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Author manuscript

*Nano Lett.* Author manuscript; available in PMC 2019 September 04.

Published in final edited form as:

*Nano Lett.* 2019 August 14; 19(8): 5443–5451. doi:10.1021/acs.nanolett.9b01943.

## Substrate Stiffness-Dependent Carbon Nanotube-Induced Lung Fibrogenesis

Kai Wang<sup>†</sup>, Lin Shi<sup>†</sup>, Will Linthicum<sup>‡</sup>, Kun Man<sup>†</sup>, Xiaoqing He<sup>§</sup>, Qi Wen<sup>‡,||</sup>, Liying Wang Rojanasakul<sup>⊥</sup>, Yon Rojanasakul<sup>§</sup>, Yong Yang<sup>\*,†</sup>

<sup>†</sup>Department of Biomedical Engineering, University of North Texas, Denton, Texas 76207, United States

<sup>‡</sup>Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, Massachusetts 01609, United States

<sup>§</sup>Department of Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia 26506, United States

<sup>||</sup>Department of Physics, Worcester Polytechnic Institute, Worcester, Massachusetts 01609, United States

<sup>⊥</sup>Allergy and Clinical Immunology Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505, United States

### Abstract

Most living tissues exhibit the specific stiffness, which has been known to have profound influence on cell behaviors, yet how the stiffness affects cellular responses to engineered nanomaterials has not been elucidated. Particularly, discrepancies exist between *in vitro* and *in vivo* nanotoxicological studies. Here, we investigated the effects of substrate stiffness on the fibrogenic responses of normal human lung fibroblasts (NHLFs) to multiwalled carbon nanotubes (MWCNTs). NHLFs were grown on polyacrylamide (PAAm) hydrogels with the stiffness comparable to that of human normal and fibrotic lung tissues, and treated with MWCNTs for various time. The fibrogenic responses, including cell proliferation, reactive oxygen species production, and collagen I expression, of NHLFs to MWCNTs were observed to be regulated by substrate stiffness in a time-dependent manner. NHLFs generally were rounded on soft hydrogels

\*Corresponding Author: yong.yang@unt.edu. Tel: +1-940-565-2982. Fax: +1-940-369-8570.

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

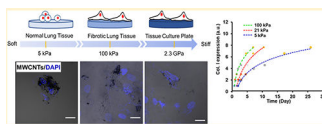
The authors declare no competing financial interest.

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.9b01943. Materials and methods. Table S1: Preparation and characterization of PAAm hydrogels. Table S2: Information and dilution of antibodies. Figure S1: Influence of substrate stiffness on the morphology of NHLFs with or without MWCNTs treatment. Figure S2: Influence of MWCNTs dosage on the proliferation of NHLFs. Figure S3: Standard curve of O.D. (640 nm) value of MWCNTs at different concentrations. Figure S4: Fluorescent images of NHLFs on different substrates with the staining of DCFDA to detect the generated ROS inside the cells with or without MWCNTs treatment. Figure S5: Western blot analysis of the F-actin expression in NHLFs on different substrates with or without MWCNTs treatment. Figure S6: YAP intracellular localization in NHLFs on different substrates. Detailed explanation of the time-stiffness superposition principle of MWCNT-induced fibrogenic responses of NHLFs (Tables S3, S4 and Figures S7-S10) (PDF)

and required a long treatment time to exhibit fibrogenic responses, while on stiff hydrogels the cells were well-spread with defined stress fibers and short-time MWCNTs treatment sufficiently induced the fibrogenic responses. Mechanistic studies showed that MWCNTs induced fibrogenesis of NHLFs through promoting expression and phosphorylation of focal adhesion kinase (FAK), while attenuating intracellular tension in the cells on stiff gels could increase MWCNTs uptake and thus elevate the induced fibrogenic responses. Moreover, we proposed a time-stiffness superposition principle to describe the equivalent effects of treatment time and substrate stiffness on nanomaterials-induced fibrogenesis, which suggested that increasing substrate stiffness expedited fibrogenesis and shed light on the rational design of *in vitro* models for nanotoxicological study.

## Graphical Abstract



## Keywords

nanotoxicity; stiffness; carbon nanotubes; lung fibrogenesis; focal adhesion kinase; intracellular tension

Nanometer-sized materials have been widely applied in electronics, energy, life science, and many other fields, yet the toxicity assessment of the engineered nanomaterials is still challenging. For example, carbon nanotubes (CNTs), a major class of engineered nanomaterials, have been used to develop various types of sensors,<sup>1,2</sup> drug delivery carriers,<sup>3,4</sup> and tissue engineering scaffolds.<sup>5</sup> The occupational and consumer exposure to CNTs can produce diverse biological and toxicological effects and thus result in public health problems.<sup>6–8</sup> *In vivo* studies have shown that CNTs can enter the respiratory tract by inhalation and cause potential damage to lung tissue.<sup>9,10</sup> The inhaled CNTs rapidly penetrate the lung lining of mice and induce lung fibrogenesis by stimulating the overproduction of collagen.<sup>11</sup> *In vitro* studies using tissue culture plastic substrates have demonstrated that CNTs can promote the differentiation of fibroblasts to myofibroblasts and induce the fibrogenic responses of fibroblasts.<sup>12–14</sup> Although both *in vivo* and *in vitro* studies have shown that CNTs can induce lung fibrogenesis, discrepancies exist in the assessment of CNTs toxicity between *in vivo* and *in vitro* studies.<sup>15</sup> For example, the fibrogenic responses of lung fibroblasts to multiwalled CNTs (MWCNTs) are observed 2 days *in vitro*,<sup>16,17</sup> but it takes weeks or months to develop fibrosis *in vivo*<sup>18</sup> after the MWCNTs exposure. Predictive *in vitro* models are thus necessary to assess the toxicity of nanomaterials *in vivo*.<sup>15</sup>

