Microfluidic platform for probing cancer cells migration property under periodic mechanical confinement

Dongce Ma, Ran Wang, Shuxun Chen, Tao Luo, Yu-Ting Chow, and Dong Sun^{a)}

Department of Mechanical and Biomedical Engineering, City University of Hong Kong, Kowloon Tong, Hong Kong

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Cancer cell migration and invasion, which are involved in tumour metastasis, are hard to predict and control. Numerous studies have demonstrated that physical cues influence cancer cell migration and affect tumour metastasis. In this study, we proposed the use of a microchannel chip equipped with a number of vertical constrictions to produce periodic compression forces on cells passing through narrow channels. The chip with repeated vertical confinement was applied on adherent MHCC-97L liver cancer cells and suspended OCI-AML leukaemia cells to determine the migration ability of these cancer cells. Given the stimulation of the periodic mechanical confinement on-chip, the migration ability of cancer cells was promoted. Moreover, the migration speed increased as the stimulation was enhanced. Both AFM nanoindentation and optical stretching tests on cancer cells were performed to measure their mechanical property. After confinement stimulation, the cancer cells possessed higher deformability and lower stiffness than non-stimulating cells. The confinement stimulation altered the cell cytoskeleton, which governs the migration speed. This phenomenon was determined through gene expression analysis. The proposed on-chip cell migration assays will help characterise the migration property of cancer cells and benefit the development of new therapeutic strategies for metastasis. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5030135>

I. INTRODUCTION

Cell migration is a complex cellular process that plays an important role in many physiological events, including cancer progression.¹ Metastatic cells migrate from a primary tumour, move through the basement membrane, enter the blood and lymphatic vessels, and finally invade various other tissues to form secondary tumours. Understanding cancer cell motility is important to cancer therapy, which necessitates research on the mechanism of tumour cell migration. Despite the remarkable progress of conventional techniques, such as wound healing assay and transwell assay,² cell migration assays continue to face numerous challenges such as large cell consumption, low throughput operation, and inability to directly observe the migration behaviour of individual cells.³

Recent developments in microfluidic technology have allowed the observation of individual cell migration with high spatial and temporal resolution.^{4–8} Several microfluidic devices have been developed to mimic the microenvironment of cell migration.^{3,5} To investigate the migration of cancer cells, many microfluidics-based studies focused on the role of soluble chemoattractant gradients^{9,10} and electrical field in directed cancer cell migration.^{11–14} On the other hand, since cancer cells physically interact with their surrounding environment during metastasis at different stages,¹⁵ an investigation of physical cues, in the form of adhesion gradients¹⁶ or patterned surfaces,¹⁷ is also important to study cell migration property.

^{a)} Author to whom correspondence should be addressed: medsun@cityu.edu.hk

Existing approaches have proven that mechanical confinement can act as a physical cue to modulate intracellular signalling, thereby altering cancer cell migration mechanisms.^{18–20} Establishment of cell migration assays in a physically confined microenvironment, especially with 3D stimulation, is important to the study of cancer progression.^{21–23} Numerous experimental models that confine impose forces on cells in well-defined microenvironments have been developed, such as microfluidic microchannel devices, grooved substrates, and micropatterned ECM lines.²⁴ However, these studies focused on cell migration under constant physical stimulation, such as confinement space, track size, and ligand density, and few study has ever investigated the influence of periodic confinement on cell migration property. In the *in vivo* extracellular microenvironment, cancer cells periodically migrate through confining pores during metastasis,^{25–27} which necessitates the study on how such periodic physical confinement stimulation affects cell migration and invasion.

