Original Research

Research of restricted migration evaluation of MDA-MB-231 cells in 2D and 3D co-culture models

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Impact Statement

In this study, we systematically analyzed the impact of different restricted degrees and different restricted environments on the evaluation of cancer cell migration. The results indicated that the restricted environments helped to distinguish the regulation of weak influencing factors on tumor migration behavior by inhibiting the migration instinct of cancer cells. In addition, the drug testing in the extracellular matrix (ECM) environment can more accurately reflect the cytotoxicity and drug resistance of drugs, compared with the 2D model. These results will advance the understanding of the limited migration behavior of cancer cells in this field and help researchers choose appropriate cancer models to study complex tumor metastasis behavior according to research needs. At the same time, we also observed pseudopodial connections among cancer cells in 3D ECM, which will lay the foundation for more complex competition-cooperation migration behavior research.

Abstract

The restricted migration evaluation is conducive to more complex tumor migration research because of the conformity with *in vivo* tumors. However, the differences between restricted and unrestricted cell migration and the distinction between different evaluation methods have not been systematically studied, hindering related research. In this study, by constructing the restricted environments on chips, the influence of co-culture conditions on the cancer cell migration capacity was studied. The results showed that the restricted channels can discriminate the influence of weak tumor environmental factors on complex tumor migration behaviors by limiting the free growth instinct of tumor cells. Through the comparison of 2D and 3D restricted migration methods, the extracellular matrix (ECM) restriction was also helpful in distinguishing the influence of the weak tumor environmental factor. However, the 3D ECM can better reflect the tortuosity of the cell migration process and the cooperative behavior among cancer cells. In the anticancer drug evaluation, 3D ECM can more accurately reflect the cytotoxicity of drugs and is more consistent with the drug resistance in the human body. In conclusion, the research will help to distinguish different evaluation methods of cancer cell migration, help researchers select appropriate evaluation models, and promote the research of tumor metastasis.

Keywords: Cancer model, cell migration, migration evaluation, microfluidic chip

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Introduction

The invasion of cancer cells into normal tissues is a key step in tumor metastasis, involving the destruction of basement membranes and the migration of cancer cells.[1,2] At this stage, the tumor cells of the original focus are gradually transformed into cancer cells with a highly invasive phenotype.[3] Their significantly enhanced migration ability accelerates the process of tumor metastasis, which seriously hinders the effective and high survival treatment of cancer.[4,5] Therefore, the research on the tumor invasion mechanism and screening anti-migration drugs through the evaluation of tumor cell migration behavior is very important.

Different from other cell biology evaluation methods, migration evaluation usually depends on the tracking observation of cell migration behavior.[6] As a typical *in vivo* model,[7] Zebrafish models are used in tumor metastasis research because of their transparency for easy observation.[8] However, it has significant limitations in flexibly controlling tumor environmental factors and could not accurately track the migration process of tumor cells at the cell scale.[9] Therefore, *in vitro* models have become indispensable methods in tumor metastasis evaluation and behavior mechanism research because of the high accuracy observation of migration behavior and the flexible regulation of tumor environmental factors.[10,11]

Various *in vitro* cell migration evaluation models have been proposed, such as wound healing models,[12,13] Transwell chamber,[14,15] and microfluidic chips.[16,17] However, in the tumor invasion process, cancer cells constantly break through the physical constraints brought by the matrix in a restricted migration environment.[18] Some 2D migration evaluation methods, such as the wound healing model[12,13] and the single cell tracking method[19] which allow cancer cells to move in an unrestricted 2D space, are only suitable for comparing the migration ability among different types of tumor cells and cannot accurately evaluate the impact of other tumor environmental factors. It is very necessary to build a restricted environment to simulate the physical restriction of the surrounding matrix on cancer cells, and previous studies have constructed 2D[20] and 3D[21] restricted environments to evaluate the impact of restricted environments on the migration behavior of cancer cells. However, there is a significant restriction difference between 2D restricted migration channels and 3D migration matrix on cancer cells. The former is rigid and the latter is flexible. Therefore, it is of great significance to clarify the differences in migration evaluation results and evaluation application occasions caused by different restricted migration environments.

In previous studies, we constructed 2D restricted migration channels[22] and 3D migration matrices[23] based on microfluidic chip technology to clarify the role of co-culture environments in the cancer cell migration ability. To further distinguish the impact of different restricted migration methods on the evaluation results, we systematically compared and analyzed the differences between restricted migration and unrestricted migration, 2D restricted migration channels, and 3D migration matrix in the study of tumor migration behavior. The relevant results will further clarify the advantages and disadvantages of relevant migration evaluation methods, facilitate researchers to select appropriate experimental models according to the actual needs, and promote the mechanism research and anticancer drug screening on tumor metastasis.