The significant deviation of the *in vitro* cellular responses to nanomaterials from the *in vivo* events is attributed to the failure to recapitulate the *in vivo* microenvironmental characteristics in current *in vitro* models. Among the microenvironmental characteristics, matrix stiffness plays a critical role in the development and maintenance of the normal function of tissues and organs,<sup>19,20</sup> the alteration in tissue stiffness could be associated with certain types of diseases.<sup>21,22</sup> For instance, the Young's modulus of the normal lung tissues

is 1–5 kPa, which is elevated to 20–100 kPa in the fibrotic lung tissues.<sup>23–25</sup> The substrate stiffness has also been shown to be a potent regulator of cell behaviors *in vitro*. For example, NIH 3T3 fibroblasts are well-spread on stiff (>180 kPa) hydrogels with mature stress fibers, whereas they exhibit a rounded shape with less prominent actin bundles on soft (~5 kPa) gels.<sup>26,27</sup> On the stiff (100 kPa) gels, the fibroblasts also exhibit higher contractility, proliferation rate, and myofibroblast differentiation efficiency than those on the soft (5 kPa) gels.<sup>28</sup> Although previous studies have suggested that the CNTs could induce substantial lung fibrogenesis,<sup>12,16,29–31</sup> less effort has been made to explore the stiffness effects on CNTs-induced lung fibrogenesis. It is worth mentioning that current *in vitro* models are mainly based on supraphysiologically stiff tissue culture polystyrene (TCPS) of 2.3 GPa in stiffness, which is several orders of magnitude higher than the stiffness of most human tissues.<sup>32</sup> Therefore, it is highly desirable to assess the toxicity of nanomaterials using the substrate of physiologically relevant stiffness.

In this study, we investigated the effects of substrate stiffness on the fibrogenic responses of normal human lung fibroblasts (NHLFs) to MWCNTs. Polyacrylamide (PAAm) gels with the stiffness comparable to the normal and fibrotic lung tissues as well as TCPS as a control were used as cell culture substrates. NHLFs are associated with collagen production during lung fibrogenesis,<sup>12,33</sup> and hence, their fibrogenic responses to MWCNTs on these substrates were assessed. Moreover, we explored the underlying mechanism of how the substrate stiffness affected cellular responses to MWCNTs. We further proposed a time-stiffness superposition principle to describe the equivalent effects of substrate stiffness and treatment time on the fibrogenic responses of the fibroblasts to MWCNTs.

## Results and Discussion.

### Substrate Stiffness Altered Cell Spreading and MWCNTs Uptake of NHLFs.

The stiffness of PAAm gels was controlled by the ratio of acrylamide to bis-acrylamide, and measured by using the atomic force microscopy (AFM) (Table S1). The soft ( $4.9 \pm 0.5$  kPa, labeled as 5 kPa) and stiff ( $100.8 \pm 2.1$  kPa, labeled as 100 kPa) gels were used to mimic the normal (1–5 kPa) and fibrotic (20–100 kPa) lung tissues, respectively. NHLFs displayed a round shape on 5 kPa gels with the significantly smaller size and lower aspect ratio than the cells on 100 kPa gels and the TCPS, where the cells were spread out with distinct stress fibers (Figures 1a-d and S1). The cell morphologies were similar to what were observed in the fibroblasts grown on the polydimethylsiloxane (PDMS) and PAAm substrates with the stiffness closed to 5 and 100 kPa.<sup>26,27</sup>

The cells were treated with MWCNTs at the concentration of 1  $\mu\text{g}/\text{mL}$  for 3 days. The MWCNTs at this dosage induced collagen I overexpression of NHLFs without causing cell death, as shown in Figure S2 and our previous work.<sup>17</sup> The MWCNTs used in this study were characterized and reported previously.<sup>17,31</sup> The MWCNTs were around 12 nm in diameter and several micrometers in length with less than 1% of contaminants. The major type of metal residues was iron (Fe), which was about 0.32% (w/w). MWCNTs were dispersed in phosphate buffered saline containing bovine serum albumin at the concentration of 1 mg/mL and further diluted in the culture medium to obtain the desired concentrations. The MWCNTs treatment did not result in significant change in the cell morphology,

compared with the control–no MWCNTs treatment (Figures 1b-d and S1). Interestingly, the overlay images in the MWCNTs groups exhibited a higher MWCNTs uptake in the cells on the soft gels than those on the stiff gels and the TCPS. The cellular uptake of MWCNTs was further quantified by measuring the turbidity of the cell lysate with referring to the standard curve (Figure S3).<sup>34</sup> The results verified that the MWCNTs uptake in the cells on 5 kPa gels was significantly higher than those of the cells grown on 100 kPa gels and TCPS (Figure 1e).

Moreover, the AFM measurement of the stiffness of NHLFs on these gels revealed that the cells on 100 kPa gels were significantly stiffer than the cells on 5 kPa gels (Figure 1f). We postulated that the softer cells had higher MWCNTs accessibility than stiffer cells. Our postulation was supported by previous studies,<sup>35,36</sup> which suggested that the higher membrane tension of the cells on stiff substrates resisted nanoparticles from entering the cells compared to the cells on soft substrates, and the soft cytoskeleton facilitated nanoparticle uptake.

### **Substrate Stiffness Modulated MWCNTs-Induced Fibro-genesis of NHLFs.**

Increased cell proliferation, reactive oxygen species (ROS) generation, and collagen I expression are the hallmarks of lung fibrosis, we herein examined these events to evaluate the effects of substrate stiffness on MWCNTs-induced fibrogenesis of NHLFs. In the absence of MWCNTs (i.e., the control groups), the cell proliferation, ROS generation, and collagen I expression of NHLFs increased with the increase in the substrate stiffness (Figure 2). Evidently, the behaviors of the fibroblasts grown on TCPS dramatically deviated from those of the cells on the gels of pathophysiologically relevant stiffness.

Treated with MWCNTs, the cells also exhibited the stiffness-dependent fibrogenic responses to MWCNTs. Strikingly, the cells grown on 5 kPa gels did not show a significant increase in the proliferation, ROS generation, or collagen I expression with MWCNTs exposure. Yet, on 100 kPa gels and TCPS, these fibrogenic responses of NHLFs significantly increased with the MWCNTs treatment. Notably, the MWCNTs-induced fibrogenic responses on the 100 kPa gels were more profound than the TCPS controls. The MWCNTs treatment increased cell proliferation and collagen expression on 100 kPa gels by 43.4% and 51.5%, respectively, higher than those on TCPS (10.9% and 29.6%, respectively; Figure 2a,c). The MWCNTs-induced increases in ROS generation of NHLFs on 100 kPa gels, and TCPS were similar (Figures 2b and S4).

