Exploration of the Effects of Substrate Stiffness on Biological Responses of Neural Cells and Their Mechanisms

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ABSTRACT: Substrate stiffness, as a critical mechanical factor, has been proven to be an important regulator of biological responses, cellular functions, and disease occurrence. However, the effects of substrate stiffness on the phenotypes and drug responses of neural cells remain largely unknown. In this study, polydimethylsiloxane (PDMS) substrates with different stiffnesses were employed to establish the mechanical microenvironment of tissues of different organs. We studied the influences of stiffness on neural cell phenotypes, including cell viability, cell cycle, cytoskeleton structures, cell stiffness, and drug responses of neural cells for hormesis and therapeutic efficacy in neurodegenerative disorders (NDD). The results showed that the greater the range of maximum stimulatory responses, the bigger the width of the stimulatory dosage and the higher the range of maximum neuroprotective activities of hormetic chemicals in neural cells grown on the soft substrate commensurable to the stiffness of the brain, indicating that neural cells on a rigid substrate are resistant to hormetic and neuroprotective effects of hormetic chemicals against 6-hydroxydopamine (6-OHDA)-induced Parkinson’s disease (PD) model. The sensitivity of neural cells on the soft substrate to drug response was attributed to the increased cell viability rate, cell cycle progression, actin stress fibers, focal adhesion formation, and decreased cell stiffness. The promoting effect of the soft substrate and the enhanced hormetic and neuroprotective effect of hormetic chemicals on soft substrates in PC12 cells were confirmed to be mediated by the upregulated EGFR/PI3K/AKT signaling pathway by RNA-Seq and bioinformatics analysis. This study demonstrates that the biomechanical properties of the neural microenvironment play important roles in cell phenotypes and drug responses of neural cells in vitro and suggests that substrate stiffness should be considered in the anti-NDD drug design and treatment.

1. INTRODUCTION

To date, the process of new drug discovery and development is still expensive and time-consuming with low success rates, which was attributed to the differences in the mechanical microenvironment of cells when grown on the rigid plastic substrate in vitro and soft tissue substrate in vivo. Currently, plastic substrates are widely used as the mechanical microenvironment for in vitro pharmacological studies. However, all cells in tissues and organs are exposed to their surrounding substrate and can sense and respond to a wide range of mechanical signals, which leads to the change of its behavior and function. Because the changes in the biomechanical factors of the tissues such as stiffness in vivo have long been overlooked and routine drug screening is still performed in plastic culture plates, the phenotypes and drug responses of cells in different mechanical microenvironments have not been adequately studied yet.

In view of biomechanical factors in drug therapy, “biomechanopharmacology” as a newly developed multidisciplinary study has been introduced into pharmacology studies in 2006, and this study demonstrated that substrate...
Stiffness could be a crucial regulator of disease occurrence,7 biological responses,4 and cellular functions.2 The impact of stiffness on cell behaviors depends on the cell type, their tissue of origin, and pathological state. Different physiological stiffnesses could induce differentiation of mesenchymal stem cells into different types of cells.8 Following injury, the tissue stiffness of the lung, liver, and kidney rapidly increased, which in turn resulted in the amplification of fibrogenesis.9 Liver fibrosis and aortic stiffness cause significant mechanical changes at the whole organ and cellular levels.10,11 As reported, the tissue stiffness of brain tissues in brain injury patients was higher than that of healthy persons.12 In addition, the stiffness factor should be considered in designing biomaterials for tissue engineering applications.13 Several studies have shown that cells regulate cell viability, cytoskeleton structures, cell stiffness, and drug response with microenvironmental stiffness.4,14,15 Therefore, particular attention should be paid to the stiffness of substrates while developing drugs for treating diseases. There are some investigations revealing that the biomechanical properties of the tumor microenvironment play important roles in the spread, viability, and migration of tumor cells in vitro, and the response of tumors to chemotherapy from clinical observations.5,16,17 However, the effects of biomechanical properties on the phenotypes and drug responses of neural cells and the specific mechanisms mediating these effects are still unknown.

Polydimethylsiloxane (PDMS) is an elastomeric polymer and well suited for biological applications because of its transparency and biocompatibility.4,18 Accordingly, in this study, we used PDMS of different stiffnesses to mimic the stiffness of different tissues (collagenous bone, mammary tumor, and adult brain) and evaluate the effects of substrate stiffness on the growth, phenotypes, and drug responses of neural cells. In this study, we show for the first time that soft substrate, with brain-like stiffness, significantly increases cell viability, cell cycle progression, actin stress fibers, and focal adhesion formation and decreases cell stiffness of neural cells.