In this study, we developed a microchannel-based cell migration chip to mimic cancer cells migrating in a microenvironment and then measured the migration stability of the cancer cells. By adding numerous vertical constrictions along the moving path on the chip, the cancer cells, when passing through the chip, received periodic mechanical confinement stimulation, in a similar way to the cancer cells migrating through narrow channels in the *in vivo* environment. Tests were performed on adherent MHCC-97L liver cancer cells and suspended OCI-AML leukaemia cells. The migration ability of single cell was evaluated by analyzing the migration distance of the cells after incubation for 9 days. We found that the cancer cells exhibited enhanced motility after on-chip periodic confinement stimulation. The cells were then isolated and collected for mechanical property characterisation via AFM nanoindentation²⁸ and optical stretching.²⁹ The results indicated that the cells after on-chip confinement stimulation were softer than cells without stimulation. Gene expression was further analysed to gain insight into the cellular and molecular mechanisms of the migrating cells. The results showed up-regulation of Rho GTPase and downstream Rho-associated kinase (ROCK), indicating the vital role of 3D mechanical stimulation during cancer cell metastasis.³⁰ The proposed on-chip cell migration assays will help characterise the migration property of cancer cells, and the results can benefit the development of new therapeutic strategies for metastasis.

II. RESULTS AND DISCUSSIONS

A. Microchannel chip design and cell migration behavior

Two kinds of microchannel chips with 5 and 10 vertical micro-construction structures were developed to mimic a 3D confined microenvironment on the basis of lithography methods. Figure 1(a) shows a schematic of the designed microchannel chip consisting of an array of microchannels, and each channel is equipped with 5-constriction structures. Figure 1(b) shows 36 microchannels on the chip (length of 2000 μm), which is between a cell docking well and a cell collecting well. Both wells have an area of 28.5 mm² for cell growth. Each microchannel has a rectangular cross section with a width of 20 μm and height of 15 μm to allow the cells to pass through. The vertical structure (length of 10 μm and height of 5 μm) reduces the height of the microchannel to 10 μm . The size of the cross-sectional area is 200 μm^2 . Adherent MHCC-97L cancer cells and suspended OCI-AML cells were seeded and cultivated in this designed chip under normal cell culture conditions. The developed chip for the cell migration assays is shown in Fig. 1(c). Figure 1(d) illustrates the spreading behavior of the MHCC-97L cells on the flat surface in the cell docking area and when the cells spontaneously migrated into the microchannels. Figure 1(e) illustrates the spreading behavior of the OCI-AML cells. The cells were compressed when they passed through the chip channels with vertical constriction structures, and such compression is periodic when a number of constriction structures exist. The confined space on the chip can induce cell protrusion and blebbing. Figures 1(f) and 1(g) show 3D reconstructions of the Z-series confocal scanning of cells when passing through the micro-constriction structure.

The migration distance was measured based on cell forward traveling displacement for every 48 h starting from day 1. Figures 2(a)–2(d) (left) show sequential images of persistent

FIG. 1. On-chip cell migration in confined microchannels. (a) Chip design. (b) Microchannels on the chip. (c) An image of the chip inside a 35 mm Petri dish. (d) On-chip migration of adherent MHCC-97L cells. (e) On-chip migration of suspended OCI-AML cells. (f) Confocal images of MHCC-97L cells under the vertical constriction structure. (g) Confocal images of OCI-AML cells under the vertical constriction structure.

cancer cell migration in the confined microchannels for over 9 days. During the first 9 days, most of microchannels have only one cell in each channel, and thus, the cell migration behaviour would not be affected by cell-cell interaction. During this time period, the cells entering the 5-constriction chip experienced confinement stimulation once, while the cells entering the 10-constriction chip were stimulated twice. Cell migration distances of five sample cells are illustrated in Figs. 2(a)–2(d) (right). The cell migration speed and 9 days cell migration distances are shown in Figs. 2(e) and 2(f). It was observed that the suspended OCI-AML cells had a faster migration speed and longer migration distance in the microchannels than the adherent MHCC-97 cells. Both types of cells showed a faster migration speed and longer migration distance in the 10-constriction microchannels than in the 5-constriction chip. Table I summarizes the testing results. Specifically, in day 9, the average migration distance of OCI-AML cells was $911.6 \pm 114.7 \mu\text{m}$ (mean $\pm$ S.D., $N=30$) in the 10-constriction chip, which was greater than $816.5 \pm 103.9 \mu\text{m}$ (mean $\pm$ S.D., $N=30$) in the 5-constriction chip and $229.3 \pm 95.1 \mu\text{m}$ (mean $\pm$ S.D., $N=20$) in the microchannels without constriction. The average migration distance of MHCC-97L cells in the 10-constriction chip was $776.3 \pm 71.2 \mu\text{m}$, which was also greater than $612.5 \pm 109.2 \mu\text{m}$ (mean $\pm$ S.D., $N=30$) of the 5-constriction chip and $230.9 \pm 84.1 \mu\text{m}$ (mean $\pm$ S.D., $N=20$) in the microchannels without confinement. OCI-AML cells cultivated in the microchannels with 10 constrictions had the highest migration speed of $96.5 \pm 14.4 \mu\text{m/day}$ (mean $\pm$ S.D., $N=30$), whereas those in the 5-constriction microchannels