Our finding that the increase in substrate stiffness enhanced the cell proliferation, ROS generation, and collagen expression of lung fibroblasts agreed with the previous study,<sup>37</sup> which showed that the mRNA expression of collagen I  $\alpha 1$  in the lung fibroblasts cultured on PAAm gels of fibrotic lung tissue-like stiffness was significantly higher than that of the cells on the gels of normal lung tissue-like stiffness. As expected, MWCNTs-induced fibrogenesis of NHLFs was also regulated by the substrate stiffness. On 5 kPa gels, despite the higher level of MWCNTs uptake observed, the fibrogenic responses of NHLFs were not sensitive to MWCNTs. However, NHLFs grown on 100 kPa gels displayed more severe fibrogenic responses to MWCNTs than those on TCPS. Previously, we evaluated the effects of substrate nanotopography on the fibrogenic response of NHLFs to the MWCNTs and found

that the cells on PDMS nanopillars of 500 nm in diameter and 560 nm in height exhibited more pronounced fibrogenic responses than the cells on PDMS flat substrates.<sup>17,38</sup> The nanopillars had a calculated stiffness of about 280 kPa,<sup>39</sup> comparable to the 100 kPa gels in the present study. The cells on PDMS flat substrates showed stiffer cytoskeleton and nucleus, accompanied by elevated fibrogenic responses; and the MWCNTs treatment only showed limited increases in induced fibrogenic responses of NHLFs.<sup>17</sup> While the distinct MWCNTs-induced fibrogenic responses of NHLFs were observed on the substrates of pathophysiologically different stiffness, it was important to understand how mechanotransduction affected the induced fibrogenesis of the fibroblasts.

### FAK Regulated MWCNTs-Induced Fibrogenic Responses of NHLFs.

Substrate stiffness regulates cell behaviors through mediating integrin activation, focal adhesion assembly, cytoskeleton organization, and nuclear deformation.<sup>40</sup> We herein examined focal adhesion kinase (FAK, a key signaling kinase in focal adhesions that link integrins and cytoskeleton), F-actin, and intracellular localization of yes-associated protein (YAP, a recognized cellular mechanosensor).

Similar trends were observed in F-actin expression and YAP intracellular localization in NHLFs on these substrates in response to the MWCNTs treatment. NHLFs on 5 kPa gels expressed a significantly lower level of F-actin than those on 100 kPa gels and TCPS, whereas the MWCNTs treatment did not significantly influence the F-actin expression for all these substrates (Figure S5). The percentage of cells with nuclear YAP on 5 kPa gels was also significantly lower than that of the cells on 100 kPa gels and TCPS, and no significant change in YAP intracellular localization was observed in all groups with or without MWCNTs treatment (Figure S6). Intriguingly, our Western blot analysis showed that, without the MWCNTs treatment, the expression and phosphorylation (tyrosine-397) of FAK increased when the substrate stiffness increased, and the levels of FAK and phosphorylated FAK (pFAK) in the cells on 5 and 100 kPa gels were significantly lower than those on the substrate stiffness. No significant change in the FAK expression and phosphorylation was detected in the cells on 5 kPa gels or TCPS after MWCNTs treatment; however, MWCNTs significantly upregulated the FAK expression and phosphorylation in NHLFs on 100 kPa gels. To further verify the role of FAK in stiffness effects on the MWCNTs-induced fibrogenic responses of NHLFs, we treated the cells with FAK inhibitor (FAK Inhibitor 14, 5  $\mu$ M) and found that the FAK inhibitor treatment profoundly downregulated the level of pFAK and collagen I, compared to the nontreated groups (Figure 3c). This indicated that inhibition of FAK expression and phosphorylation attenuated the MWCNTs-induced fibrogenesis.

The expression and phosphorylation of FAK are associated with the mechanotransduction of cells in response to the extracellular physical signals such as stiffness and regulate many cell behaviors such as cell proliferation and differentiation.<sup>41,42</sup> It has been reported that substrate stiffness regulated the idiopathic pulmonary fibrogenesis through activating the FAK/ Akt pathway,<sup>37</sup> and increasing substrate stiffness enhanced the FAK expression and phosphorylation in different types of cells.<sup>42-44</sup> Our results showed that the FAK expression and phosphorylation in NHLFs increased with the increased substrate stiffness (Figure 3),

concurring with previous studies. Moreover, our results demonstrated that the MWCNTs treatment significantly upregulated FAK expression and phosphorylation in the cells cultured on 100 kPa gels, but not on 5 kPa gels or TCPS. Similarly, a previous study showed that single-walled CNTs (SWCNTs) treatment for 5 days did not alter the FAK expression or phosphorylation in human epithelial cells cultured on TCPS.<sup>45</sup> Yet, the MWCNTs treatment at a high concentration (50  $\mu\text{g}/\text{mL}$ ) was shown to reduce the mRNA expression of FAK in human dermal fibroblasts on TCPS.<sup>46</sup> The difference between this study and our observation was likely to be caused by a much lower MWCNTs concentration (1  $\mu\text{g}/\text{mL}$ ) in the present study. Our results manifested the important role of FAK expression and phosphorylation in the mechanosensitive fibrogenic responses of NHLFs to MWCNTs. Further, we explored the underlying mechanism of how the MWCNTs-induced fibrogenic responses were affected by the substrate stiffness at the pathophysiological range.

### **Time-Dependent MWCNTs-Induced Lung Fibrogenesis.**

Animal studies showed that the lung fibrogenesis induced by the inhaled MWCNTs was a subchronic or chronic process.<sup>11,18,47</sup> Although MWCNTs treatment did not induce significant fibrogenic responses of NHLFs on the normal lung-mimicking (5 kPa) substrates after 3 days of exposure (Figure 2), we extended the treatment time to 10 days without noticing evident cell death and evaluated the expression of collagen I, FAK, and pFAK in NHLFs on 5 kPa gels in response to MWCNTs. Shown in Figure 4a, increased MWCNTs uptake was observed in NHLFs over time. At 10 days post-MWCNTs treatment, NHLFs expressed a higher level of collagen I compared with the control (i.e., without MWCNTs treatment). Moreover, the increased levels of both expression and phosphorylation of FAK were detected as well (Figure 4b), which further confirmed that FAK played an important role in regulating the fibrogenic responses of NHLFs to MWCNTs.

