), cell division cycle 42 (CDC42), and diaphanos-related formin-1 (mDia1) (Figure 7f), which was consistent with the result of gene microarray. The inhibited expression of these proteins could partially account for the change of the morphology and the oriented movement of cells. TGFβ pathway is known to be the dominant pathway that regulates scar formation. Therefore, we further investigated the effect of ACNTs on TGFβ pathway. The data also showed that ACNTs could suppress the expression of TGFβ1, TGFβ2, and SMAD4 and could inactivate the phosphorylation of SMAD2 and SMAD3 (Figure 7f). TGFβ signaling is mediated through SMAD and non-SMAD pathway, including RhoA, ROCK, Ras to regulate cell cytoskeleton, cell cycle, cell proliferation, and motility. These results indicated that ACNTs could interfere with the TGFβ pathway to affect the cell behavior.

**CONCLUSION**

In summary, the ACNTs were discovered to be capable of affecting cell behavior and ECM components via suppressing cell proliferation, guiding their growth direction, and inhibiting collagen deposition. The underlying mechanism was revealed to be related to the regulation of TGFβ pathway and specific genes. Moreover, as a feasible bulk material, such freestanding ACNTs exhibited an ideal inhibitory effect on hypertrophic scar formation in vivo. These findings may provide an alternative strategy of applying ACNTs in the bioengineering field.
MATERIALS AND METHODS

Preparation of ACNT. A catalyst with a layered structure was prepared using iron as a catalyst and aluminum oxide as a buffer layer. The substrates were Si (110) wafer with a 300−1000 nm-thick silica surface. First, the substrates went through a polished treatment, and aluminum oxide (thickness of 3 nm) and iron (thickness of 1.1−1.2 nm) were subsequently deposited on the substrates by electron beam evaporation at a vacuum pressure of $5 \times 10^{-4}$ Pa. Specifically, the substrate was first put over the target material with the polished surface downward, and the shutter was closed before deposition. When the vacuum reached $5 \times 10^{-4}$ Pa, the high-energy electron beams bombarded the target material to evaporate it. When the deposition rate stabilized at the target value, the shutter was opened to deposit the aluminum oxide vapor on the substrate. The iron was deposited on the surface.

Figure 6. ACNTs inhibited the scar formation in vivo. (a) Photographs of the wound during the healing procedure. (b) Histological appearance of scars harvested on day 35 for ACNTs and control groups stained with H&E (b-1, b-3) and Masson’s (b-2, b-4), respectively (insets, magnified photographs). (c) Comparison of the wound-healing time indicated no significant difference ($n=15$, $p>0.05$). (d) Comparison on SEI. Dermal hypertrophy was measured by SEI on day 35 according to the H&E stained sections ($n=15$, **$p<0.01$). (e−j) Histological sections stained with Sirius red. (e−g) Photographs observed under polarization microscope for the control, ACNTs, and normal groups, respectively. (h−j) Magnified images of the control, ACNTs, and normal group, respectively. The arrows indicate Type-I collagen stained with red or orange, and the asterisks indicate Type-III collagen stained with yellow or green.
substrate in the same way. Afterward, the ACNT arrays were synthesized by CVD in a tube furnace with argon and hydrogen gases as the carrier gases and ethylene as the carbon source. First, oxygen and water were purged from the tube by flowing argon gas, followed by flowing argon/hydrogen gas while the temperature ramped up from room temperature to 740 °C. After the temperature reached 740 °C, catalyst iron particles were formed, and the carbon precursor gas flowed into the tube. With further heating, the carbon precursor decomposed, dissolved into the catalyst particles, and separated out after saturation and nucleation. After growth at 740 °C for 10 min and cooling down to room temperature, the aligned CNT array was obtained. ACNT films can be directly pulled out from the array due to the strong interactions between CNTs.

**Characterization.** For SEM observation, the ACNTs film was directly put on carbon tape and observed under a field emission scanning electron microscope (Zeiss Ultra S5, Germany) operated at 3.0 kV (Ultra S5, Carl Zeiss, Germany). To prepare transmission electron microscope samples, we directly pulled the CNT films out from the array, spread them on the copper mesh, and then dropped dimethyl sulfoxide for 4 h at room temperature. Then, the cells were vacuum freeze-dried after washing twice with water and then deposited with platinum using a sputter coater. They were then observed under a field emission scanning electron microscope (FE-SEM) operated at 3.0 kV (Ultra S5, Carl Zeiss, Germany).

