



# Netrin-1 promotes the collective cell migration of liver cancer cells in a 3D cell culture model

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Received: 19 July 2018 / Accepted: 4 August 2019 / Published online: 12 August 2019  
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## Abstract

Collective cell migration plays an important role in embryonic development, wound healing, and cancer metastasis. We aimed to investigate the expression, role, and mechanism of Netrin-1 in collective cell migration using a 3D culture model. An immunohistochemical study showed that certain cells invaded surrounding tissue by collective migration and that Netrin-1 expression in these cells was increased, especially at the invasive front. In the 3D culture model, collective cell migration was clearly observed, as leader cells were followed by cells migrating along a canal. N-cadherin-mediated cell junctions were observed in collective cell migration, and Netrin-1 expression was elevated in these cells. Netrin-1 did not affect the expression of N-cadherin in 2D-cultured cells; however, in 3D culture, the overexpression of Netrin-1 increased N-cadherin and promoted the collective migration of Huh7 cells, while the knockdown of Netrin-1 decreased N-cadherin and inhibited collective migration in SK-Hep-1 cells. Interestingly, N-cadherin knockdown in Huh7 cells significantly diminished Netrin-1-promoted collective cell migration, while the overexpression of N-cadherin restored collective migration in Netrin-1-knockdown SK-Hep1 cells. These results suggest that Netrin-1 enhances N-cadherin junctions to promote liver cancer cell collective migration in 3D cell culture and may subsequently increase liver cancer metastasis.

**Keywords** Netrin-1 · Liver cancer · Collective cell migration · 3D culture · Metastasis

## Introduction

Liver cancer is the most common and most lethal tumor for males under 60 years of age in China [5]. Early intrahepatic

metastasis and postoperative recurrence are the main reasons for the poor prognosis of liver cancer. Current theories regarding how cells escape the primary tumor to initiate metastasis fit into two broad categories as follows: single cell and collective cell migration [4, 8]. Previous studies have focused on single-cell migration and revealed that cancer cells invade tissues through EMT (epithelial to mesenchymal transition), which is the classic mode of single-cell migration. However, the concept of EMT has been challenged; for example, Kyra Camp Bell and Jordi Casanova proposed a different view [3] that collective cell migration versus single-cell migration and epithelial versus mesenchymal characteristics might combine in variable degrees, not only in different migratory events but also at different times in a single migratory process. However, the mechanism that promotes liver cancer cell collective migration remains unclear.

We have proven that Netrin-1 induces EMT and promotes liver cell invasiveness in a hypoxic environment [32]. Furthermore, we observed Netrin-1 overexpression in liver cancer cells, especially at the invasive front. A recent study has suggested that guidance signaling regulates leading-edge behavior during collective cell migration of cardiac cells in

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*Drosophila* [25], and Slit- and Netrin-mediated signals maintain the balance between epithelial and mesenchymal characteristics of migrating cardioblasts. Therefore, we speculated whether Netrin-1 could induce collective cell migration in liver cancer cells.

Adhesion molecules play an important role in cancer metastasis; among them, N-cadherin has been extensively studied in the context of individual and collective cell migration [2, 22, 31]; however, its precise role is still debated [22] and could depend on its pro-migratory or inhibiting role, cell type (in vivo versus in vitro), research approach (2D versus 3D assay), and type of migration. For example, N-cadherin is considered to promote differentiation as well as migration of granule cells [26], but its expression negatively controls the invasive behavior of gliomas [24]. The role of N-cadherin in the collective migration of liver cancer cells requires further study.

In this study, we investigated whether Netrin-1 used an N-cadherin-dependent mechanism to regulate collective cell migration in liver cancer cells. We showed that Netrin-1 was upregulated in liver cancer cells, especially at the interface front of the tumor. In the 3D cell culture model, Netrin-1 increased liver cancer cell collective migration, and the knock-down of N-cadherin in Huh7 cells significantly diminished Netrin-1-promoted liver cancer cell collective migration. These results suggest that Netrin-1 enhances N-cadherin junctions and promotes liver cancer cell collective migration, subsequently increasing liver cancer cell metastasis.

## Materials and methods

### 2D cell culture and transfection

Human Huh7 and SK-Hep1 cells were cultured in DMEM medium containing 10% FBS (HyClone, USA) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin and incubated in a 5% CO<sub>2</sub> incubator at 37 °C. The method of Netrin-1 and N-cadherin plasmid lentiviral transfection was performed as described previously [17].