### **Intracellular Tension Modulated Fibrogenic Responses of NHLFs to MWCNTs.**

Because the fibroblasts grown on 100 kPa gels were stiffer and exhibited a lower MWCNTs uptake (Figure 1e,f), yet more significant fibrogenic responses to MWCNTs than the cells on 5 kPa gels (Figure 2), we postulated that the intracellular tension influenced the fibrogenic responses of NHLFs to MWCNTs. We therefore treated the cells on 100 kPa gels with a Rho kinase (ROCK) inhibitor (Y27632, 10  $\mu\text{M}$ ) to assess the effects of intracellular tension on MWCNTs-induced fibrogenic responses. After treated with Y27632, the actin stress fibers were attenuated, and the cells exhibited a lower stiffness (Figure 4c,d), both suggesting a lower intracellular tension after Y27632 treatment. A higher MWCNTs uptake was observed in the Y27632 treated cells (Figure 4c) compared to the no-treatment group, which was further confirmed quantitatively (Figure 4e). The observations suggested that a lower intracellular tension favored MWCNTs uptake, being consistent with the previous finding (Figure 1). Intriguingly, while the Y27632 treatment did not cause any evident change in collagen I expression in NHLFs in the control groups, the MWCNTs exposure resulted in ~2.4-fold increase in collagen I expression in the Y27632-treated cells, significantly higher than the ~1.8-fold increase in the cells without the ROCK inhibition (Figure 4f). ROCK was recently shown to be related to the uptake efficiency of cells.<sup>48,49</sup> For example, ROCK inhibition by Y27632 promoted the microglial uptake of dextran.<sup>48</sup> Our results suggested that reducing the ROCK activity and consequently lowering the intracellular tension could

enhance the cellular accessibility to MWNCTs and thus elevate the fibrogenic responses of NHLFs to MWCNTs.

### Time-Stiffness Superposition of MWCNTs-Induced Lung Fibrogenesis.

Our results suggested the equivalent effects of substrate stiffness and MWCNTs treatment time on the fibrogenic responses of the fibroblasts to MWCNTs. As illustrated in Figure 5a, on the normal lung-mimicking (5 kPa) substrates a short-term MWCNTs treatment did not induce evident fibrogenic responses, yet extending the treatment time increased the MWCNTs uptake and subsequently induced the fibrogenesis through promoting expression and phosphorylation of FAK. These observations were in line with the subchronic or chronic process of MWCNTs-induced fibro-genesis in the animal studies. By contrast, on the fibrotic lung-mimicking (100 kPa) substrates a short-term MWCNTs treatment was sufficient to induce the acute fibrogenic responses of NHLFs through the FAK and ROCK signaling cascades. Attenuating intracellular tension could increase the MWCNTs uptake and thus elevate the fibrogenic responses. It was reasonable to speculate that, while MWCNTs induced collagen overproduction and consequently stiffened the substrate, the fibrogenesis would be expedited through the positive feedback, by which the stiffer substrate accelerated collagen production.

The fibrogenic responses such as collagen I expression were observed as the functions of substrate stiffness and treatment time as illustrated in Figure 5b. Upon the same MWCNTs treatment time, increasing the substrate stiffness would enhance collagen I expression of NHLFs (Figure 5c). Similarly, when the cells were grown on the substrates of the same stiffness, increasing the MWCNTs treatment time would increase the collagen I expression (Figure 5d). Both the mechanistic understanding and experimental assessment suggested that the MWCNTs treatment time and substrate stiffness had equivalent influences on the MWCNTs-induced fibrogenesis of NHLFs.

To correlate the equivalent effects of MWCNTs treatment time and substrate stiffness on the fibrogenic responses of NHLFs to MWCNTs, we proposed a time-stiffness superposition principle. This principle is analogous to the time– temperature superposition principle in polymer rheology, which has been applied to describe the equivalent effects of time and temperature on the viscoelastic properties of polymers, and thus the viscoelastic data measured at one temperature could be superimposed on the data measured at a different temperature by shifting one set of data along the time axis.<sup>50</sup> By the proposed time-stiffness superposition principle, a set of MWCNTs-induced fibrogenic responses of the fibroblasts measured on one substrate of stiffness  $E$  could be horizontally shifted by using a shift factor  $\alpha_E$  upon a reference stiffness  $E_0$  as follows:

$$\log \alpha_E = \frac{-C_1(E - E_0)}{C_2 + (E - E_0)}$$

where  $C_1$  and  $C_2$  were constants. The superposition process from the normalization of fibrogenic response data to the estimation and determination of  $\alpha_E$  as well as  $C_1$  and  $C_2$  were described in Chart S1 and the “Time-stiffness superposition of MWCNT-induced

fibrogenic responses of NHLFs” section in the Supporting Information. After determining  $\alpha_{ES}$ ,  $C_1$ , and  $C_2$ , a “master curve” could be generated to predict the MWCNTs-induced fibrogenic response of interest at a specific stiffness over a broad time scale.

We herein demonstrated the time-stiffness superposition principle by taking example of the collagen I production as the fibrogenic response of interest. In addition to the PAAm gels of 5 and 100 kPa in stiffness and TCPS, NHLFs were also grown on 21 kPa gels that mimicked the fibrotic lung tissue at the early stage. The cells were treated with MWCNTs for 2 and 3 days, and the collagen I expression was measured by Western blotting. MWCNTs treatment for 3 days induced a higher level of collagen I expression than that of 2 days of treatment on all substrates (Figure 5e). By taking the collagen I production on the 5 kPa gels ( $E_0 = 4.9$  kPa) as the reference, the measurement on different substrates was shifted with a shift factor specific to the substrate  $E$  along the time axis. By applying the least-squares method, the  $\alpha_{ES}$  were determined (see detailed description in the Supporting Information, Figures S7-10 and Tables S3,S4), and then the fibrogenesis data on the reference substrate and the shifted data sets from other substrates formed a master curve as indicated by the broken curve (Figure 5e). By following the same process, the master curves that referred to the substrate of a different stiffness, for instance 21 and 100 kPa gels, were generated (Figure 5f). Figure 5f showed that, at the same MWCNTs treatment time, increasing substrate stiffness would enhance collagen I production, and a longer time was required to reach the same level of collagen I expression on a softer substrate than a stiffer substrate.

