A Potential Model for Detecting Crowding-induced Epithelial Cell and Cancer Cell Extrusion*

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Summary: Overcrowding and cell deformation lead to the shedding of apoptotic and live cells to maintain homeostasis in the epithelium. Recent studies have attempted to explain the effect of extrusion on epithelial homeostasis and tumor metastasis, but lack the requisite quantitative models for testing extrusion. Here, we designed a petri dish inversion model to detect the extrusion ability of both normal epithelial cells and epithelial cancer cells. Firstly, we found cell extrusion was observed in both normal epithelial cells (LO2 cells) and cancer cells; in confluent LO2 cell culture, certain cells were surrounded by their neighbors, suffered "collective attack", and were then made round in shape. Green fluorescent protein (GFP)-labeled cancer cells were also found to be squeezed by normal LO2 cells. Using the petri dish inversion model, we quantified the number of extrusion cells, and demonstrated that the ability of cancer cell extrusion was related to the metastatic potential of cancer cell lines. Our findings provide a novel model to detect crowding-induced epithelial cell and cancer cell extrusion. This novel model provides a quantitative method for research into apoptotic and cancer cell extrusion, particularly in human hepatocellular carcinoma.

Key words: hepatocellular carcinoma; metastasis; epithelial homeostasis; crowding; extrusion

Epithelial homeostasis is critical for proper maintenance of barrier, defense, and transport functions of epithelia. Loss of epithelial homeostasis is found to be associated with different pathological events, such as pathogen infection and structural damage[1]. Previous studies have confirmed that cell extrusion is an important homeostatic mechanism to eliminate apoptotic or harmful cells from crowded areas of epithelia[2–4]. Recent studies have revealed new roles of extrusion in controlling developmental morphogenesis and maintaining homeostatic cell numbers[5, 6]. In crowded regions of the tissue, a proportion of cells undergo a serial loss of cell-cell junctions; meanwhile, crowding induces neighboring cells to activate Rho-mediated assembly and contraction of an intercellular actomyosin ring, eventually squeezing out excessive live cells[7, 8]. Normally, live extruded cells eventually die through a process termed anoikis, or apoptosis due to loss of survival signaling. More recently, research has verified that transformed cells, such as oncogenic KRAS and APC mutant cells, can also shed from the epithelia by way of live cell extrusion[9–11]. However, in contrast to apoptotic cell-induced apical extrusion, oncogenic mutant cells prefer to extrude basally[11–14]. Meanwhile, in contrast to other epithelia, liver tissue has an advantage in the metastasis of extruded cancer cells, regardless of the direction of extrusion: hepatocellular carcinoma (HCC) cells extruded apically could cause hematogenous metastasis; on the other hand, basally extruded HCC cells might invade basement membranes, potentially initiating lymphatic metastasis.

Although an increasing number of studies have focused on exploring the role of extrusion in physiology and pathology, and visually observing extrusion in cells and tissues, it is hard to quantitatively detect and compare the extrusion ability and number of different cell lines. In this study, we observed cell extrusion both in normal epithelial cells and HCC cells. Meanwhile, we designed an *in vitro* petri dish inversion model, and detected the extrusion ability of normal epithelial cells and epithelial cancer cells.

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1 MATERIALS AND METHODS

1.1 Cell Culture and Transfection

Human LO2 cells and HCC cell lines Huh7, SMMC-7721, and SK-Hep1 were all cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, USA), supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin and incubated in a 5% CO_2 incubator at 37°C. Green fluorescent protein (GFP)-labeled plasmid lentiviral transfection was performed as described previously[15].

1.2 GM130 Fluorescent Staining

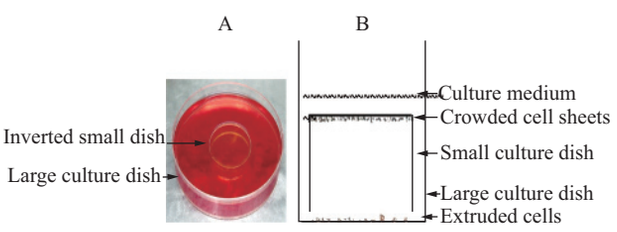
Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for 10 min on ice, then blocked with 1% BSA for 60 min, and incubated with GM130 antibody (1:100, Abcam, UK) diluted in 1% BSA at 4°C overnight. This was followed by three washes with phosphate buffered saline (PBS), and finally incubation with secondary Alexa Fluor 594-conjugated anti-rabbit antibody for 1 h at room temperature. After three washes with PBS, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for detecting nuclei. Cells were mounted and examined under an Olympus FluoView FV1000 confocal microscope.