FIG. 2. Persistent migration of cancer cells under mechanical confinement stimulations. (a) Displacement of MHCC-97L cells and five sample migration distances for 9 days in the 5-constriction chip. (b) Displacement of MHCC-97L cells and five sample migration distances for 9 days in the 10-constriction chip. (c) Displacement of OCI-AML cells and five sample migration distances for 9 days in the 5-constriction chip. (d) Displacement of OCI-AML cells and five sample migration distances for 9 days in the 10-constriction chip. (e) Average migration velocities in the microchannel chip without constriction and the microchannel chips with 5- and 10-constrictions. (f) Histogram of cell migration distances for 9 days in constriction chips (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

had an average migration speed of $84.7 \pm 19.7 \mu\text{m}/\text{day}$ (mean $\pm$ S.D., $N = 30$) and $22.7 \pm 13.4 \mu\text{m}/\text{day}$ (mean $\pm$ S.D., $N = 20$) without constriction. MHCC-97L cells that migrated through the 10-constriction microchannels had a speed of $79.8 \pm 13.6 \mu\text{m}/\text{day}$ (mean $\pm$ S.D., $N = 30$) in comparison with $59.6 \pm 15.9 \mu\text{m}/\text{day}$ (mean $\pm$ S.D., $N = 30$) in the 5-constriction chip and $28.1 \pm 14.3 \mu\text{m}/\text{day}$ (mean $\pm$ S.D., $N = 20$) in the microchannels without constriction. The average migration speed was calculated using $v = s/t$, where v is average speed of the cell during 9 days, s is the total cell migration distance measured in day 9, and t is the total migration time. It was also observed that in the 5- and 10- construction chips, both types of cells had a higher migration speed for the first two days and decelerated and accelerated periodically in the remaining days; while the cells migrating in the chip without construction stopped migration

TABLE I. Average migration speed and migration distance for 9 days in no constriction, 5-, and 10-constriction chips.

Cell type	Number of constrictions	Average migration velocity ($\mu\text{m}/\text{day}$)	Average migration distance (μm)
MHCC-97L	No constriction	22.7 ± 13.4	229.3 ± 95.1
	5 constrictions	59.6 ± 15.9	612.5 ± 109.2
	10 constrictions	79.8 ± 13.6	776.3 ± 71.2
OCI-AML	No constriction	28.1 ± 14.3	230.9 ± 84.1
	5 constrictions	84.7 ± 19.7	816.0 ± 103.9
	10 constrictions	96.5 ± 14.4	911.6 ± 114.7

after day 9. We thus hypothesized that the cell accelerated under the vertical confinement in microchannel and decelerated without the stimulation. The cells passing through the 10-constriction chip accelerated around day 5, whereas the cells passing through the 5-constriction chip maintained the decreasing trend of velocity. Given that the cancer cells reached the second chokepoint structure in the 10-constriction chip around day 5, the increased migration speed was possibly due to the further vertical confinement stimulation induced by the structure. The velocity drop of 97L-10 cells was probably due to the lack of vertical confinement stimulation from day 7 to day 9. The similar velocity drop of 97L-5 cells was also observed. The 97L-5 cells kept deceleration from day 3 to day 9, because there was no confinement stimulation until day 9.

The above results indicated that the cancer cells migrate faster and longer through the use of vertical constriction in microchannels to produce periodic mechanical confinement on cells. Repeated stimulations on cells would increase cancer cell migration ability.

B. Cell stiffness

Cell biological behaviour is closely correlated with cell mechanical properties such as stiffness.³¹ As reported in the literature, cell stiffness is regarded as a biomarker of metastatic potential.^{29,32} In this study, we further characterised the mechanical property of the cancer cells after they migrated through the designed microchannel chip. After incubation for about 21 days, the cells in the collecting and docking wells were retrieved and expanded for cell deformability tests.