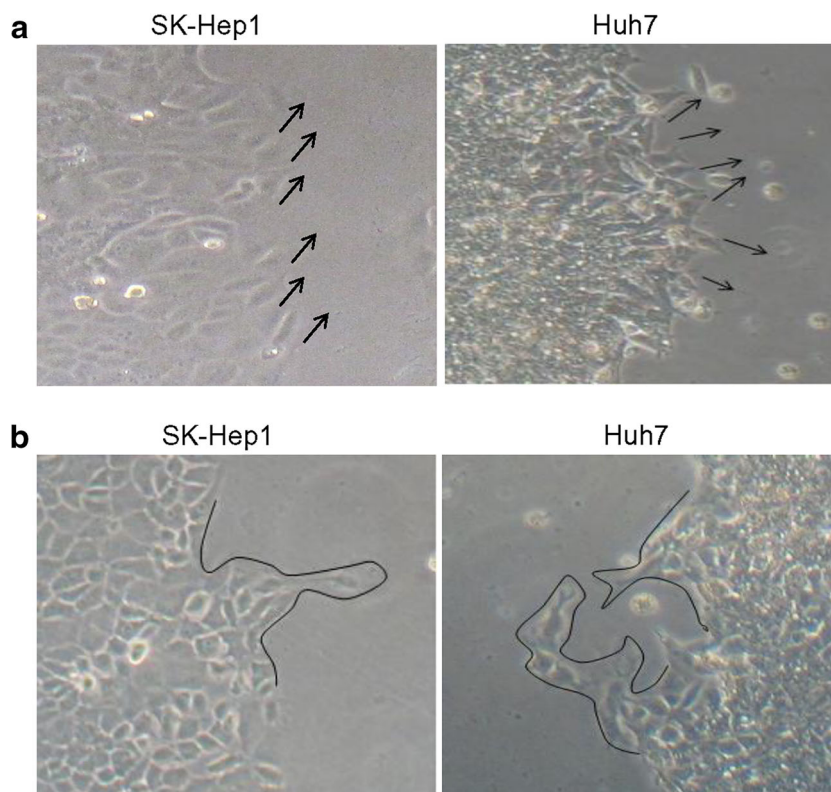
### Scratch wound healing study

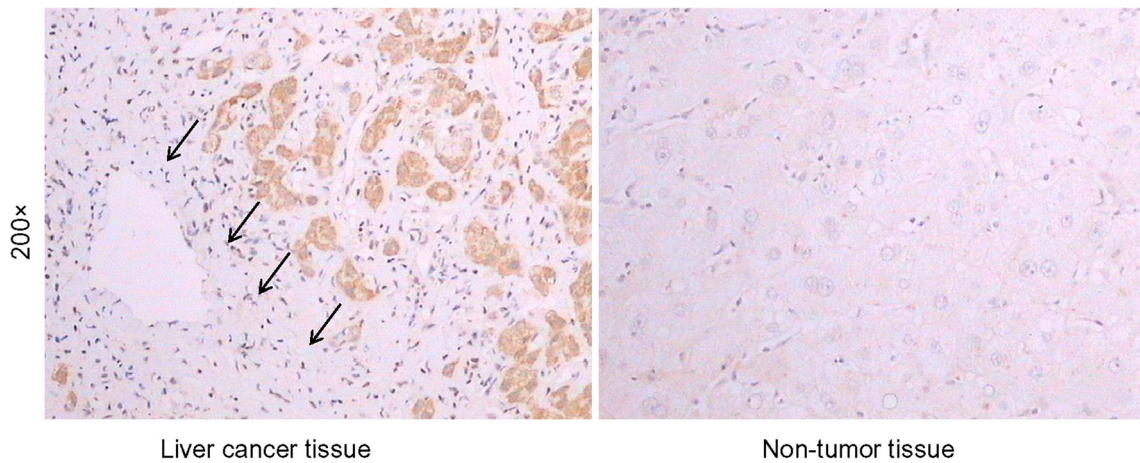
The scratch wound healing was performed as described previously [18].

### Immunohistochemistry

Human liver cancer samples were collected at the time of surgical resection at Tongji Hospital. This study was performed according to the guidelines of the Ethics Committee of the Tongji Hospital and approved in accordance with the ethical standards of the World Medical Association Declaration of Helsinki. The immunohistochemistry assay was performed as described previously [32].