Materials and methods

Construction of migration models and selection of microfluidic chips

Before tumor cells get onto the blood vessel, cancer cells need to constantly make a breakthrough of the constraints of surrounding environments and migrate in ECM pore space (as shown in Figure 1(a)). To study the influence of 2D restricted migration channel and ECM migration environment (Figure 1(b)) on the evaluation results of cancer cell migration behavior, 2D and 3D co-culture migration environments were constructed based on the microfluidic chips that have been designed in the existing studies.[22,23] The migration ability changes with and without co-culture were the basis for evaluating different migration evaluation methods.

The 2D co-culture chip was mainly composed of a polydimethylsiloxane (PDMS) structure and a 200-μm-thick glass sheet, forming two cell culture regions for 2D co-culture. The two cell culture regions were connected by 27 restricted migration channels, and the wide, the long, and the height of each channel were 60, 300, and 10/15 μm respectively. Other design parameters of the 2D co-culture chip were detailed in the previous report.[22] The 3D co-culture microfluidic device was also made of a PDMS layer and a 200-μm-thick glass sheet, forming four microchannels for 3D migration environment construction and nutrition supply. The two channels in the middle were used to load cells containing collagen solution to form 3D gel structures (Figure 1(c)), and their sizes were both 120 μm high, 440 μm wide, and 3.2 mm long. Other design parameters of the 3D co-culture chip were detailed in the previous report.[23] All the PDMS structures were manufactured using standard soft-lithography methods.[24,25]

Cell culture and fluorescent protein transfection

The MDA-MB-231 (ATCC) cells were maintained in the L15 medium. HMEpiC (ScienCell) cells were used as normal cells for the 2D co-culture model and were cultured in a mammary epithelial cell medium (MECM, ScienCell). MCF-10A (ScienCell) cells used as normal cells for the 3D co-culture model were cultured in DMEM/F-12 medium (HyClone). Human Umbilical Vein Endothelial Cells (HUVECs) (ScienCell) used for the 3D co-culture model were cultured in Roswell Park Memorial Institute 1640 medium (HyClone). All types of the cells were cultured in an incubator at 37°C. To trace the cancer cells, the Hanbio lentiviral green fluorescence protein (lentiviral production, Hanbio™) was used to transfect MDA-MB-231 cells with GFP based on the supplier's principle. Fluorescent-labeled cancer cells were named MDA-MB-231-GFP cells.

Realization of 2D and 3D co-culture models

Before realizing the cancer models, the chips were placed into a sterilizing pan to sterilize at 120°C for 20 min and then the chips were dried by airing in a sterile biosafety cabinet. For the 2D co-culture model, the HMEpiC cells and the MDA-MB-231 cells were loaded into the normal cell region and the cancer cell region, respectively; the cell concentrations of both types of cells were 2×10^6 cells mL^{-1}. Because of the surface tension phenomenon at the interface between the restricted migration channels and the cell regions, the cell suspensions could not get into restricted migration channels. After cell adhesion for 6 h, the fresh medium was loaded into the chips for co-culture.

For the 3D co-culture model, Rat Tail Collagen I (Corning®) was used to construct the 3D ECM. The cell densities of MDA-MB-231 cells and MCF-10A cells in hydrogel cell solutions were both 4×10^6 cells mL^{-1}. The two cell-containing hydrogel solutions were loaded into the corresponding channels (Figure S1), and the gel structures were formed at 37°C for 40 min. Then the fresh medium was loaded into a culture medium channel beside the normal cell gel and the HUVECs cell suspension was loaded into another culture medium channel beside the cancer cell gel, and the density was 3×10^6 cells mL^{-1}, and then the chip was put into the cell incubator for 6 h. Finally, all the medium channels were loaded with fresh medium for co-culture and replaced fresh medium every 12 h.