Recent evidence has demonstrated that biomechanical factors can directly influence the effects of anticancer drugs, such as lapatinib, cisplatin, and taxol.4,17 In our previous studies, we demonstrated that several topo inhibitors, such as camptothecin (CPT), doxorubicin hydrochloride (DOX), etoposide (ETOP) at low doses, exhibited strong hormetic and neuroprotective effects in PC12 cells through hormetic mechanisms.19 A hormetic response is an adaptive mechanism generally activated by low doses of physical or chemical stressors and have long-term beneficial effects on cells or organisms and protect them from further damage, while at the

Figure 1. Cell viability and cell cycle analysis of neural cells on substrates with different stiffnesses. Neural cells were grown on substrates with different stiffnesses for 24 h. (A and D) are MTT, (B and E) are CCK-8, and (C and F) are WST-1 cell viability assay results. (A, B, and C) PC12 and (D, E, and F) N2a cell growth profiles on different substrates. (G) Distributions of G1 (first peak) and G2 (second peak) in the cell cycle of PC12 cells. (H) is the quantified results of (G). Values represent the means ± standard deviation (SD) of at least three independent experiments. *p < 0.05 and **p < 0.01 vs cell viability rate or rate of cells on the soft substrate (0.1 kPa) by one-way analysis of variance (ANOVA) analysis.
higher dose the toxic effect prevails. The hormesis concept has been receiving increasing attention in neural science, and a diverse range of chemicals have been reported to exhibit hormetic responses in neuronal models. In this study, we further investigated whether different substrate stiffness also influenced the responses of PC12 cells to topo inhibitors. Meanwhile, we performed high-throughput mRNA sequencing (RNA-Seq) and bioinformatics analysis to determine the underlying mechanisms mediating these effects in neural cells.

We found that softer substrates could significantly enhance the hormetic and neuroprotective effects of low-dose topo inhibitors in neural cells, which were attributable, at least partially, to the upregulation of the EGFR/PI3K/AKT pathway.

2. RESULTS

2.1. Cell Viability Analysis of Neural Cells on Substrates with Different Stiffnesses. To investigate the effects of substrate stiffness on neural cell viability, we prepared PDMS substrates with different stiffnesses. Young's modulus of the PDMS of different stiffnesses was found to be 46.7 ± 2.1 kPa (60:1), 5.3 ± 0.2 kPa (80:1), and 0.1 kPa (100:1), which are equivalent to the stiffness of collagenous bone, mammary tumor, and adult brain parenchyma, respectively. Soft matrices that mimic the brain are neurogenic. Meanwhile, tissue culture plates (TCPs) (~10^6 kPa) were used as the usual cell culture microenvironment in vitro. The cell viability of PC12 or N2a cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell counting Kit-8 (CCK-8), and WST-1 assays. The results showed that the cell viability of PC12 and N2a cells grown on the soft substrate (0.1 kPa) increased by 85.6−106.9% (Figure 1A−C) and 66.9−86.7% (Figure 1D−F), respectively, than those of cells grown on TCPs, indicating that the viability rate of neural cells was increased on the soft substrate. Since the PC12 cell line was the most sensitive to the stiffness of substrates among the tested neural cell lines, we chose the PC12 cell line for the subsequent study.

2.2. Cell Cycle Analysis of Neural Cells on Substrates with Different Stiffnesses. We further examined whether changes of the cell cycle on different substrates caused altered cell viability. As shown in Figure 1G,H, a 7.0% decrease of G1 phase cells but a 6.7% increase of G2/M phase cells on the soft substrate (0.1 kPa) increased by 85.6−106.9% (Figure 1A−C) and 66.9−86.7% (Figure 1D−F), respectively, than those of cells grown on TCPs, indicating that the viability rate of neural cells was increased on the soft substrate. Since the PC12 cell line was the most sensitive to the stiffness of substrates among the tested neural cell lines, we chose the PC12 cell line for the subsequent study.