1.3 *In Vitro* Petri Dish Inversion Model

As shown in fig. 1, cells grown to approximately 100% confluence in a small culture dish (60×15 mm, Corning, Oneonta, USA) were placed, inverted in a large culture dish (150 mm×25 mm, Fisher Scientific, Pittsburgh, USA) with enough culture medium to make sure the medium in the large dish could inundate the bottom of the small dish, using an aseptic bending needle to pull out the gas in the small dish. Live cells extracted from the small culture dish could live in the large culture dish below. Twenty-four hours later, the small culture dish was gently removed. The cells shedding in the large culture dish were able to grow for 7 days; cells were then stained with crystal violet to count clone formation. All steps were aseptic, and we avoided touching the bottom of the small dish.

1.4 Statistical Analysis

Results are expressed as mean±standard deviation (SD) and representative of at least three independently performed experiments. Student's *t*-test for parametric variables was used for statistical analysis. All tests were two-sided and $P<0.05$ was considered statistically significant. Analysis was performed using SPSS software (version 18).



In vitro petri dish inversion model

Fig. 1 *In vitro* petri dish inversion model
A: a picture of the petri dish inversion model; B: a sketched picture of the petri dish inversion model

2 RESULTS

2.1 Observation of Extrusion in Cultured Liver Cells and HCC Cells

Some studies have observed cell extrusion in epithelial cells, including apoptotic cell extrusion and transformed cell extrusion. However, there are few reports on cancer cell extrusion, particularly in HCC. Firstly, we observed cell extrusion in cultured monolayer LO2 cells (normal liver cells). In confluent LO2 cell culture, certain cells were surrounded by their neighbors and suffered "collective attack", changing the attacked cells to a rounder shape (fig. 2A). This phenomenon is in accordance with recent reports of extrusion in other cells[16]. To assess the fate of HCC cells cultured in a monolayer with normal LO2 cells, Huh7, SMMC-7721, and SK-Hep1 cells were labeled with GFP plasmids, and then co-cultured with LO2 cells at a ratio of 1:100. Interestingly, HCC cells were surrounded and morphologically squeezed by normal LO2 cells (fig. 2B). As the location of the Golgi complex reflects the direction of cell movement, to examine the direction of force and migration in neighboring cells, HCC cells were fixed and stained with the Golgi matrix protein GM130 antibody. Encouragingly, these data clearly showed that the direction of force and migration in neighboring cells pointed towards the HCC cells, acting to squeeze them out of the monolayer (fig. 2C).

2.2 Successful Creation of a Petri Dish Inversion Model

To simulate a simple and compelling model for detecting crowding-induced extrusion, we constructed a model we called the "petri dish inversion model". In this model, cells grown to approximately 100% confluence in a small culture dish were placed, inverted, in a large culture dish with sufficient culture medium. When apoptotic cells extruded from the small culture dish, they would float in the culture medium, while live cells extruded from the small culture dish into the culture medium could live in the large culture dish below. When cells grew to new clones, they were counted after staining with crystal violet. As shown in fig. 3, we found that the clone numbers of extruded SK-Hep-1, SMMC-7721, and Huh7 cells were 126±10, 89±8, and 64±8, respectively. These were significantly greater than that of the normal LO2 cells (19±6, $P<0.01$), which was in line with the metastatic potential of cell lines[17]. Next, we co-cultured Huh7, SMMC-7721, and SK-Hep1 cells labeled with GFP