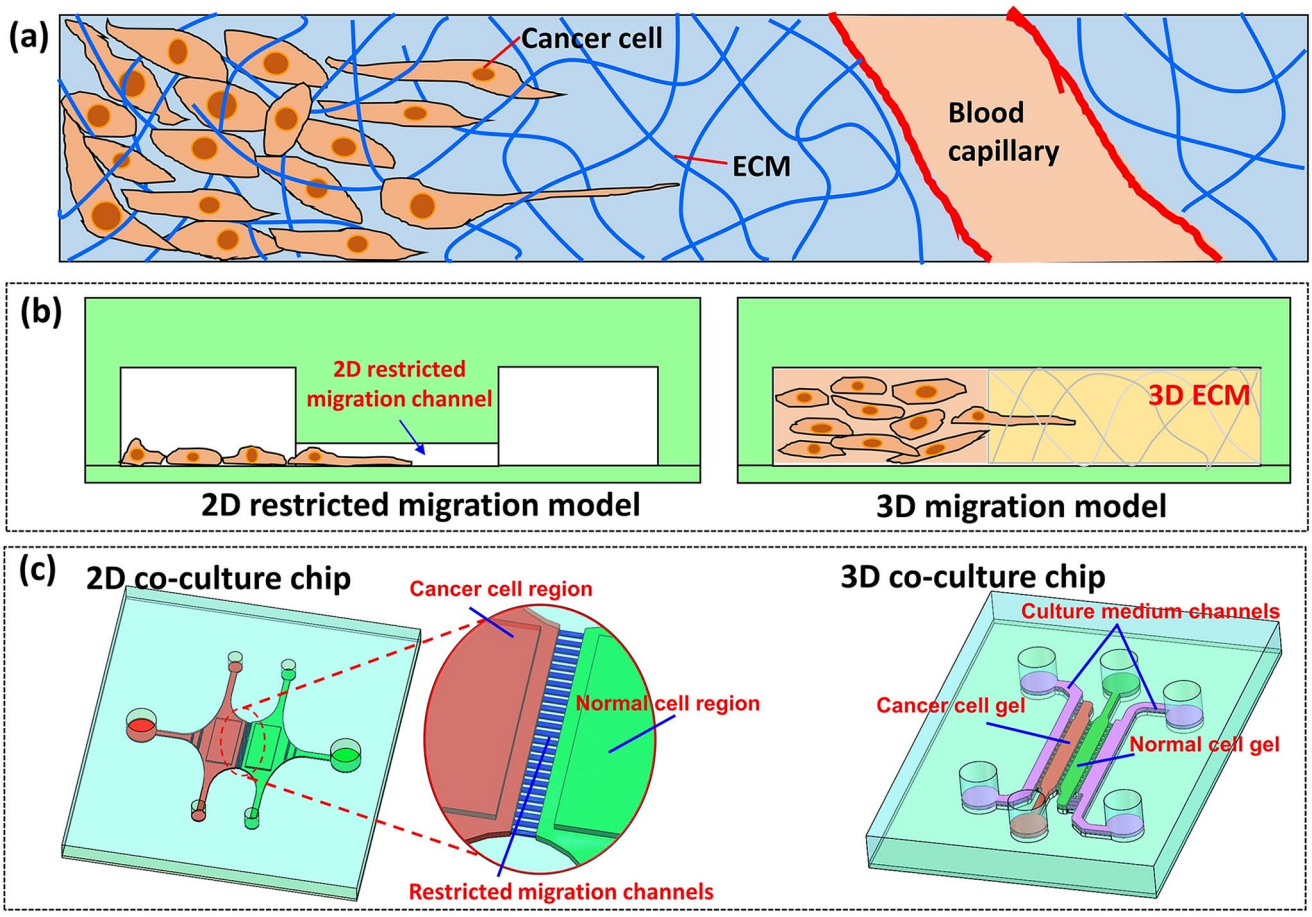

Figure 1. 2D and 3D co-culture models for cancer cell restricted migration evaluation. (a) Schematic diagram of cancer cells invading the surrounding matrix. (b) Schematic diagrams of 2D restricted migration channel and 3D ECM migration environment. (c) 2D and 3D co-culture chips for cancer cell migration evaluation.

Cytotoxicity test of paclitaxel

For the efficacy evaluation of anticancer drugs, Paclitaxel (Sigma Aldrich) was chosen to deal with the cancer models. After co-culturing the cancer models for one day, different concentrations of paclitaxel were acted on the cells. After one day of drug treatment, we analyzed the cell viabilities of normal cells and cancer cells using the Live/Dead Cell Imaging Kit (Life Technologies Corporation), according to the principle of the supplier.

Data processing

Fluorescent images were acquired through the fluorescence microscope. Image-pro software was used to obtain migration data. We used SPSS V19.0 software to process data. The relevant statistical data showed mean ± standard deviation. We used Origin software to draw curves. The asterisks (*) meant the statistically significant P values that were below 0.05.

Results

Evaluation of the restricted migration behavior in the 2D co-culture model

To clarify the impact of the restricted migration environment on the evaluation of the cancer cell migration behavior, we designed 2D co-culture chips (as shown in Figure 1(c)) that contained the restricted migration channels with different heights (10 and 15 μm). Different heights of the restricted migration channels were assumed to be different degrees of restriction on cancer cells. Because the co-culture realization depended on the surface tension formed by the fluid at the interface between the migration channels and the cell regions,[22] the surface tension formed by migration channels higher than 20 μm too weak to form a stable co-culture environment. Therefore, we chose only the 15 μm high migration channels for the comparative analysis. Figure 2 shows the migration images of the restricted migration channels with different heights, and it was very intuitive that the tumor cell migration ability was stronger in the 15 μm high channels than in the 10 μm high channels. This indicated that the different restrictions caused by different channel heights indeed had a very significant impact on the migration evaluation of cancer cells.