Figure 2. Cytoskeleton structures and cell stiffness of neural cells on substrates with different stiffness. (A) Cytoskeleton structures of PC12 cells (nuclear blue, vinculin green, and F-actin red), the magnification is 20×, scale bars = 5 μm. Cell stiffness of PC12 (B) and N2a (C) cells measured by atomic force microscopy (AFM). Values represent the means ± SD of at least three independent experiments. *P < 0.05 and **P < 0.01 vs stiffness of cells on the soft substrate (0.1 kPa) by one-way ANOVA analysis.
speculate that the increase of cell viability of the PC12 cells on softer substrates was related to the rapid cell cycle progression.

2.3. Cytoskeleton Structures of Neural Cells on Substrates with Different Stiffnesses. Cytoskeleton structures of PC12 cells on substrates with different stiffnesses were further investigated via a fluorescence microscope. As shown in Figure 2A, cells grown on TCPs only formed the dispersed stress fibers at the outer edge and spot-like expression of vinculin. In contrast, cells grown on the soft substrate exhibited prominent stress fibers throughout the cytoplasm and rod-like expression of vinculin. These results indicated that neural cells grown on soft substrates exhibit increased actin stress fibers and focal adhesions than cells on TCPs.

2.4. Elastic Modulus of Neural Cells on Substrates with Different Stiffnesses. We next examined whether the degree of stiffness of neural cells was also correlated with the different substrate stiffnesses. To assess the elastic modulus of neural cells, we used an AFM instrument, which uses a microcantilever that is extremely sensitive to weak forces. As shown in Figure 2B,C, AFM measurements demonstrated that Young’s modulus of PC12 and N2a cells grown on the soft substrate was less than that of cells grown on TCPs. The median values of cell stiffness of PC12 cells in the 0.1 and ~106 kPa groups were 0.33 and 1.33 kPa, respectively. These results suggested that cell stiffness of neural cells tended to decrease with the decrease in substrate stiffness.

2.5. Responses to Topo Inhibitors in PC12 Cells Grown on Substrates with Different Stiffnesses. We then studied whether different substrate stiffnesses also influenced the responses of PC12 cells to topo inhibitors, which are widely used to treat cancers at high dosage and could induce hormetic and neuroprotective effects at low dosage. To investigate the hormetic effects of topo inhibitors, PC12 cells were treated with different concentrations of CPT (0.01–3.5 μM), ETOP (0.39–100 μM), or DOX (0.08–20 μM) for 24 h. The cell viability of these topo inhibitors was assessed by MTT assay. From Figure 3, we can see that the responses to three topo inhibitors were significantly different in softer and stiffer substrates. CPT (0.22 μM) increased the cell viability of PC12 cells by 34.1 and 78.7% grown on TCPs and the soft substrate (0.1 kPa), respectively (Figure 3A). ETOP (1.56 μM) increased the viability of cells grown on TCPs and the soft substrate (0.1 kPa) by 22.9 and 53.3%, respectively (Figure 3B). DOX (0.32 μM) increased the viability of cells grown on TCPs and the soft substrate (0.1 kPa) by 27.1 and 55.3%, respectively (Figure 3C). In contrast, 3.5 μM CPT, 100 μM ETOP, and 20 μM DOX, significantly reduced cell viability of PC12 cells on stiffer substrates (46.7 kPa and TCPs) and slightly reduced cell viability on softer substrates (0.1 and 5.3 kPa), indicating the greater range of maximum stimulatory responses and the bigger width of the stimulatory dosage of topo inhibitors in neural cells grown on softer substrates than on stiffer substrates. These results demonstrated that softer substrates could significantly enhance the hormetic effects of low-dose topo inhibitors in neural cells.

2.6. Neuroprotective Effects of Topo Inhibitors in PC12 Cells on Substrates with Different Stiffnesses. We hypothesized that substrate stiffness could also influence the neuroprotective effects of low-dose topo inhibitors against a 6-OHDA-induced PD model. To test this hypothesis, PC12 cells were treated with 0.01–0.22 μM CPT, 0.39–1.56 μM ETOP, or 0.08–1.25 μM DOX for 24 h and then incubated with or without 250 μM 6-OHDA for an additional 24 h. As shown in Figure 3, the responses of PC12 cells to neuroprotection of three topo inhibitors were significantly different in softer substrates and stiffer substrates. CPT (0.22 μM) inhibited the cytotoxicity of 6-OHDA in cells grown on TCPs and the soft...
substrate (0.1 kPa) by 20.4 and 54.9%, respectively (Figure 3D). ETOP (1.56 μM) inhibited the cytotoxicity of 6-OHDA in cells grown on TCPs and the soft substrate (0.1 kPa) by 15.4 and 39.9%, respectively (Figure 3E). DOX (0.32 μM) increased the viability of cells grown on TCPs and the soft substrate (0.1 kPa) by 17.9 and 36.7%, respectively (Figure 3F), indicating that the range of maximum neuroprotective activities of topo inhibitors in neural cells grown on softer substrates is higher than that on stiffer substrates. These results indicated that softer substrates could remarkably enhance the neuroprotective effects of low-dose topo inhibitors against 6-OHDA-induced neurotoxicity. The degree of neuroprotection was dependent on the stiffness of substrates.