**Fig. 1** Migration of liver cancer cells in a 2D monolayer culture induced by wound healing. Serum-starved SK-Hep1 and Huh7 cell monolayers were cultured and scratched, and they were imaged by phase microscopy after 24 h. Notably, some cells migrated by single cell migration (a), while other cells migrated by collective migration (b). This phenomenon was observed in both SK-Hep1 and Huh7 cells





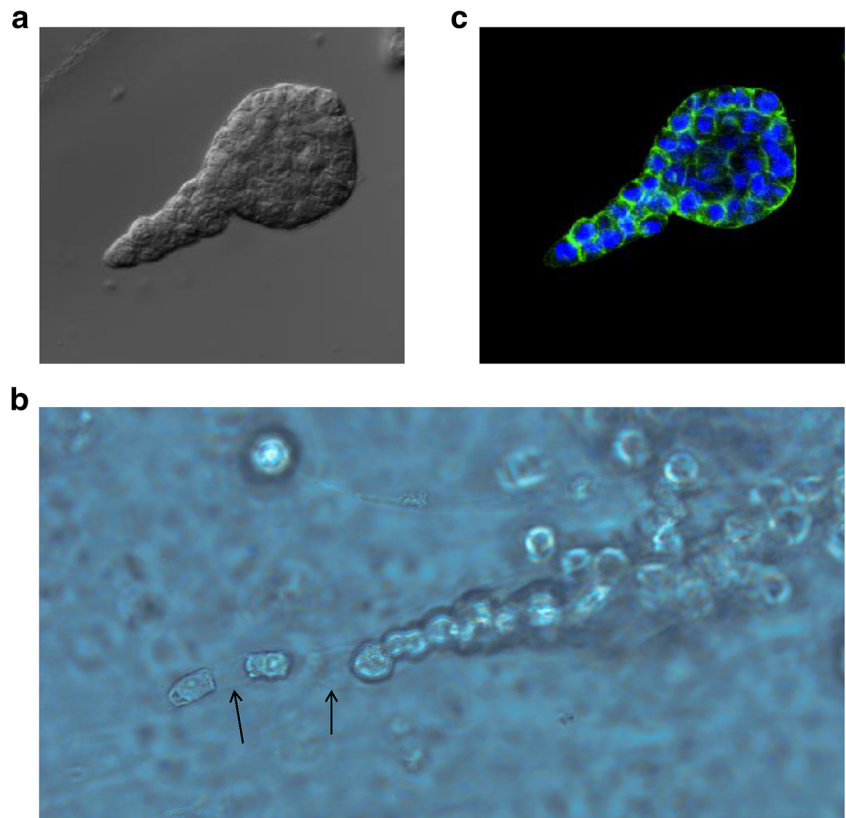
**Fig. 2** Netrin-1 expression in collectively invaded liver cancer tissues. Collective migration was frequently observed in the front region of liver cancer tissues (a). Netrin-1 was clearly upregulated in liver cancer tissues (a) compared with nontumor tissues (b)

**3D cell culture and cell collection**

The 3D cell culture assay was performed using a hydrogel solution (BeaverNano™, China) as described previously [17]. Briefly,  $1 \times 10^5$  cells were resuspended in 120  $\mu$ l 10% sucrose solutions and quickly mixed with an equal volume of 0.5% hydrogel solution. The cells were then immediately seeded in a glass-bottom cell culture dish (NEST, USA). The medium was changed every other day, and the cells were

allowed to grow for several days and imaged by microscopy. To collect the cells in the hydrogel, 600  $\mu$ l of cell culture medium was added to the hydrogel, repeatedly agitated with a pipette to destroy the overall structure of the hydrogel and to completely break it apart. Then, the cells were centrifuged for 5 min at 3000 rpm, and the supernatant was removed. Then, 600 ml of cell culture medium was added, and the abovementioned steps were repeated. Finally, the cell suspension was centrifuged, and sedimented cells were collected for subsequent western blotting.