Based on fluorescence images, we counted the total migration distance and the number of migrated cells in the restricted channels and divided the total migration distance by the number of migrated cells to evaluate the average migration ability. As shown in Figure 3, relevant statistical results showed that in 15 μm high migration channels, cancer cells had higher migration ability compared with 10 μm high migration channels. Further statistical analysis also

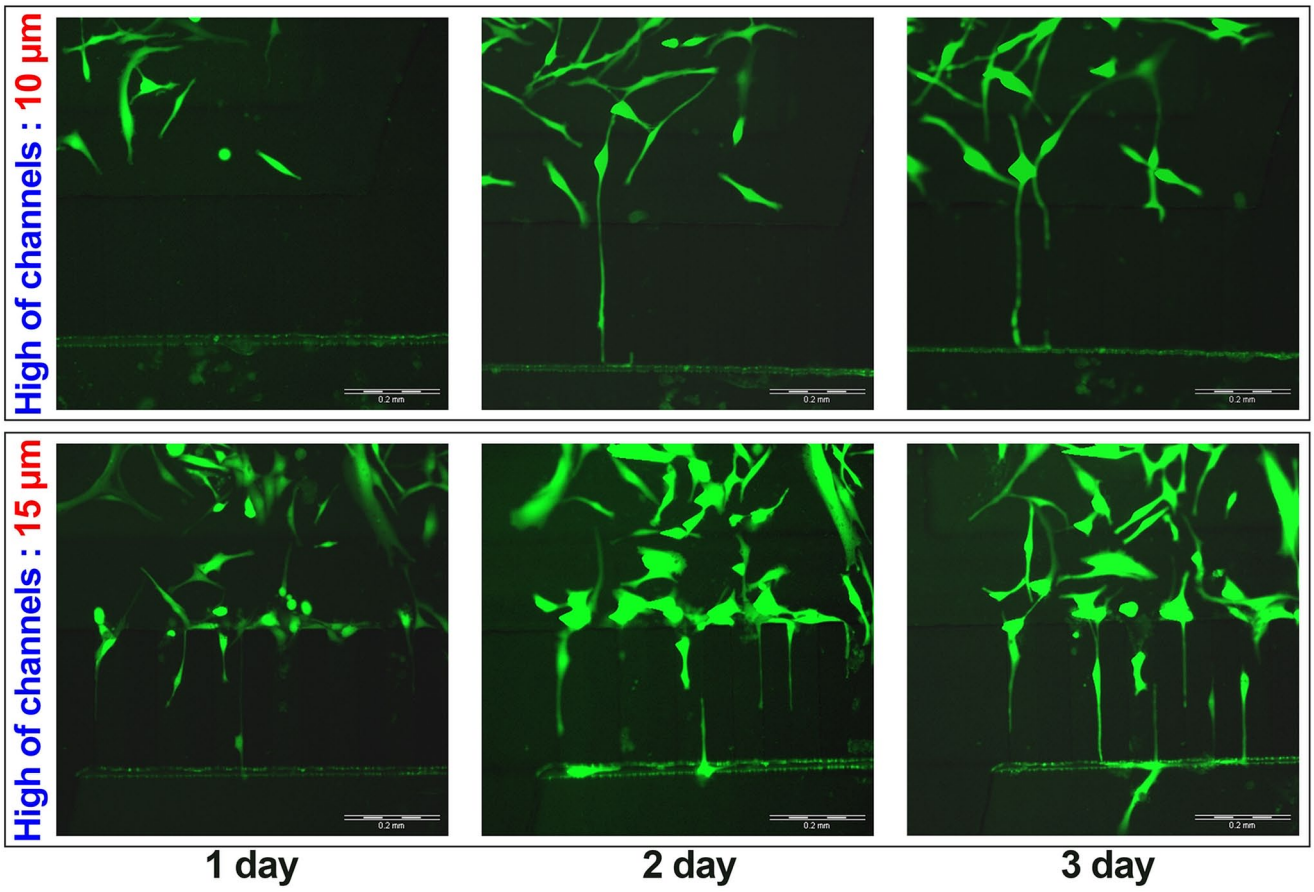

Figure 2. Migrating images of 10 μm high migration channels and 15 μm high migration channels at different days, MDA-MB-231-GFP cells were in green; scale bar: 200 μm.

shows that the evaluation results of cell migration ability have significant differences in migration channels with different heights (Figure S2). Meanwhile, compared with the chip with 10 μm high channels, the significant increase of total migration distance in the chip with 15 μm high channels was not only interrelated to the number increase of migrated cancer cells but also positively related to their average migration capacity. This fully indicated that the structure size of restricted migration channels has a great impact on migration evaluation results, and the smaller the channel size was, the more obvious the restriction on cancer cell migration was.