2.7. Gene Ontology (GO) Functional Enrichment and Pathway Enrichment Analysis of DEGs. To elucidate how stiffness influenced the effects of PC12 cells, we performed RNA-Seq analysis of the RNA samples from different groups. After filtering by DESeq2, 146 significant DEGs were identified in soft substrate samples compared with TCP samples, including 124 upregulated and 22 downregulated genes. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed to reveal the potential biological roles of these DEGs. In total, 146 DEGs were enriched in 212 GO terms, with 141, 32, and 39 genes being annotated as biological process, cellular components, and molecular functions, respectively. Among all terms, “cellular process” and “biological process” were the most abundant in the biological process group (Figure 4A), DEGs with “membrane part” and “neuron part” in the cellular components group were highly represented (Figure 4B), and DEGs with “receptor activity”, “molecular transducer activity”, and “signal transducer activity” in the molecular function category were significantly enriched (Figure 4C). The pathway enrichment results for DEGs (Figure 4D) showed that the pathways with higher enrichment levels between the soft substrate group and the TCP group included the focal adhesion, cell adhesion molecules, regulation of actin cytoskeleton, Ras signaling pathway, and PI3K/AKT signaling pathway.

2.8. Validation of RNA-Seq Data by qRT-PCR. By comparing the results of GO and KEGG analysis, we observed that the Ras signaling pathway played a significant biological role in substrate stiffness regulating phenotypes of neural cells. There were 7 DEGs belonging to the Ras signaling pathway. These 7 DEGs were selected to confirm the reliability of the RNA-Seq results by qRT-PCR (Figure 5A,B). These genes were mostly involved in the Ras signaling pathway, PI3K/AKT signaling pathway, and regulation of actin cytoskeleton, which may explain the increased cell viability, stress fiber, and focal adhesions formation of PC12 cells grown on softer substrates compared with those of cells grown on stiffer substrates.
substrates. The expression trends of all selected genes were highly consistent between the two methods. Thus, qRT-PCR results validated the reliability of the RNA-Seq data.

2.9. Neural Cells Grown on Softer Substrates Upregulated the EGFR/AKT Pathway. Since Ras signaling pathway locates upstream of other top-ranked pathways, the PI3K/AKT pathway plays pivotal roles in the cell growth/survival/cycle response and is downstream of the Ras signaling pathway. Meanwhile, RNA-Seq and qRT-PCR results indicated that the expression of EGFR, KRAS, and PI3K genes in the soft substrate group increased compared to the TCP group, we hypothesized that the EGFR/PI3K/AKT signaling pathway in PC12 cells was involved in the enhanced viability and neuroprotective effects induced by softer substrates. We examined the phosphorylated and total protein levels of EGFR and AKT in PC12 cells grown on substrates with different stiffnesses by Western blotting assay. Our data showed that the levels of p-EGFR and p-AKT were significantly increased in the softer substrate groups compared to those in stiffer substrate groups (Figure 5C,D). Moreover, both EGFR inhibitor AG1478 and PI3K/AKT inhibitor LY294002 significantly reversed the increased phosphorylation of AKT (Figure 6A−D). These results indicated that the influence of substrate stiffness on the pharmacological responses of neural cells is through the activation of the EGFR/PI3K/AKT signaling pathway.

2.10. Inhibition of the EGFR/PI3K/AKT Pathway Attenuated the Hormetic and Neuroprotective Effects of CPT in Neural Cells on Softer Substrates. To further validate the role of the EGFR/PI3K/AKT pathway in the viability-enhancing effect of softer substrates on PC12 cells, we tested whether the pathway inhibitors could reverse soft substrate-induced cell growth stimulation in PC12 cells. The MTT colorimetric assay revealed that the viability of PC12 cells grown on the soft substrate increased by about 100%, which was consistent with the previous result in Figure 1A. However, preincubation with 1 μM AG1478 or 10 μM LY294002 partially abolished the growth stimulation of PC12 cells on the soft substrate (Figure 6E), suggesting that the
EGFR/PI3K/AKT pathway is involved, at least partially, in the viability-enhancing effect of the soft substrate on PC12 cells.