**Fig. 3** Collective cell migration in the 3D cell culture model. Representative micrograph of the collective migration of liver cancer cells in the 3D culture model (a). Intercellular connections were observed between the front cells (b). Immunofluorescence suggested that N-cadherin expression was retained between leading cells and invasive cells along (c) (blue, nuclei; green, N-cadherin)



## Western blot analysis

Cells were lysed in RIPA buffer (Promoter Company, China). Samples were then separated in an SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were immunoblotted with antibodies against N-cadherin (Abcam, UK) and Netrin-1 and  $\beta$ -actin (Santa Cruz Biotechnology, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence using an ECL detection reagent. Protein quantitative analysis was performed by ImageJ software.

## Immunofluorescence staining

3D-cultured cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 30 min on ice. Cells were blocked with 1% BSA for 60 min and incubated with primary antibodies (1:100 dilution for Netrin-1 and 1:500 dilution for N-cadherin) diluted in 1% BSA at 4 °C overnight, followed by 3 washes with BSA solution and finally incubated with Alexa Fluor 594-conjugated anti-rabbit secondary antibody, Alexa Fluor 488-conjugated anti-mouse secondary antibody, or FITC-labeled secondary antibody for 3 h at room temperature. After 3 washes with BSA solution, cell nuclei were stained with DAPI. Cells were mounted and examined under an Olympus FluoView FV1000 confocal microscope.

## Statistical analysis

The results are expressed as the mean  $\pm$  SEM and are representative of at least three independently performed experiments. For statistical analysis, unpaired *t* test was used for analyzing the protein expression of Netrin-1 and N-cadherin; two-way ANOVA was performed to analyze the length of migration in 3D cell cultures at different days. All tests were two-sided, and  $p < 0.05$  was considered statistically significant. Analysis was performed using GraphPad Prism 5.0.

## Results

### Netrin-1 was increased in collectively invading liver cancer cells

Previously, we observed that Netrin-1 expression was upregulated in liver cancer tissues and cell lines. The overexpression of Netrin-1 promoted the invasion and migration of liver cancer cells [17, 32]. In this study, we speculated whether Netrin-1 is involved in the collective migration of liver cancer cells. First, we observed the movement of liver cancer cells by

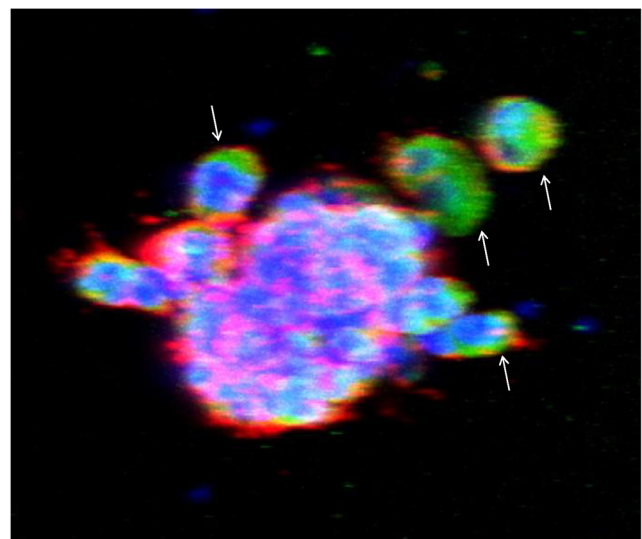
a wound healing assay in 2D cell culture. As shown in Fig. 1 a, some cells migrated through single-cell migration, while certain cells migrated by collective migration (Fig. 1b). These phenomena were observed in both Huh7 cells and SK-Hep1 cells. Furthermore, collective cell migration was also observed in liver cancer tissues. Interestingly, Netrin-1 expression was increased in liver cancer tissues compared with nontumor tissues, especially in collectively invading liver cancer cells (Fig. 2).

### Collective migration was observed in liver cancer cells in a 3D culture

Investigating in vivo tumor migration may be better represented by a 3D environment [14]. Therefore, we cultured liver cancer cells in a 3D hydrogel to form spheroids. As shown in Fig. 3 a, collective migration was clearly observed in the 3D culture. Certain liver cells invaded the hydrogel as leader cells, forming a canal, and cancer cells followed along the canal. Intercellular connections were observed between the leader cells (Fig. 3b). This confirmed that this 3D cell culture model could be applied to observe the action of collective cell migration of liver cancer cells. Using immunofluorescence, we observed N-cadherin staining between leading cells and invasive cells along cell-cell junctions (Fig. 3c), suggesting that liver cancer cells could invade through N-cadherin-dependent collective migration.