Next, we compared the cancer cell migration ability in chips with and without co-culture. As shown in Figure 3(a) to (c), the total migration distance after co-culture showed significant differences ($^*P < 0.05$) at day 2 and day 3, compared with monoculture. In Figure 3(c), the results also demonstrated that cell co-culture helped improve the migration ability of cancer cells. In the previous research,[22] we proved that the high expression of interleukin (IL)-6 after culturing with normal cells helped to the improvement of cancer cell migration capacity. However, in the chip with 15 μm high migration channels, the results showed no statistical difference with and without co-culture (Figure 3(d) to (f)). This indicated that MDA-MB-231 cells could show very strong migration ability in unrestricted or less restricted migration environments so it was very hard to distinguish the influence of weak factors such as the co-culture condition on the tumor cell migration capacity. When the height of the migration channels was 5 μm, it was difficult for cancer cells to invade the channels (Figure S3).

Comparison of restricted migration evaluation between 3D and 2D environments

To evaluate the differences between 2D and 3D restricted migration evaluation methods, we carried out systematic experiment analysis and comparisons. Compared with Figures 2 and 4(a), it can be seen that the migrated cells in 2D environment and 3D ECM were conformed with the morphological characteristics of the mesenchymal cell migration, and the formation of cell pseudopods was observed. From the perspective of migration evaluation results (Figure 4(c) to (e)), in 3D ECM, the difference in co-culture conditions was also reflected in the migration evaluation results. Therefore, in terms of simulating the migration process of tumor cells and evaluating the influence of other environmental factors on cancer cell migration, the 2D restricted migration channels and the 3D ECM environment showed consistent results.

However, there were obvious differences in migration assessment between the 2D restricted channels and the 3D ECM. In the 3D ECM, the migration directions of cancer cells

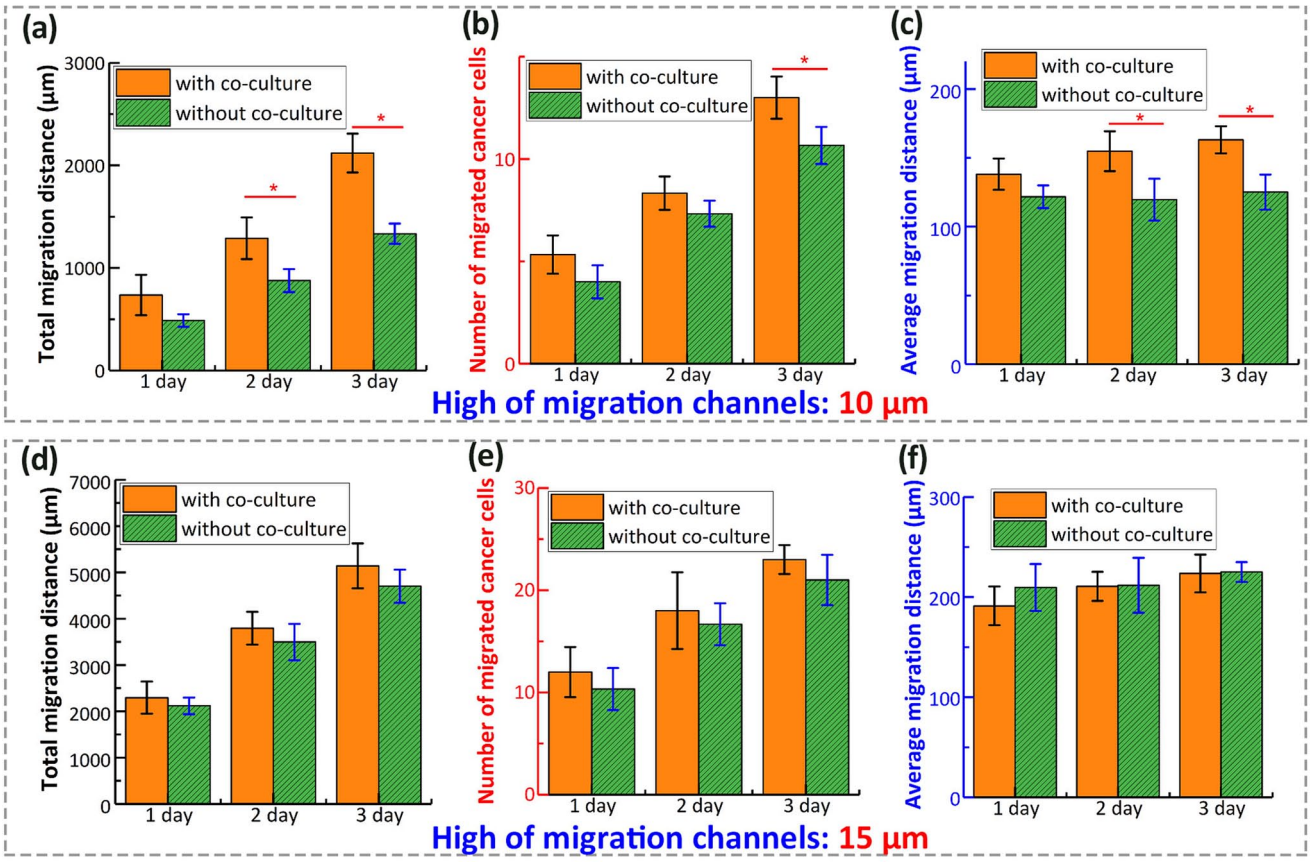

Figure 3. Evaluation of cancer cell migration ability in the 2D chip. (a) to (c) Evaluation of cell migration ability with and without co-culture in the chip with 10 μm high migration channels. (d) to (f) Evaluation of cell migration ability in the chip with 15 μm high migration channels.
Source: Data cited from Mi *et al.*[22]
*$P < 0.05$.