We further investigated whether the EGFR/PI3K/AKT pathway participates in enhancing the hormetic effect of low-dose CPT in PC12 cells on the soft substrate. As shown in Figure 6E, the MTT colorimetric assay indicated that 0.22 μM CPT increased the viability of PC12 cells on the soft substrate by about 80% compared to the control group, which is similar to the result shown in Figure 3A. However, co-treatment with low-dose CPT and AG1478 or LY294002 partially abolished the growth stimulation by a low-dose of CPT in PC12 cells on the soft substrate compared to CPT treatment alone. We further assessed if the neuroprotective effect of CPT against 6-OHDA-induced cell death was affected by the EGFR/PI3K/AKT pathway inhibitors in PC12 cells. The results of MTT colorimetric assay (Figure 6G) revealed that 0.22 μM CPT exhibited about 55% neuroprotective activity on the soft substrate compared to the group treated with 6-OHDA alone, which was consistent with the previous result in Figure 3D. However, AG1478 or LY294002 partially abolished the inhibitory effect of low-dose CPT on 6-OHDA-induced cell death on the soft substrate compared to co-treatment of CPT and 6-OHDA. These results demonstrated that the EGFR/PI3K/AKT pathway was involved, at least partially, in the soft substrate compared to standard culture on rigid substrates (Figure 1A–F), suggesting that the softness of neural cells correlates well with substrate stiffness. Accumulating evidence suggests that matching the substrate stiffness with the in vivo target tissue stiffness facilitates cell viability.5,16,18 Adult mesenchymal stem cells form more neurons on soft substrates than on stiff substrates.8 Our results from different cell viability assays showed that the viability of neural cells on substrates with stiffness similar to the stiffness of the brain increased two times compared to standard culture on rigid substrates (Figure 1A–F), suggesting that a strong relationship was observed between decreasing substrate stiffness and increasing neural cell viability. In addition, cell cycle progression of neural cells was significantly more rapid on softer substrates than on stiffer substrates (Figure 1G,H), which was inconsistent with the previous study that cell cycle progression of MCF-7 cells was increased on a rigid substrate.4 Overall, these findings indicate that soft substrate, with brain-like stiffness, promote neural cell viability, which was associated with the increase in cell cycle progression, actin stress fibers, and focal adhesion formation and a decrease in cell stiffness.

Recent studies reported that mechanical changes in substrates during tumor progression affect the response to chemotherapeutics.5,17 MCF-7 cells are resistant to the cytotoxicity of antitumor drugs on soft substrates.4 We speculate that the substrate stiffness could also regulate the response of neural cells to chemotherapeutics. In the current study, we found that neural cells respond to drug hormesis (Figure 3A–C) and therapeutic efficacy (Figure 3D–F) differently on substrates with unequal physiological stiffness, indicating that substrate stiffness is a crucial parameter affecting drug treatment efficacy of neural cells. Accordingly, we speculate that agents proven to be ineffective or less effective in treating neurodegenerative disorders (NDD) on TCPs in vitro might be efficient in vivo. It is possible that matching the substrate stiffness used for pharmacological studies in vitro to the stiffness of the native tissues in vivo creates an environment conducive to drug screening. The influence of substrate stiffness on the phenotypes and drug responses of neural stem cell-derived neurons need further investigation.