**Cell Lines Culturing and EdU Proliferation Assay.** Human dermal fibroblast cells were cultured in a fibroblast medium supplemented with 2% FBS and 1% each of l-glutamine, penicillin, and streptomycin. Passages 3–8 were used in all experiments. NIH-3T3 fibroblast cells were cultured in Dulbecco's modified Eagle media (DMEM) supplied with 10% fetal bovine serum and 1% penicillin and streptomycin. They were incubated at 37 °C in a humidified environment supplied with 5% CO₂ and the culture medium was changed every day. Cell proliferation was determined by EdU after a 3 day culture. In brief, after incubating on the ACNTs for 3 days, the cells were stained as previously reported in the protocol and visualized with a digital microscope system (IX81, Olympus, Tokyo, Japan). 76

**Fluorescence Staining.** α-Tubulin and F-actin were stained with α-tubulin and phalloidin, respectively. Specifically, the cells were seeded on cover glasses with or without the ACNTs, cultured for 3 days, washed with PBS, and followed by fixation with 4% paraformaldehyde for 20 min. The cells were then permeabilized with 0.2% Triton X-100 for 15 min and blocked with 5% bovine serum albumin (BSA) for 60 min at room temperature. After washing twice with PBS, they were co-incubated with phalloidin, α-tubulin, and 4′,6-diamidino-2-phenylindole (DAPI). Analysis was performed under a Zeiss LSM-710 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

**Time-Lapse Imaging.** ACNTs and glasses were placed in 24-well plate and sterilized under UV light for 30 min. Then, about 10⁵ cells were seeded on plates, and the plate was moved into a microincubation chamber on an Axiovert 200 imaging system (Zeiss), which was equipped with a 5% CO₂ supply and maintained at 37 °C. Time-lapse observation was then conducted automatically at multiple locations on the plate every 160 s. The series of photographs were then converted into videos using Adobe Premiere Pro CC software (Adobe Systems Inco., CA, USA).

**Scanning Electron Microscope Observation.** After being cultured on the ACNTs for 3 days, both HDF and NIH3T3 cells were fixed using 2.5% glutaraldehyde at the temperature of 4 °C overnight. Washed twice with water, the cells were treated with 20% dimethyl sulfoxide for 4 h at room temperature. Then, the cells were vacuum freeze-dried after washing twice with water and then deposited with platinum using a sputter coater. They were then observed under a field emission scanning electron microscope (FE-SEM) operated at 3.0 kV (Ultra S5, Carl Zeiss, Germany).

**Microarray Analysis.** Total RNA (approximately 40 μg) from HDF cells cultured on glass and ACNTs for 3 days was extracted using Trizol reagent. Then, the samples were sent to Shanghai Biotechnology Corporation for microarray expression analysis using an Agilent 8×60K human microarray. The results were analyzed using GO enrichment analysis. Of all the changed genes, we chose genes that were related to scar formation, such as genes related to the ECM (COL1A1, COL3A1, COL5A1 and COL15A1), cell proliferation (NFIB, CYP7B1 and NOG), cell cytosome, and cell motility (TNS4, KCNAB2, ARHGAP26, MYOZ2, PDPN, and SPAG16) to further reveal the possible scar formation mechanisms.

**Real-Time Quantitative PCR.** Total RNA from HDF and NIH3T3 cells cultured on glass and ACNTs was extracted with Trizol reagent. A PrimeScript RT reagents Kit was applied for total RNA reverse transcribing. Identical quantities of cDNA were then subjected to real-time quantitative PCR (qPCR) using SYBR green as the fluorescence dye. Each reaction was conducted in triplicate. 