AFM-based nanoindentation was used to probe the centre of the adherent MHCC-97L cells attached on the surface of a glass cover. The indentation of cells under the loading force of the AFM tip was calculated, and the corresponding force-indentation curve was obtained [Fig. 3(a)]. The curve was fitted using the Sneddon model,³³ based on which Young's modulus of the MHCC-97L cells was calculated. The histogram of the measured Young's modulus from 24 sample indentation curves is illustrated in Fig. 3(b). The average Young's modulus of the cells in the docking well (control group) was 2.01 ± 0.6 kPa (mean $\pm$ S.D., $N = 30$). The average Young's modulus of the cells migrating through the 5- and 10-constriction microchannel chips

FIG. 3. Results of cell stiffness characterisation. (a) Force-indentation relationship of representative MHCC-97L cells by AFM. (b) Young's modulus of non-migrating and migrating MHCC-97L cells. (c) Force-deformation relationship of OCI-AML cells under optical stretching. (d) Young's modulus of non-migrating and migrating OCI-AML cells (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

was 1.70 ± 0.7 and 1.4 ± 0.7 kPa (mean $\pm$ S.D., $N = 30$), respectively. These results indicated that MHCC-97L cells were softer than the non-migrating cells after migrating through the microchannels with vertical constrictions, and the cells passing through the 10-constriction channel were softer than those passing through the 5-constriction channel.

The optical stretching method was further employed to measure the deformability of the suspended OCI-AML cells. Each cell was stretched by two optical traps, with a stretching force ranging from 7 pN to 43 pN. Figure 3(c) shows the cell deformation results under different stretching forces. The Young's modulus was calculated based on the stretching force that induces the largest deformation of cells, as shown in Fig. 3(d). OCI-AML cells collected from the cell docking well (control group) possessed an average stiffness of 396.9 ± 74.5 Pa (mean $\pm$ S.D., $N = 30$). OCI-AML cells migrating through the 5- and 10-constriction microchannel chips were soft, which had an average stiffness of 187.5 ± 56.7 and 170.5 ± 31.9 Pa (mean $\pm$ S.D., $N = 30$), respectively. Furthermore, cells migrating through the 10-constriction microchannel were softer than those migrating through the 5-constriction microchannels.

Both AFM nanoindentation and optical stretching tests revealed that the cancer cells possessed decreased stiffness after migrating through the confined microchannels. Notably, cancer cells migrating through the 10-constriction microchannel chip were softer than those migrating through the 5-constriction chip. This result suggested that the enhancement of periodic mechanical confinement stimulations on cells could affect the cell stiffness. The reduced cell stiffness may further facilitate cancer cells to change cell shape and move through the much confined space.³⁴ Some soft cancer cells have extremely high tumorigenic ability.^{35,36} Our findings indicated that periodic mechanical stimulation may contribute to tumour progression.

C. Gene expression analysis

To further explore the cellular and molecular mechanism of the migration cells passing through the confined microchannels with vertical constrictions, expression levels of cytoskeleton regulation and cell migration related genes were measured. The expression level of two membrane proteins, encoded by F2r and CD11a gene,^{37,38} and the expression level of small GTPase protein and kinase, encoded by RhoA and ROCK gene, were measured. RhoA GTPase is primarily associated with cytoskeleton regulation and functions as a central regulator of contractility. ROCK is a key regulator of the actin cytoskeleton acting downstream of the RhoA GTPase, which is also associated with cancer progression.^{30,39}