RNA-Seq technology plays an important role in gene expression analysis. In this study, we performed RNA-Seq to observe the changes in gene expression in neural cells on substrates with different stiffnesses. RNA-Seq and qRT-PCR results showed that a large number of DEGs were altered under different substrate stiffnesses. We determined the underlying mechanisms of how substrate stiffness influenced the phenotypes and drug responses of neural cells based on this bioinformatics analysis. PAK2, a PAK family protein, has a profound effect on the reorganization of the actin cytoskeleton in a variety of organisms.31,52 AF6, a connector of intercellular...
adhesion molecules and the actin cytoskeleton. It can control integrin-mediated cell adhesion and belongs to a cell–cell adhesion system.32,33 Kwon et al. reported that AKAP12 is essential for the integrity of endothelium by regulating the actin cytoskeleton through PAK2 and AF6 in zebrafish.35 The EGFR/P13K/AKT pathway plays pivotal roles in the cell viability/survival/cycle.34 In response to stress, the activated EGFR activates PI3K, generating phosphorylatedinositol 3,4,5-trisphosphate, which in turn activates AKT.35 Lin et al. found that ADAM17 regulates prostate cancer cell viability and cell cycle progression by the activation of the EGFR/P13K/AKT pathway.56 Bordeleau et al. reported that substrate stiffness regulates cell functions through its impact on the PI3K-AKT pathway.56 In the present study, we found that softer substrates upregulated the mRNA expression levels of cell growth/survival/cycle-associated genes of EGFR and P13K, and regulated the actin cytoskeleton-associated gene PAK2 and cell–cell junction-associated gene AF6 in neural cells (Figure 5A,B). Additionally, the levels of p-EGFR and p-AKT were significantly increased in neural cells grown on softer substrates compared with those on stiffer substrates (Figure 5C,D). These results demonstrated that the effects of substrate stiffness on the phenotypes of neural cells were through, at least partially, the activation of the Ras pathway. Additionally, the viability-enhancing effect of soft substrates and the enhanced homeric and neuroprotective effects of low-dose CPT on soft substrates in PC12 cells were significantly attenuated by pretreatment with the EGFR inhibitor AG1478 or the P13K/AKT inhibitor LY294002 (Figure 6), suggesting that EGFR/P13K/AKT played a significant biological role in substrate stiffness regulated drug responses of neural cells.

In conclusion, in response to changes in the mechanical microenvironment, neural cells regulate their phenotypes, such as cell viability, cell cycle, cytoskeleton structures, and cell stiffness, which further influence drug responses for hromesis and therapeutic efficacy in NDD. Thus, this work provides evidence that mechanical intervention of the neural microenvironment should be considered in the anti-NDD drug design and treatment. In addition, a matrix that more closely mimics the native cellular environment should be developed for anti-NDD drug screening systems in vitro instead of the currently used TCPs. Apparently, more efforts need to be made in biomachanopharmacology in the future. Meanwhile, we also found that the integral regulation of the neural mechanical microenvironment combined with beneficial hometic effects of drugs may be a better approach for NDD treatment. In addition, our results may also contribute to increasing the understanding of the development and treatment of NDD, as well as advancing in vitro pharmacological studies for NDD.

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. CPT, DOX, ETOP, type I collagen, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), rhodamine-phalloidin, paraformaldehyde (PFA), Triton X-100, 6-hydroxydopamine (6-OHDA), and rabbit antivinculin polyclonal antibody were purchased from Sigma-Aldrich Co (St. Louis, MO). PDMS Sylgard 184 Silicone was obtained from Dow Corning (Midland, MI). F-12K medium, Dulbecco’s modified Eagle’s medium (DMEM)/F12, phosphate-buffered saline (PBS), and penicillin–streptomycin (PS) were purchased from Gibco (Carlsbad, CA). Fetal bovine serum (FBS), horse serum (HS), Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody, rhodamine-phalloidin, TRIZol reagent, and oligo (dT) magnetic beads were obtained from Invitrogen (Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Molecular Probes (Eugene, Oregon). Hoechst 33342 staining kit, WST-1 cell viability and cytotoxicity assay kit, cell counting kit-8 (CCK-8), antifade mounting medium, cell cycle and apoptosis analysis kit, and AG1478 and LY294002 were obtained from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China). Ultrapure RNA extraction kit, SuperRT cDNA First-Strand Synthesis Kit, and UltraSYBR Mixture (with ROX Reference Dye) were purchased from CWBIO Co., Ltd (Beijing, China). Primary antibodies against phospho-EGFR, EGFR, phosphor-AKT, AKT, β-actin, horseradish peroxidase (HRP)-conjugated Goat anti-Rabbit IgG, and secondary antibodies were obtained from Proteintech (Chicago, IL) or Cell Signaling Technology (Danvers, MA).