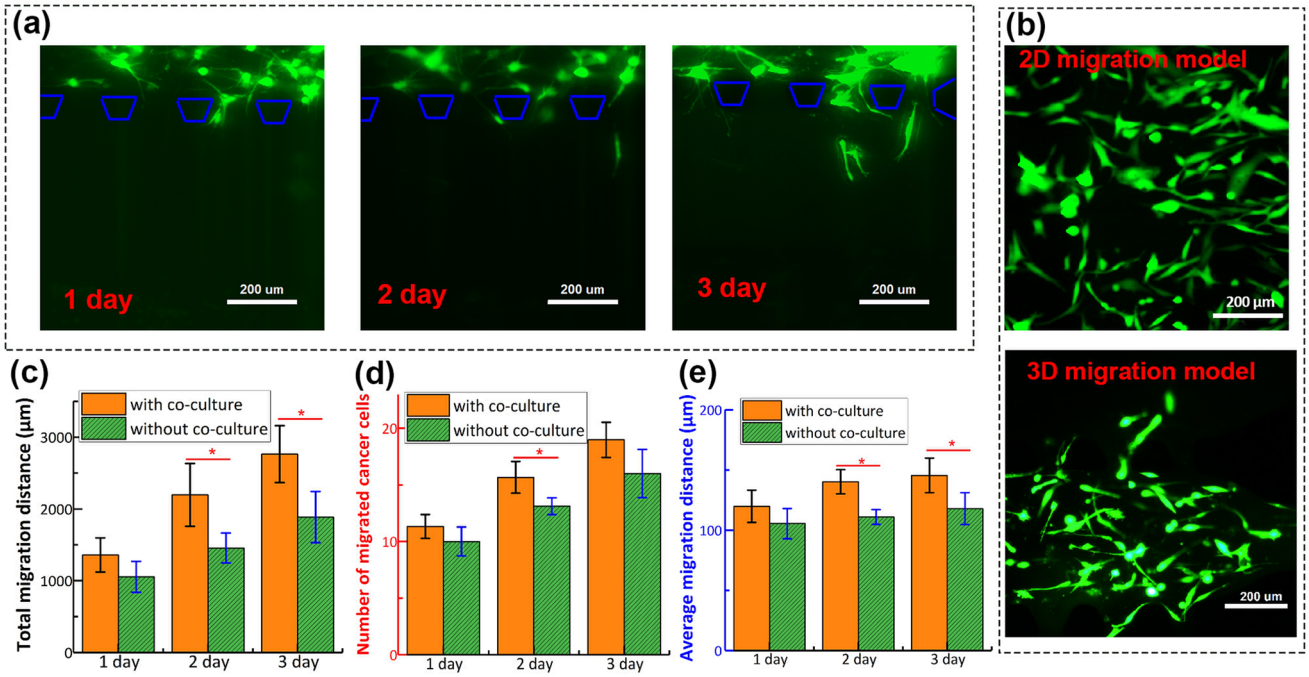

Figure 4. Evaluation of cancer cell migration ability in the 3D chip. (a) The migration images of the 3D co-culture model, MDA-MB-231-GFP cells were in green and the scale bars were 200 μm. (b) Morphological characteristics of cancer cells in the 2D and 3D models; scale bar: 200 μm. (c) to (e) Migration evaluation of cancer cells in the 3D cancer model.
*$P < 0.05$.