Figure 4 illustrates the results of mRNA expression level evaluated by quantitative RT-PCR, indicating that RhoA and ROCK expressions in the migrating cells significantly upregulated with the increased expression of F2r and CD11a in both MHCC-97L cells and OCI-AML cells. Both RhoA and ROCK upregulation levels of cells migrating through the 10-constriction chip were higher than that of cells migrating through the 5-constriction chip and much higher than that of non-migrating cells. The high expression level of RhoA indicated that the adherent MHCC-97L cells might adopt a pressure-based lobopodial migration model, which was dependent on RhoA-ROCK-myosin II-mediated contractility.⁴⁰ For the non-adherent OCI-AML cells, the high expression level of RhoA indicated that the leukemia cell might migrate using high-contractility amoeboid migration driven by membrane blebbing.⁴¹ These results confirmed that periodic mechanical confinement stimulation can greatly affect the RhoA/ROCK pathway for both suspended and adherent cells. Our studies on carcinoma and leukemia cells revealed that metastatic cells expressed higher levels of ROCK compared to non-metastatic cells, which is in accordance with the reports on the role of Rho/ROCK in cellular invasion of 3D culture.^{42,43}

III. MATERIALS AND METHODS

A. Cell culture

Human acute myeloid leukaemia cell line (OCI-AML) was cultured in MEM Alpha (Gibco) with 10% (v/v) foetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (PenStrep, Gibco) in a 35 mm culture dish (Falcon, Corning). Human hepatocellular carcinoma

FIG. 4. Gene expression test results. (a) Related migration and invasion pathways. (b) Gene expression of MHCC-97L cells and MHCC-97L cells migrating through the 5- and 10-constriction microchannel chips. (c) Gene expression of OCI-AML cells and OCI-AML cells migrating through the 5- and 10-constriction microchannel chips (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

cell line (MHCC-97L) was cultured in Dulbecco's Modified Eagle Medium (Gibco) with 10% (v/v) FBS and 1% PenStrep in a 35 mm culture dish. All cells were maintained in a humidified atmosphere of 5% CO_2 /95% air at 37 °C provided by the direct heat CO_2 incubator (Forma Steri-Cycle i160, Thermo Scientific). The diameters of OCI-AML cell and MHCC-97L cell are about $13.5 \pm 0.65 \mu\text{m}$ and $13.97 \pm 0.73 \mu\text{m}$ (mean $\pm$ S.D., $N = 30$) in suspension condition, respectively. The adherent MHCC-97L cells were sub-cultured using 0.25% trypsin-EDTA (Gibco) as suggested by the manufacturer.

B. Chip fabrication

The microfluidic chip was manufactured by casting polydimethylsiloxane (PDMS, Sylgard 184) on a microstructure mould. The microstructure mould was fabricated using two-step photolithographic technology. Before fabrication, two photomasks with microchannels and micro-constrictions were designed and printed on a transparency with a high-resolution printer. For mould fabrication, a silicon wafer was coated with a 5 μm -thin layer of SU-8 photoresist (GM 1050, Gersteltec Sarl). Micro-constriction structures were patterned following the standard protocol recommended by the manufacturer with the first layer of photomask. The second layer of SU-8 photoresist (GM1070, Gersteltec Sarl), with thickness of 15 μm , was coated on the same wafer and aligned to the first layer to define the microchannels, loading area, and collection area and achieve peripheral connections. Precise alignment was achieved using a mask aligner (model200, OAI). The mould was placed in a Petri dish and covered with freshly prepared PDMS (1:10 w/w mixing with the curing agent). After degassing and thermally curing at 70 °C, the cast PDMS was peeled off from the SU-8/silicon mould and trimmed to a proper size, followed by punching two holes at the cell docking area and collecting area. The trimmed PDMS was cleaned and bonded to a cover glass surface after exposure for 25 s to oxygen plasma treatment (PDC-002, Harrick Plasma). After plasma bonding, chips were baked for 5 min at 75 °C.

C. On-chip cell migration assays

Prior to the experiments, the microchannel chip was sterilised with 75% ethanol and UV irradiation. The chip was rinsed with PBS and filled with cell culture medium. After negative

pressure degassing, the chip was checked under a microscope to ensure that all bubbles inside the channel were removed. A single cell suspension was achieved by treating adherent cells with 0.25% trypsin-EDTA or by direct pipetting of suspended cells. The single cell solution was centrifuged at $300\times g$ for 3 min and resuspended in culture medium at a concentration of 5×10^4 cells/ml. About 150 μ l of cell solution was transferred into the cell docking well, and 150 μ l of culture medium was added into the cell retrieving well. The entire chip was placed into a cell culture incubator, and the culture medium was added every 48 h. During incubation, single cells spontaneously migrated into the microchannels and moved towards the cell collecting well. Migration distance was measured based on the cell traveling distance after incubation for 9 days, where only the first cell that entered the channel was counted. The velocity of cells was measured by imaging the cells' positions every 24 h. After 31 days of incubation, cells in the collecting well were collected and expanded for further tests.