4.2. Substrate Preparation. The substrates used were either tissue culture plates (TCPs) or PDMS samples with different stiffnesses, which were prepared by modifying the ratio of the “base” and “curing agent” as follows: 100:1 (w/w; 0.1 kPa), 80:1 (w/w; 5.3 kPa), and 60:1 (w/w; 46.7 kPa) as previously described.37 After degassing under vacuum for 1 h, PDMS gels were transferred into 6/24/96-well TCPs and cured at 80 °C for 24 h. Before using for cell culture, all PDMS substrates were exposed to UV light for 24 h. All plates (TCPs and PDMS) were coated with type I collagen (0.1 mg/mL) for 24 h at 37 °C, and then rinsed with PBS three times. Next, the substrates were soaked in the cell culture medium prior to the addition of cells.

4.3. Cell Culture and Drug Treatments. Rat adrenal pheochromocytoma PC12 cells and mouse neuroblastoma Neuro-2a (N2a) cells were obtained from American Type Culture Collection (Manassas, VA). PC12 cells were cultured in ATCC-formulated F-12K medium supplemented with 15% HS, 2.5% FBS, and 1% antibiotics (100 units/mL PS). N2a cells were maintained in DMEM/F12 medium containing 10% FBS and 1% PS. PC12 and N2a cells were incubated in a humidified atmosphere containing 5% CO2 at 37 °C. For all in vitro assays, topo inhibitors CPT, DOX, and ETOP were dissolved in DMSO to prepare stock solutions at a final DMSO concentration of less than 0.1%. The working solutions were freshly diluted in the basal medium.

4.4. Cell Viability and Cytotoxicity Analysis. PC12 or N2a cells were seeded into 96-well TCPs coated with or without PDMS substrates of different stiffnesses. For cell viability analysis, the cells were plated at a density of 6 × 103 cells/well, and cultured for 24 h. Cell viability was determined by MTT,20 CCK-8,37 and WST-138 assays as described in our previous paper. For cytotoxicity measurements, the cells (6 × 103 cells/well) were treated with a wide range of concentrations of topo inhibitors for 24 h. Cell cytotoxicity was measured using the MTT method. 6-OHDA is widely used as a neurotoxin to generate the experimental cell model of PD.39 For neuroprotection analysis, the cells (1 × 104 cells/well) were pretreated with indicated concentrations of topo inhibitors for 24 h prior to the treatment of 250 μM 6-OHDA for an additional 24 h. MTT assay was used to determine the neuroprotective effects of low-dose topo inhibitors against 6-OHDA-induced cell death.

4.5. Cell Cycle Analysis. For cell cycle analysis, PC12 (1 × 105 cells/well) was seeded in 6 well TCPs with substrates of different stiffnesses. After two days, the cells were collected and processed for cell cycle analysis as previously described.37 After degassing under vacuum for 1 h, PDMS gels were transferred into 6/24/96-well TCPs and cured at 80 °C for 24 h. Before using for cell culture, all PDMS substrates were exposed to UV light for 24 h. All plates (TCPs and PDMS) were coated with type I collagen (0.1 mg/mL) for 24 h at 37 °C, and then rinsed with PBS three times. Next, the substrates were soaked in the cell culture medium prior to the addition of cells.
washed with cold PBS, fixed with ice-cold 70% ethanol at −20 °C for at least 24 h, and then stained with propidium iodide at 37 °C for 30 min. The cells were detected by FACS Canto (BD, CA), and cell cycle analysis was performed using FlowJo software version 7.6.1.

4.6. Immunostaining and Imaging. After growing on substrates of different stiffnesses for 48 h, PC12 cells were washed with PBS and fixed with 4% PFA at 25 °C for 30 min, permeabilized with 0.25% Triton X in PBS at 15 min at 4 °C, blocked with 2% BSA for 1 h at 37 °C. Immunofluorescence staining was performed at 4 °C by incubating with the rabbit anti-vinculin polyclonal antibody overnight, washing three times with PBS, and then incubating with goat anti-rabbit Alexa Fluor 488 IgG antibody and rhodamine-phalloidin for 2 h at 37 °C. DAPI was used for counterstaining of nuclei. Finally, the samples were mounted on microscope slides and imaged by fluorescence microscopy (Eclipse Ti-S, Nikon) equipped with a confocal system (UltraVIEW VoX, PerkinElmer).

4.7. Atomic Force Microscopy (AFM). AFM instrument (S500; Keysight) was used to measure the elastic modulus of the PC12 or N2a cells, which were cultured on substrates with different stiffnesses for 48 h. Five sites in the cell nucleus region of each cell were selected for the measurement. The cantilevers (TLC150; Nanosensor) with a force constant was used to record force curves at a frequency of 1 Hz based on our previous method. In the study, Young's modulus of the PC12 or N2a cells is calculated by the Hertz’s model. The spring constant of the cantilever can be determined before each experiment using the thermal tune method.