While the time-stiffness superposition principle was proposed to correlate the time- and stiffness-dependent MWCNTs-induced lung fibrogenesis, it was likely to be applicable to other engineered nanomaterials and stiffness relevant fibrosis. Moreover, other cell types such as lung epithelial cells and macrophages were involved in lung fibrogenesis upon MWCNTs exposure. The lung epithelial cells underwent the epithelial–mesenchymal transition (EMT) after MWCNTs treatment and transdifferentiated to myofibroblasts, therefore promoting fibrogenesis.<sup>13,51</sup> The EMT could be driven by increasing substrate stiffness.<sup>52</sup> The macrophages could also be activated by MWCNTs treatment and stiff substrates to promote pulmonary fibrosis.<sup>53,54</sup> It is reasonable to speculate that the time-stiffness superposition principle is applicable to those events. The validation of the principle in those applications required further investigation.

## Conclusion.

This study investigated the effects of substrate stiffness in the pathophysiological range on the fibrogenic responses of NHLFs to MWCNTs. The substrate stiffness profoundly affected cell spreading and cell stiffness and, consequently, the uptake of MWCNTs. The MWCNTs-induced fibrogenic responses were not evident on the normal lung tissue mimicking hydrogels (5 kPa) upon short-term exposure, yet gradually increased for extended time. However, the induced fibrogenesis was significant on the fibrotic lung tissue-mimicking gels (100 kPa). The mechanical study suggested that the MWCNTs induced lung fibrogenesis through the FAK and ROCK signaling cascades. Based on the demonstrated equivalent effects of substrate stiffness and treatment time on the fibrogenic responses of the fibroblasts to MWCNTs, we proposed a time-stiffness superposition principle to correlate these



dependences and to predict MWCNTs-induced fibrogenic responses over a broad time scale. Moreover, the superposition principle provided insight into the design of pathophysiologically relevant *in vitro* platforms for nanotoxicology study.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

This work is partly supported by National Institutes of Health R15GM122953 (to Y.Y.) and R01-EB018857 (to Y.R.).