D. Cell stiffness measurement

Stiffness of the suspended OCI-AML3 cells was measured by optically stretching the cells using an optical tweezers system (BioRys 200, Arryx).²⁹ Microbeads were attached to the cell membrane through streptavidin–biotin binding, which functioned as handles for optical stretching. Bead displacements under different stretching forces were measured to calculate cell stiffness. For adherent MHCC-97L cells, cell stiffness was measured by AFM-based nanoindentation (Bioscope, Bruker, USA). The AFM tip was employed as a nanoindenter to deform the cell to a specific depth. The collected force-indentation curve was fitted using the Sneddon model, which was used to calculate Young's modulus with the published protocols.²⁸

E. RNA isolation and real-time RT-PCR analysis

The relative quantity of mRNA expression was tested by real-time RT-PCR. Total RNA of cells was extracted using Trizol reagent (Invitrogen). The mRNA was reverse transcribed to cDNA using iScript™ cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR amplification was performed with SsoAdvanced SYBR Green Supermix kit (Bio-Rad) in CFX96 Real-time System (Bio-Rad, USA). The specific primers used in the analyses are listed in Table II. The primer sequences were obtained from the reference or primer bank. In the sample analysis, the threshold was set based on the exponential phase of amplified products, and the cycle threshold (CT) for each target gene was determined. The expression of the target gene was normalized to that of the 18s housekeeping gene.

F. Imaging measurement

Cell migration inside the chip was imaged on a Zeiss Axio Vert A1 microscope equipped with a CMOS camera (AxioCam 105 colour). ZEN software and Image J software were used to measure the migration distance. 3D scanning was performed on a Leica TCS SP8 confocal laser scanning platform with $40\times$ water immersion objective (NA=1.1).

TABLE II. Forward and reverse primer sequences for qRT-PCR.

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
F2r	GCCCGCAGGCCAGAATCAAA	TCCTCATCTCCCAAAATGGTTCA
ROCK	ACCTGTAACCCAAGGAGATGTG	CACAATTGGCAGGAAAGTGG
CD11a	TGAGAGCAGGCTATTTGGGTAC	CGGCCCATGTGCTGGTAT
RhoA	GCAGGTAGAGTTGGCTTTATGG	CTTGTGTGCTCATCATT CCGA
18s	TGCATGGCCGTTCTTAGTTG	AGTTAGCATGCCAGAGTCTCGTT

G. Statistical analysis

All data were reported as the mean $\pm$ standard deviation (S.D.) from at least three independent experiments. The data of AFM forces and stiffness were processed by NanoScope Analysis. Student's t-test was applied to compare two distinct groups. Statistical significance was set at $P < 0.05$. Statistical analysis was carried out with Microsoft Excel software.

IV. CONCLUSIONS

This paper presents the use of novel on-chip cell migration assays to sense cancer cell migration under periodic mechanical confinement stimulation. The chip is equipped with an array of vertical constrictions, which can produce periodic compression force on cells when cells pass through microchannels on the chip. Cell migration experiments were performed on adherent MHCC-97L cells and suspended OCI-AML cells. The experimental results revealed that the migration ability of cancer cells was promoted via periodic mechanical constriction on cancer cells. The cancer cells became soft, which indicated enhancement in cancer progression. The increased number of vertical constriction installed in the microchannel chip could directly increase the migration ability, which might also cause the decrease in cell stiffness. The cell cytoskeleton was reorganised under such mechanical confinement stimulation, which could promote persistent migration of cancer cells. Furthermore, the confined constrictions may act as a mechanical cue to upregulate expression of RhoA and ROCK genes thus activate the RhoA/ROCK signalling pathway, which may increase the cancer cell invasion ability. Successful development of on-chip cell migration assays in this study will benefit the establishment of new experimental tools to characterise the metastasis mechanism and cancer cell invasion, thereby contributing to the development of new therapeutic strategies for cancers.

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