4.8. RNA Extraction, Library Preparation, RNA Sequencing, and Data Analysis. After culturing on substrates with different stiffnesses, the total RNA obtained from the PC12 cells was isolated using the TRIzol reagent. The purity, concentration, and integrity of the total RNA were determined. Total RNA was treated with DNase I and mRNA with poly(A) tails were purified using oligo (dT) magnetic beads. Then the mRNA was fragmented using a fragmentation buffer. Then target RNA was fragmented and reverse transcribed to ds-cDNA (double-stranded cDNA) using the N6 random primer. Ds-cDNA ends were repaired with the phosphate group at the 5′ end and a sticky “A” at the 3′ end, and then an adapter was ligated to a sticky “T” at the 3′ end. After this, the ligated product was amplified using specific primers. The PCR products were denatured, and the single-stranded DNA was cyclized. Eventually, the cDNA library was sequenced using a BGISEQ-500 Transcriptome platform. Differentially expressed genes (DEGs) between PDMS substrates and TCP substrates were screened out by DESeq2 to have a false discovery rate (FDR) < 0.05 and absolute Log2 (fold change) ≥ 1.5. Gene ontology (GO) term enrichment was analyzed using WEGO software to determine the potential biological functions of the DEGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for DEG pathway enrichment analysis. Significance was accepted after Benjamini–Hochberg correction and cut-offs of FDR ≤ 0.01 were enforced.

4.9. Validation of DEGs Using qRT-PCR. The RNA samples were the same as those used for RNA-Seq. Total RNA (1 μg) was reverse transcribed using the cDNA Synthesis kit. The real-time PCR reaction was performed with the UltraSYBR Mixture and the primers used are as follows: EGFR: 5′-GCCATCTGGGCAAGATACC-3′ (sense) and 3′-GTCTTCGCTGACATAGG-C-3′ (antisense); KRAS: 5′-TGCTTCTCATCATCGTGAAGTTG-3′ (sense) and 3′-CAAGTGAGCTGTGGTCTCA-3′ (antisense); PI3K: 5′-CGAGGAGTGTGCACAGCTGTC-3′ (sense) and 3′-TGTTCCGTTCACAAACAC-3′ (antisense); PKA: 5′-AACGGAGAGTCAGGAAGACAG-3′ (sense) and 3′-TGGACAAAGAGCAGGTTGTT-3′ (antisense); PRKCA: 5′-GTATTCCCCGACCACGACT-3′ (sense) and 5′-GGGCCATGAATTGGTTGCTTT-3′ (antisense); AF6: 5′-AAGCTGGCCGA-CATCTTCA-C-3′ (sense) and 5′-GCTTGCTAGAGACTCGGAATTACA-3′ (antisense); SYT6: 5′-TGACGATGAGACCTGTGGC-3′ (sense) and 5′-CCA-CACACA-ATAACTACCGG-3′ (antisense); GAPDH: 5′-TGAAGCTTCATTCTTGCCTGATGA-C-3′ (sense) and 5′-GCCCTCTCTGTGGTCTCAGTA-3′ (antisense). The amplification procedures were as follows: hot-start activation, 95 °C for 3 min; denaturation, 40 cycles of 95 °C for 10 s; and annealing, 60 °C for 30 s. GAPDH was used as reference controls. The 2−△△Ct method was used to calculate expression changes.

4.10. Western Blotting. The method of Western blotting was the same as our previous reports. Briefly, after culturing on substrates with different stiffnesses, the total proteins of the cell samples were isolated using RIPA lysis buffer. The sample protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit. Equal amounts of proteins from each group were separated by appropriate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto methanol-activated polyvinylidene fluoride (PVDF) membranes. After being blocked with skimmed milk, the PVDF membranes were incubated with the indicated primary antibodies, followed by incubation with the corresponding secondary antibodies. Protein bands were visualized using Bio-Rad ChemiDoc (Hercules, CA).

4.11. Statistical Analysis. All data were expressed as the mean ± standard deviation (SD) of three independent experiments. One-way analysis of variance analysis with Tukey's post hoc test was used to assess the difference in mean values between multiple groups. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). P < 0.05 was considered statistically significant.

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Notes
The authors declare no competing financial interest.

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