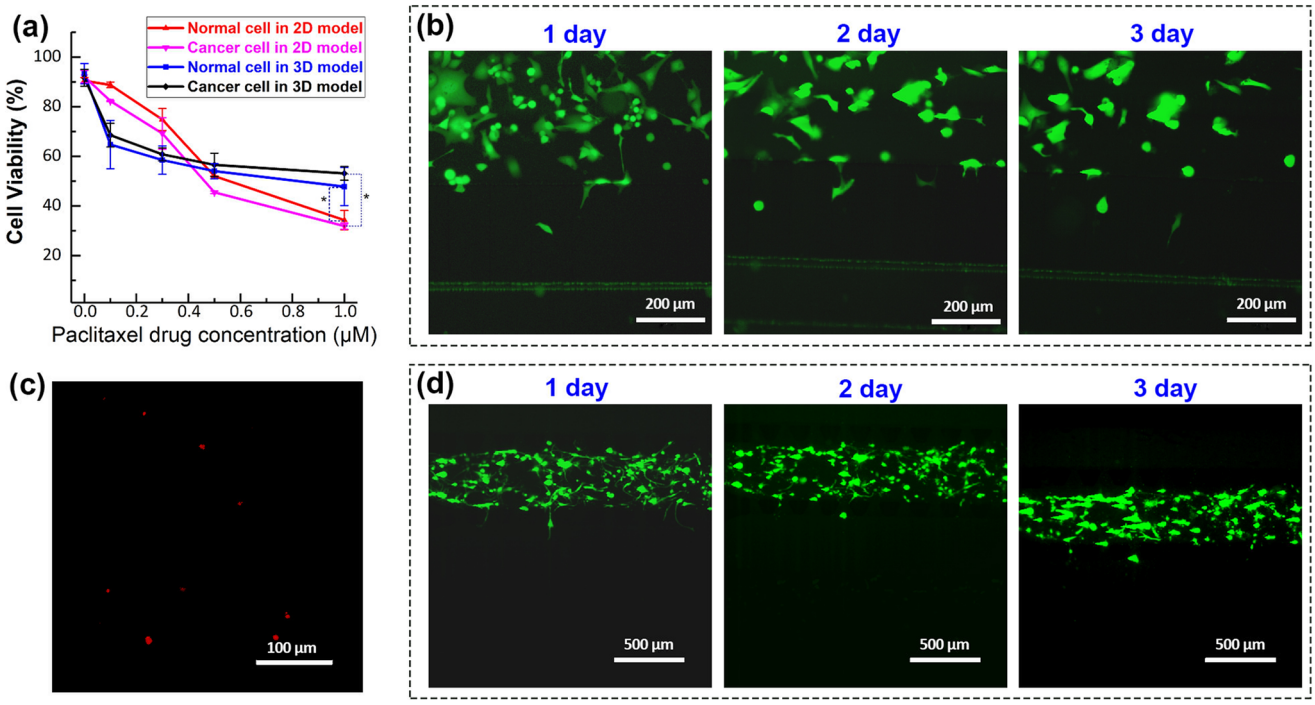

Figure 5. Evaluation of the anticancer drug in the 2D and 3D chips. (a) The cell viability of different cells in 2D and 3D co-culture models after one day of treatment with the drug. (b) Migrating images of MDA-MB-231-GFP cells in the 2D model, we started treating with 0.3 μM paclitaxel after co-culture for 24 h; scale bar: 200 μm. (c) Fluorescence image of dead cells (in red) that were collected from the experimental waste liquid of the 2D chip; scale bar: 100 μm. (d) Migrating images of cancer cells in the 3D model, we started treating with 0.3 μM paclitaxel after co-culture for one day; scale bar: 500 μm.
Source: Data cited from Mi *et al.*[22] and Du *et al.*[23]
*$P < 0.05$.

showed variability (Figure S4), while the migration directions in 2D channels were completely fixed. By tracing the 3D migration process of migrated cells, we found that several cells (the cells in red, blue, and yellow dashed ellipses, Figure S4) established some connections through pseudopods. This indicated that in the invasion process, the behavior of cancer cells was not only the competition for nutrition and living space but also some cooperation.

In addition, cancer cells migrated on the rigid matrix in the 2D model, while on the flexible matrix in the 3D model. However, they all showed consistent cell morphology (Figure 4(b)). The construction method of cell-contained ECM was to mix cells with the material first and then conduct the material cross-linking. This caused the cells to be completely wrapped by gel after cross-linking, and the cells needed to constantly break through the wrapping of ECM, to extend their morphology and to migrate. The most direct proof was that we mixed cancer cells with sodium alginate solution (2 wt%) and cross-linked the mixture with calcium chloride (3 wt%). We found that after culturing for two days, the morphology of cancer cells had not expanded and the cell migration was also not observed (as shown in Figure S5), which meant that the cells were completely wrapped by the gel and could not break through the binding of high cross-linking strength materials. Meanwhile, it also indicated that the pore size, concentration, and cross-linking strength of gel materials will greatly affect the evaluation results of the cancer cell migration in the 3D ECM.

Evaluation of the anticancer drug in different co-culture models

To find out the distinctions between the 2D and 3D models in the anticancer drug evaluation, we cited the published data[22,23] for further analysis. Figure 5(a) shows the curve of the cell viability of different cells with the increase in paclitaxel concentration. When the concentration of paclitaxel overtook 0.5 μM, the cell viability in 3D ECM was higher than that in the 2D model. It was generally believed that the tolerance of cancer cells in the ECM was stronger than that in the 2D model, and the ECM environment could be more consistent with the drug metabolism pattern *in vivo*.[26,27] In our previous study,[28] the cell viability in the ECM was also higher than that in the petri dish. But when the concentration of paclitaxel was below 0.5 μM, the curve in Figure 5(a) did not conform to this tolerance law.