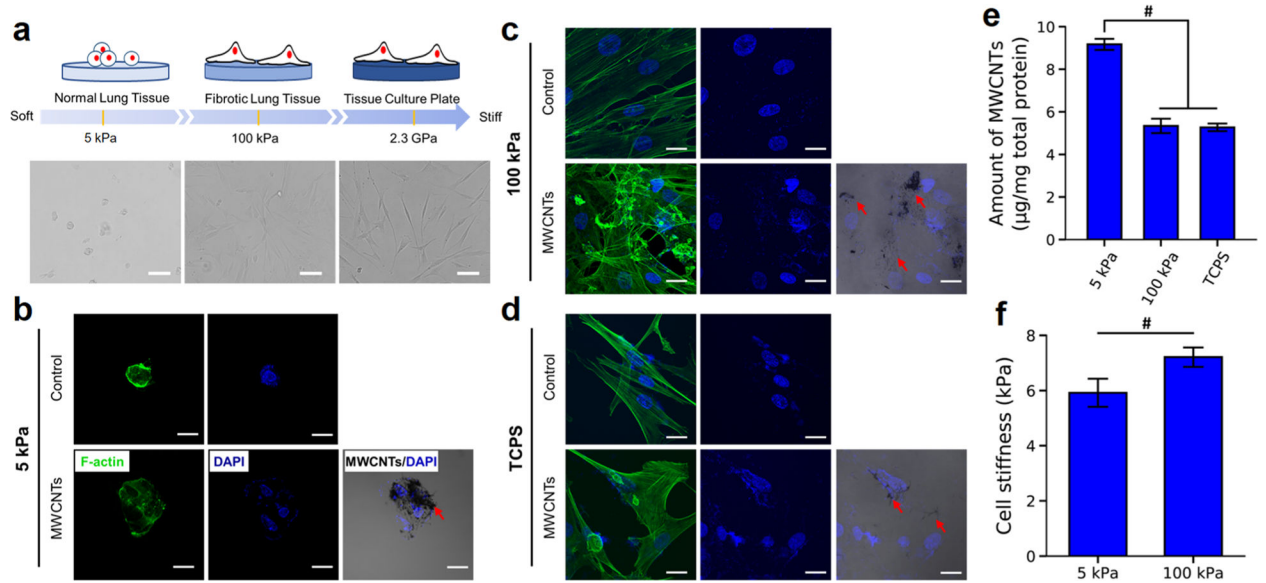
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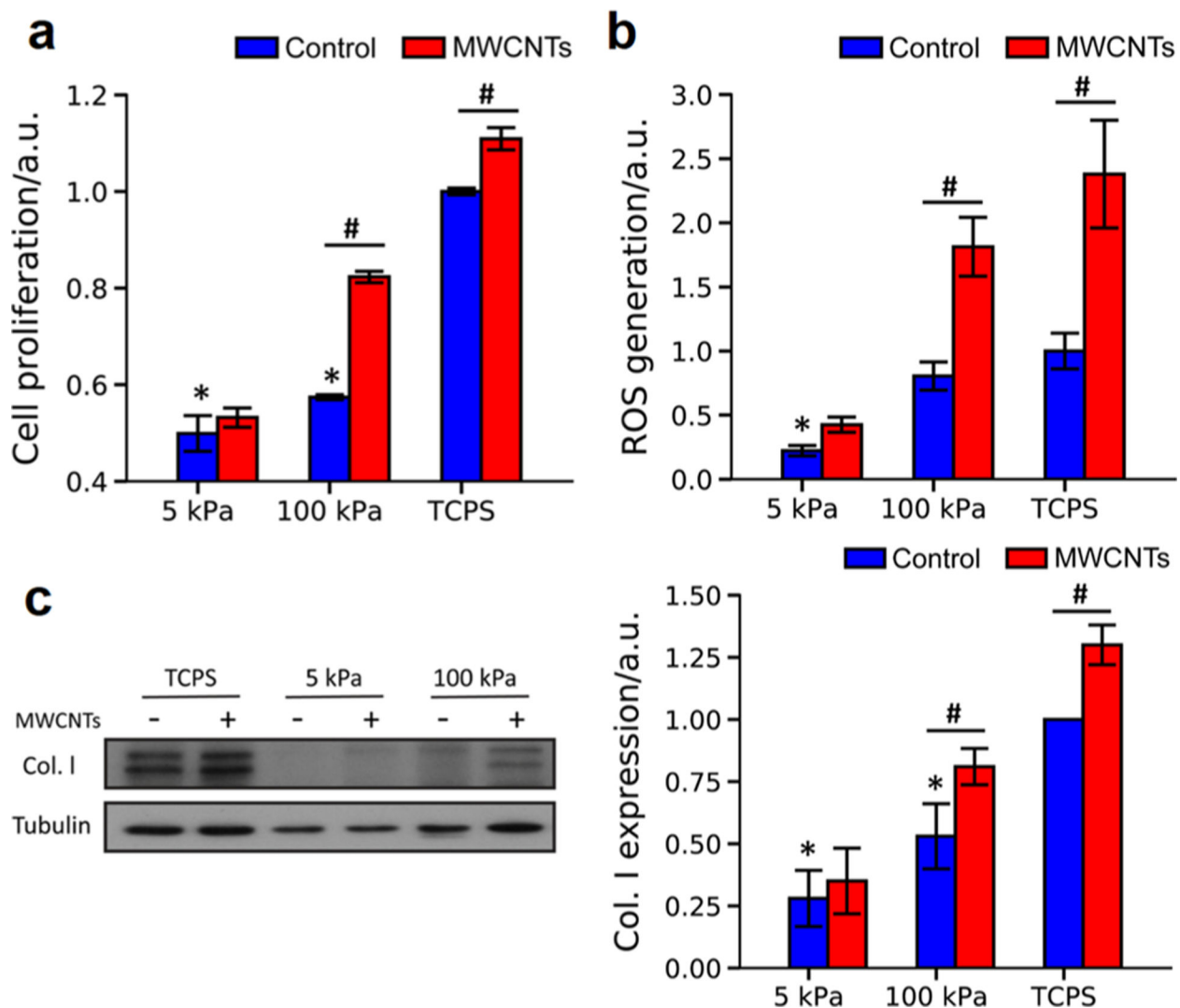
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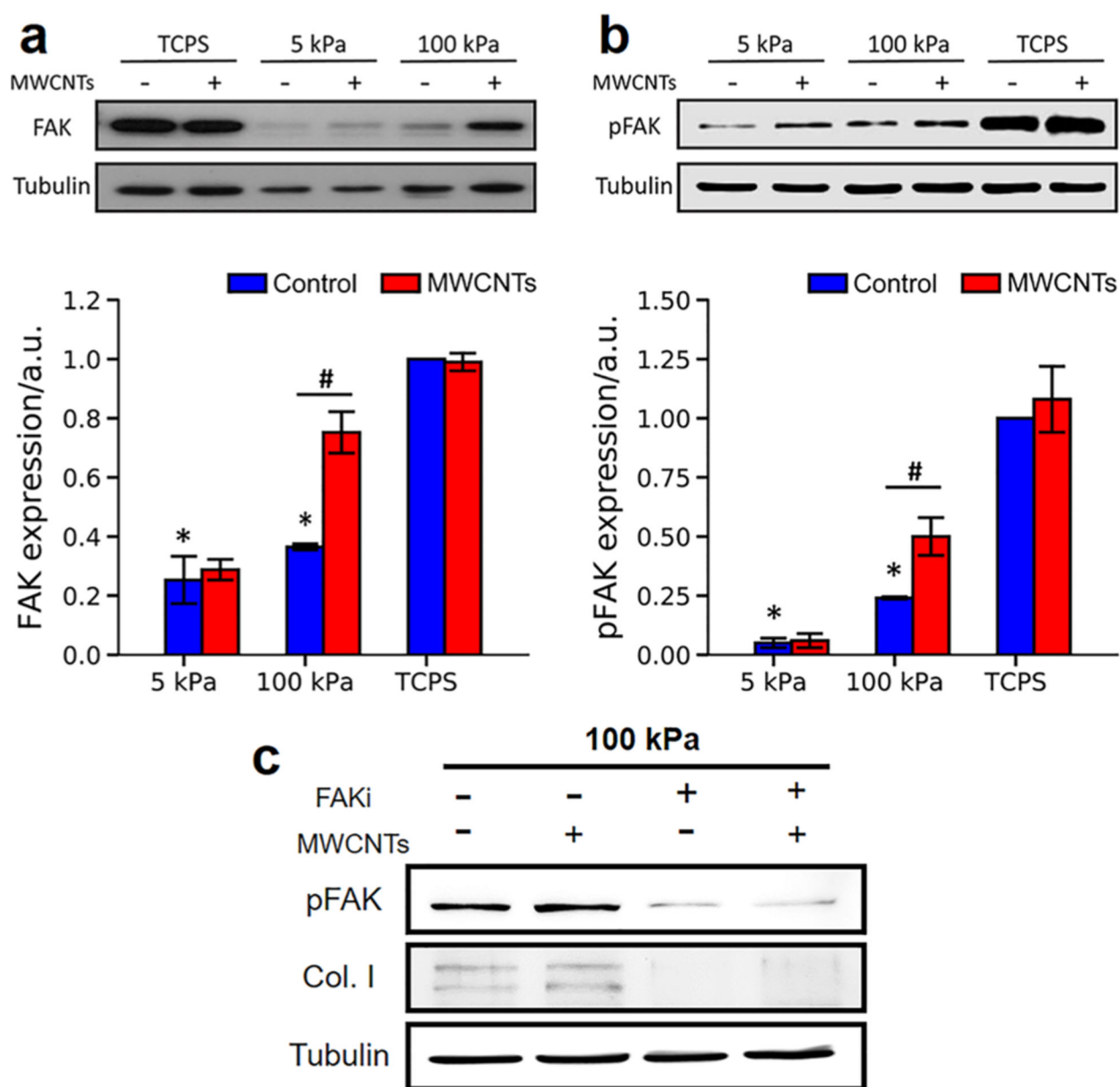