![Figure 7. Mechanism of ACNTs suppressed hypertrophic scar formation. (a) Differently expressed genes presented in a volcano plot. The red and blue dots indicate up-regulated and down-regulated genes, respectively. Accordingly, the threshold fold changes were 2 and 0.5. (b) Microarray data of the cells cultured on ACNTs presented in heatmap. (c, d) Screened gene expressions of HDF and NIH3T3 cells cultured on ACNTs confirmed via RT-PCR, respectively. (e) Screened gene expressions of HDF and NIH3T3 cells verified by Western blot. (f) ACNTs inhibited TGFβ pathway.](image-url)
membranes were then co-incubated with the indicated primary antibodies at different dilutions at 4 °C overnight according to the manufacturer’s instructions. The membranes were then incubated with horseradish-peroxidase-conjugated antibodies for 2 h at room temperature after washing three times with tris-buffer solution-Tween (TBS-T). Washed three times in TBS-T, the membranes were incubated with secondary Bio-Rad ECL Western blotting solution-Tween (TBS-T) for 1 h and imaged on a FluorChem 8050 imaging system (ProteinSimple, Santa Clara, CA, USA). The density of the bands was quantified by ImageJ software.

Hypertrophic Scar Model Establishment. All animal studies were performed adhering to the NIH guidelines for care and use of laboratory animals and with the approval of the Animal Experiment Centre of the Second Military Medical University. Adult New Zealand rabbits, male, aged 3 months, were obtained from Slac Laboratory Animal Corporation. The 30 rabbits were randomly divided into two animal groups: ACNTs and control groups (n = 15). After anaesthetization with 1% (10 mg/mL) pentobarbital sodium (1 mg/kg), the hypertrophic scar model was established according to the previous method. Specifically, surgical procedures were conducted under sterile conditions; an epidermal wound on the ventral side of the ear with a diameter of 1 cm was established down to the cartilage with a punch biopsy. To ensure the removal of epidermis, dermis, and perichondrium, an operating loupe was applied during the procedure.

Animal Groups and Administration. The ACNTs films were dry-drawn from a CNT array. Two-layered ACNTs were then co-incubated with the indicated primary antibodies at different dilutions at 4 °C overnight according to the manufacturer’s instructions. The membranes were then incubated with horseradish-peroxidase-conjugated antibodies for 2 h at room temperature after washing three times with tris-buffer solution-Tween (TBS-T). Washed three times in TBS-T, the membranes were incubated with secondary Bio-Rad ECL Western blotting solution-Tween (TBS-T) for 1 h and imaged on a FluorChem 8050 imaging system (ProteinSimple, Santa Clara, CA, USA). The density of the bands was quantified by ImageJ software.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b07439.

Cell growth curve of multiple cells; cell apoptosis assay determined by flow cytometry; the activation of caspase3 and caspase 8 using Western blot; the oriented growth of HUVEC, HSMC, and MC3T3 cells on ACNTs; schematic diagram of the SEI routine; histological evaluation of the normal group; top 30 categories of GO enrichment analysis; focal adhesion pathway changes in ACNTs group; primer sequences used for RT-PCR; detailed materials and methods (PDF)

Video S1: Fibroblast cells movement (AVI)

Video S2: Fibroblast cells movement (AVI)

Video S3: Fibroblast cells movement (AVI)

Video S4: Fibroblast cells movement (AVI)

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**Author Contributions**

*W.W., S.H., and H.S. contributed equally to this work. J.S., W.W., and H.P. conceived and designed the research. S.H., W.W., H.S., X.L., Y.H., J.C., Q.Z., and L.C. performed the experiments. W.W., H.S., S.H., and J.S. analyzed the data and prepared the figures. W.W., H.S., and H.P. wrote and revised the manuscript.*

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was funded by Foundation of Ministry of Science and Technology of the People’s Republic of China (2016YFA0203302, 2011CB932503), National Natural Science Foundation of China (8141101156, 21225417, 31271031, and 31211282), Natural Science Foundation of Shanghai (15ZR1412500), Science and Technology Commission of Shanghai Municipality (12 nm0503200, 15XD1500400), Shanghai Municipal Science and Technology Commission Key Program (154119S0600), the Fok Ying Tong Education Foundation, and Municipal Human Resources Development Program for Outstanding Leaders in Medical Disciplines in Shanghai (2017BR011).

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DOI: 10.1021/acsnano.7b07439
ACS Nano 2018, 12, 7601–7612

7611