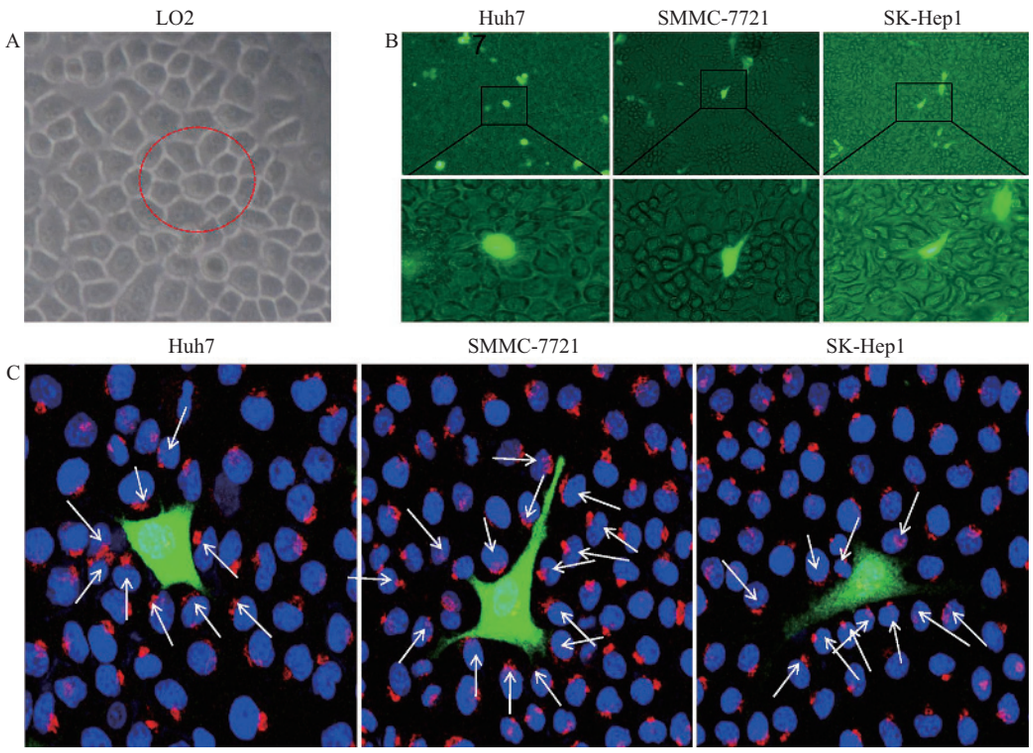


Fig. 2 Extrusion in liver cells and HCC cells
A: a representative picture for extrusion in confluent LO2 cells (×200). Certain cells were surrounded and suffered "collective attack" by their neighbors. B: Huh7, SMMC-7721, and SK-Hep1 cells labeled with GFP were surrounded and morphologically squeezed by LO2 cells (×40). The bottom of panel B shows locally amplified pictures of the inserts in the top panel. C: Golgi matrix protein GM130 (the arrows) reflected the direction of force and migration of cells (×200). Red, GM130; blue, nuclei; green, GFP labeled HCC cells

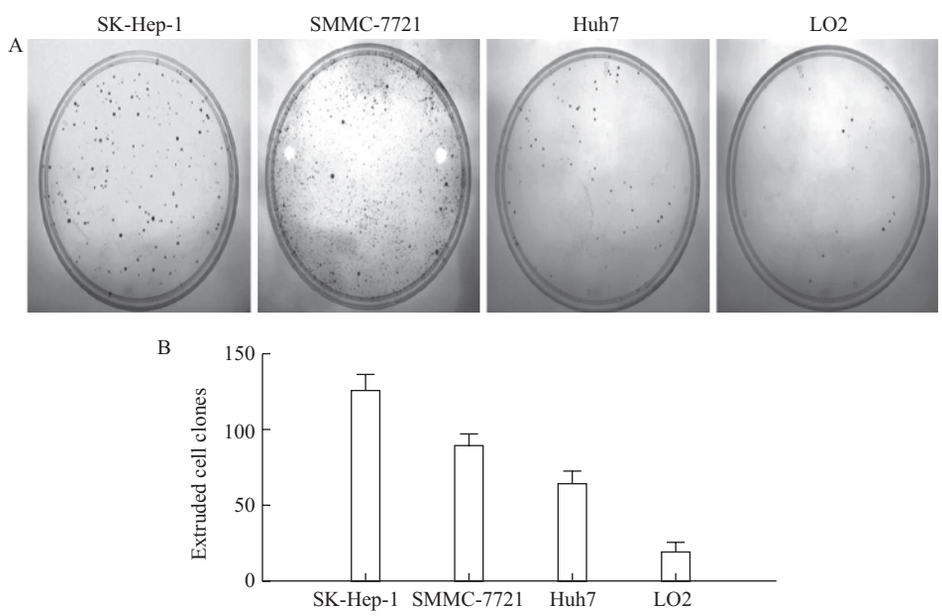


Fig. 3 A: cell clones in the large culture dish of extruded SK-Hep-1, SMMC-7721, Huh7, and LO2 cells in the petri dish inversion model; B: the clone numbers of extruded SK-Hep-1, SMMC-7721, Huh7 and LO2 cells were 126±10, 89±8, 64±8, and 19±6, respectively (P<0.01) (Assay shown is representative of three experiments with similar results.)

with LO2 cells at ratio of 1:100, and found that SK-Hep1 cells were more likely to be squeezed out by LO2 cells than Huh7 and SMMC-7721 cells, as shown in fig. 4. Remaining cell number in the small dishes for SK-Hep1 cells (33±6) was less than that for both Huh7 (74±9) and SMMC-7721 cells (143±12) (P<0.01).