**Figure 1.**

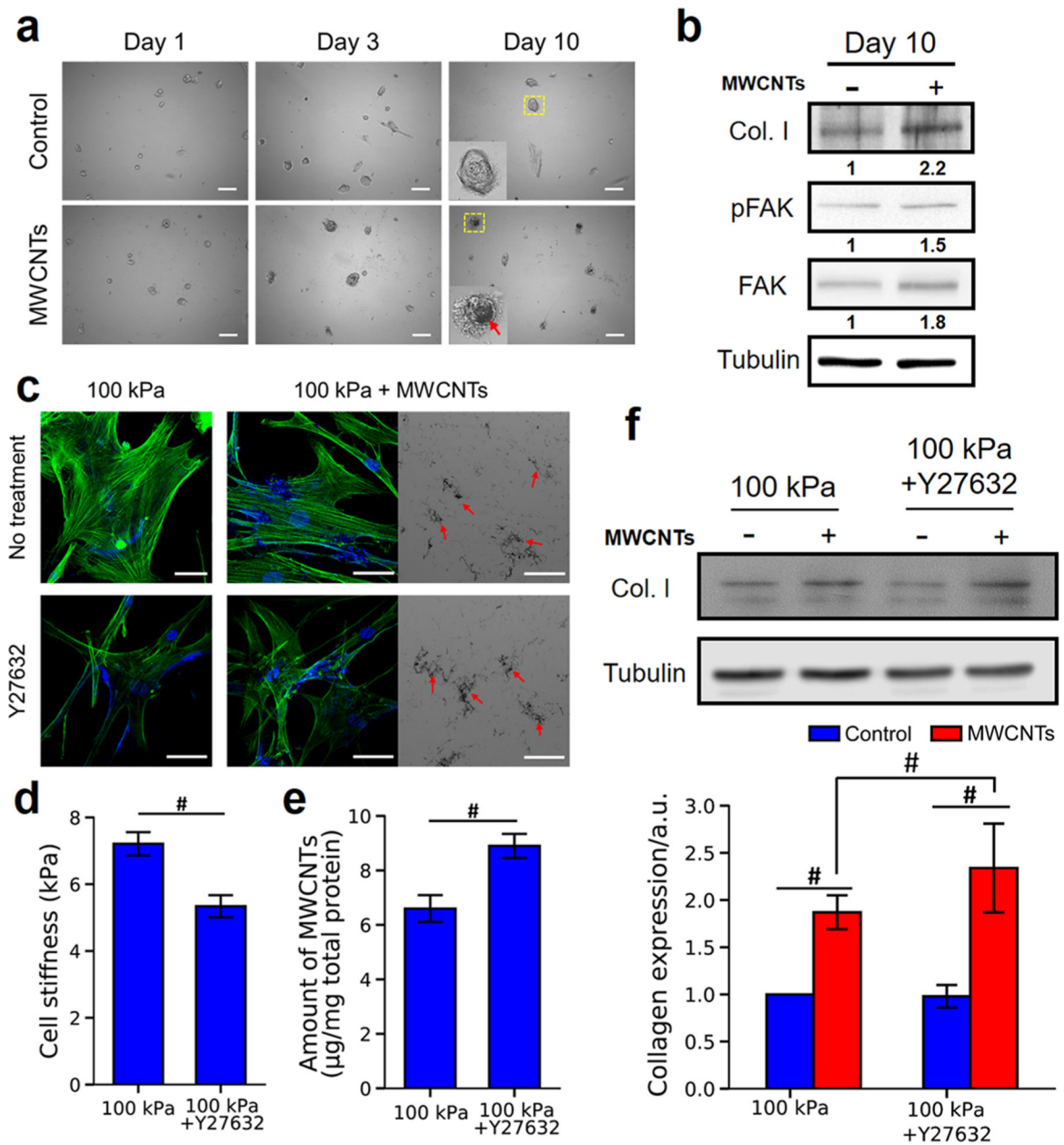
Substrate stiffness effects on the morphology, MWCNTs uptake, and cell stiffness of NHLFs. (a) Illustration and bright field images of NHLFs on substrates of various stiffnesses. The scale bars are 100  $\mu\text{m}$ . (b–d) Confocal fluorescent images of NHLFs on 5 kPa (b) and 100 kPa (c) gels and TCPS (d) with or without MWCNTs treatment. Green, F-actin; blue, nuclei. The overlay images showed the MWCNTs (indicated by the red arrows) localized in the cells. The scale bars are 20  $\mu\text{m}$ . (e) Quantification of MWCNTs uptake in NHLFs on different substrates ( $n = 3$ ). The total amount of MWCNTs was normalized by total protein concentration. (f) AFM measurement of the stiffness of NHLFs on soft and stiff gels ( $n > 50$ ). # $p < 0.05$ .



**Figure 2.** Fibrogenic responses of NHLFs to MWCNTs on PAAm gels and TCPS. (a) Cell proliferation ( $n = 3$ ), (b) ROS generation ( $n = 10$ ), and (c) collagen I expression ( $n = 3$ ) were measured to assess the fibrogenic responses of NHLFs to MWCNTs. Control: without MWCNTs treatment. MWCNTs: with MWCNTs treatment. All the data were normalized to the mean value of the TCPS group without MWCNTs treatment. Negative and positive signs in (c) indicated the cells were treated without and with MWCNTs, respectively. \*significant difference ( $p < 0.05$ ) from the TCPS controls (no MWCNTs treatment); #significant difference ( $p < 0.05$ ) between groups.



**Figure 3.** Role of FAK in MWCNTs-induced fibrogenic responses of NHLFs. Expression of (a) FAK ( $n = 3$ ) and (b) phosphorylated FAK (pFAK) ( $n = 3$ ) in NHLFs on different substrates in response to the MWCNTs treatment. Control: without MWCNTs treatment. MWCNTs: with MWCNTs treatment. All the data were normalized to the mean value of the TCPS group without MWCNTs treatment. (c) Expression of pFAK and collagen I in NHLFs on 100 kPa gels with MWCNTs and FAK inhibitor treatment. Tubulin was used as the loading control. Negative and positive signs indicated the cells were treated without and with MWCNTs or FAK inhibitor. \*significant difference ( $p < 0.05$ ) from the TCPS controls (no MWCNTs treatment); #significant difference ( $p < 0.05$ ) between groups.