After analysis, we thought in the 2D model, dead cells were easy to detach off the substrate and then were taken out during the washing process, which led to falsely high cell viability. To verify the conjecture, we collected the liquid in 2D model during the experiment and observed dead cells under the microscope (Figure 5(c)) after centrifuging and re-suspending procedures. In the 3D ECM, dead cells were not detected in the washing process due to the encapsulation of the gel, which can more accurately reflect the cytotoxicity of anticancer drugs. By observing the migration of cancer cells, the results (Figure 5(b) and (d)) showed that the cancer

cell pseudopods disappeared after the drug treatment. The changes in cell morphological characteristics of cancer cells were positively correlated with the changes in migration ability; this was consistent with the previous results.

Discussion

In previous studies,[29,30] it was also found that the height of restricted migration channels affects the migration ability of cancer cells, but it was only for studying the cancer cells themselves. For analyzing other tumor environmental factors (such as normal cells) on tumor cell migration, the influence of the restriction degree on evaluation results has not been clarified. However, the cancer cells *in vivo* migrated in a restricted environment.[31] It was beneficial to limit the migration capacity of cancer cells themselves by reducing the height of migration channels for the influence evaluations of complex tumor environmental factors (especially weak factors) on migration ability. Our results fully demonstrated that the limited migration of 2D or 3D environments was beneficial for clarifying the effects of other environmental factors on the migration behavior of cancer cells. Moreover, our models can flexibly adjust the restricted degree of cancer cells, such as adjusting the height of 2D restricted migration channels, which can more flexibly meet the needs of migration evaluation.

About the reason that cancer cells were difficult to migrate in below 5 μm high migration channels, we thought this was related to the more difficult deformation of the cell nucleus because the cell nucleus is the hardest organelle in a cell.[32] Despite the migration ability of cancer cells in channels below 5 μm being decreased significantly, cell migration could still occur, because the cell nuclear damage caused by the extreme cell deformation promoted the heterochromatin formation and then facilitated the cell nuclear transfer, thus allowing the cell migration.[33]

Based on the 2D and 3D models, the key role of cell pseudopods in the migration behavior of cancer cells was observed, and the relevant results are consistent with the morphological changes of tumor cells during migration.[34] The Rat Tail Collagen I was more conducive to the mesenchymal cell morphology of MDA-MB-231 and the formation of cell pseudopods, compared with other ECM materials, such as calcium alginate gel,[35] gelatin,[36] and UV-curable hydrogel.[37] Meanwhile, through the observation of cell pseudopods, the cooperative behavior of cancer cells during migration was also observed. However, recent research has always focused on competition among cancer cells, and cooperation among cancer cells has rarely been reported.[38] Archetti and Pienta[39] analyzed the cooperation among cancer cells from the perspective of game theory. It was the first time that we had directly observed this cooperation among cancer cells during migration on the chip, although the relevant mechanism needs to be further studied. In a word, the 3D ECM can better reflect the tortuosity of the cancer cell invasion and the complexity of their migration behavior.

We confirmed that the migration ability of tumor cells had a significant positive correlation with this kind of cell pseudopods; this was consistent with existing reports.[40] This indicated that the inhibition of anticancer drugs for the migration ability was directly related to the change in cell morphological characteristics. Moreover, the longer the drug acts on cancer cells, the more obvious the disappearance of the characteristics of mesenchymal cells. In conclusion, after the drug treatment, the morphological change trends of tumor cells in different cancer models were consistent.

Conclusions

We systematically evaluated the crucial role of the restricted migration environment in the research of complex tumor migration behaviors. Through the comparison of the 2D restricted channels and 3D ECM environment in the migration evaluation, the selection significance of migration evaluation methods in tumor metastasis research was clarified. Relevant results showed that the 2D restricted channels (10 μm high) could well inhibit the instinct of cancer cells that grew rapidly into the surrounding space, which helped study the role of the co-culture condition in the tumor cell migration ability. But when restricted channels were less than 5 μm, the cancer cell behavior migrating into the channels was completely constrained. The 3D ECM environment also came true with similar restrictions, which was able to evaluate the impact of other weak factors (compared with the strong migration instinct of tumor cells) on their migration ability. Meanwhile, the 3D ECM environment could reflect the variability of the tumor cell migration direction, which was conducive to observing and analyzing the cooperation among cancer cells under the competitive migration environment. It should be noted that the selection of ECM materials will seriously affect the evaluation results of cell migration, and the concentration and proportion of ECM materials need to be strictly controlled. In addition, the 3D ECM environment could reflect more accurate cytotoxicity and drug resistance of anticancer drugs, which was more helpful for the screening and evaluation of anti-tumor metastasis drugs.

AUTHORS' CONTRIBUTIONS

Design and implementation of the processes: ZD, SY, and SM. Protocol development: QG, ZL, and GX. Writing of the article: ZD. Editing of the article: ZD and SM.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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