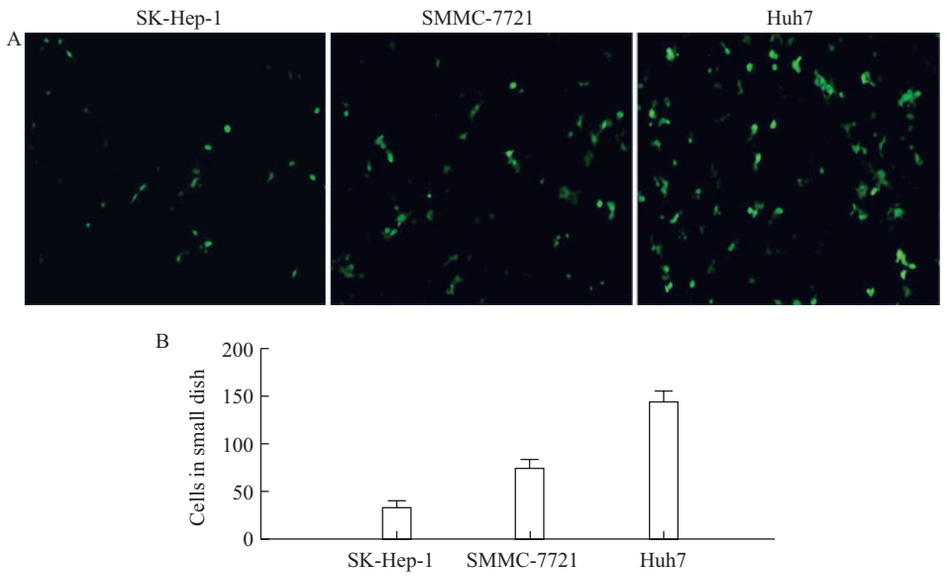


Fig. 4 Cells remained in the small dish.

A: GFP-labeled HCC cells were mixed with LO2 cells at a ratio of 1:100 to grow to confluence in the petri dish inversion model. The cells remained in the small dish, indirectly showing the number of extruded HCC cells (×40). The data showed the remaining HCC cells in the small dish per field of vision. B: Remaining cell number in the small dishes of SK-Hep1, SMMC-7721 and Huh7 was 33±6, 74±9, and 143±12, respectively (P<0.01). Bars represent the means±SD of three independent experiments.

This suggests that the extruded SK-Hep1 cells were greater in number than Huh7 and SMMC-7721 cells, in accordance with the metastatic potential of HCC cell lines. Once again, this suggests that the petri dish inversion model was sufficient to examine the extrusion ability in crowded cell conditions, and that this model may provide another method for the research of epithelial cell function and cancer cell metastasis.

3 DISCUSSION

Cell extrusion is considered to be an important way for the epithelium to remove apoptotic and harmful cells[12]. In 2012, Jody Rosenblatt and Buzz Baum first demonstrated that epithelial tissues could "throw away" extra live cells in overcrowded areas by live cell extrusion[7, 8]. Since then, more studies have focused on exploring the role of extrusion in physiology and pathology, particularly in the metastasis of cancer cells[18, 19]. However, since most studies used immunofluorescence methods to investigate extrusion in cells and tissues, it is hard to quantify the extruded cells and compare their extrusion abilities. Jody Rosenblatt designed a simple model to simulate overcrowding observed *in vivo*[8], growing cells to confluence on a silicone membrane stretched to 28% of its original length, then releasing it from stretching to produce artificial overcrowding conditions with a density increased by 1.3-fold. They quantified live and apoptotic extruding cells 2 h after overcrowding, and regrew the extruded cells collected at 2 h post-crowding. This model is clever and practical, but inevitably leads to artificially induced cell shedding from the monolayer when the silicone membrane is released, although the authors confirmed the tight and adherens junctions were still intact by ZO-1 and β-catenin staining. In our study, we demonstrated extrusion in liver cells and HCC cell lines in culture by immunofluorescence method, and then devised a novel model to simulate overcrowding conditions. In this model, cells naturally grown in the small culture dish can be extruded into the culture medium, live in the large culture dish below, or undergo non-artificial apoptosis. As the bottom of the small dish is equal to or lower than the liquid level in the large dish, cells in the small dish will not be disturbed by gravity when they fall into the large dish. Using this model, we also demonstrated the extrusion ability of cancer cells according to the metastatic potential of HCC cell lines. This petri dish inversion model is simple and compelling: all of the extrusion steps are non-artificial, therefore minimizing human influence. Liver tissue has an advantage in the metastasis of extruded cancer cells regardless of the direction of extrusion, so we can further investigate metastasis of HCC cells, which may provide another avenue for research into epithelial cell function and the metastasis of HCC cells.

Conflict of Interest Statement

The authors have no conflicts to disclose.

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