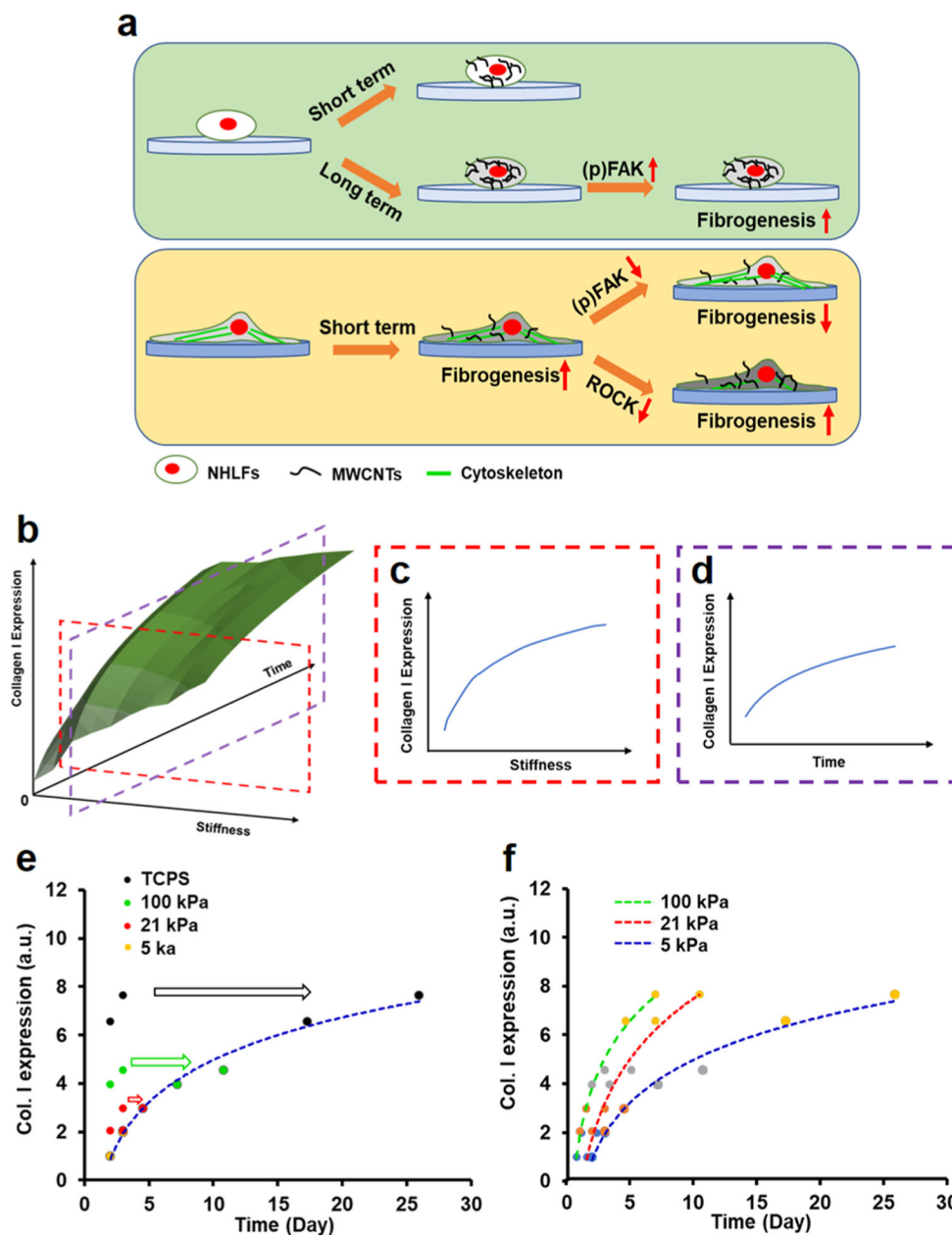


**Figure 4.**

Effects of treatment time and ROCK inhibition on MWCNT-induced fibrogenesis of NHLFs. (a) Bright field images of NHLFs on 5 kPa gels with or without MWCNTs treatment at Days 1, 3, and 10. The insets were enlarged boxed areas, and the red arrow pointed to the MWCNTs. Scale bars are 100  $\mu\text{m}$ . (b) Expression of collagen I, pFAK, and FAK in NHLFs on 5 kPa gels with (+) or without (-) MWCNTs treatment for 10 days. Tubulin was used as the loading control. The relative protein band intensities were normalized to the tubulin control and presented underneath the protein bands. (c) Confocal images of NHLFs on 100 kPa gels with or without Y27632 treatment. Y27632 (10  $\mu\text{M}$ ) was added 2 h before NHLFs were treated with MWCNTs for 3 days. Green, F-actin; blue, nuclei. The bright field images showed the MWCNTs localized in the cells. The red arrows



indicated the MWCNTs. Scale bars are 50  $\mu\text{m}$ . (d) Stiffness of NHLFs on 100 kPa gels with or without Y27632 treatment ( $n > 50$ ). (e) Quantification of the MWCNTs uptake of NHLFs on 100 kPa gels with or without Y27632 treatment ( $n = 3$ ). (f) Western blot analysis of collagen I in NHLFs ( $n = 3$ ). Tubulin was used as the loading control. Negative and positive signs indicated the cells were treated without and with MWCNTs. #significant difference ( $p < 0.05$ ) between groups.



**Figure 5.** Time-stiffness superposition of MWCNTs-induced lung fibrogenesis. (a) Mechanosensitive fibrogenic responses of NHLFs to MWCNTs. (b) Three-dimensional plot of the stiffness and time effects on MWCNTs-induced collagen I expression. Collagen I expression of NHLFs increased as the function of (c) substrate stiffness while the treatment time was fixed and (d) MWCNTs treatment time while the substrate stiffness was fixed. (e) Generation of a master curve referring to the 5 kPa gel. (f) Master curves generated by referring to the substrate of a specific stiffness.