### Netrin-1 regulated liver cancer cell collective migration

To determine whether Netrin-1 is involved in liver cancer cell collective migration, we used immunofluorescence to detect



**Fig. 4** 3D reconstruction of immunofluorescence images showing Netrin-1 expression in the 3D cell culture model. The arrows indicate the leader cells. Blue, nuclei; red, N-cadherin; green, Netrin-1

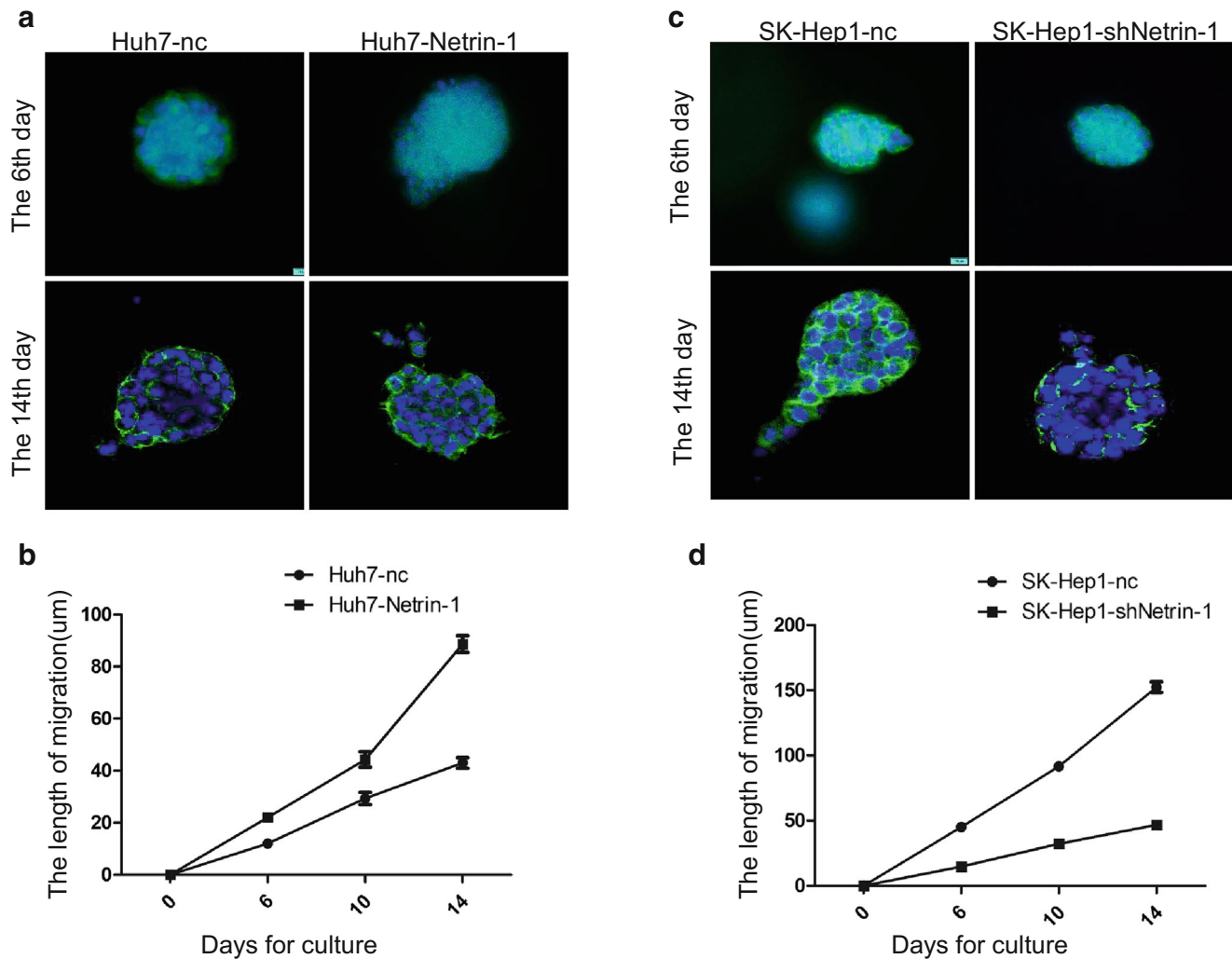
the expression of Netrin-1 in the 3D culture model, and we found that Netrin-1 was significantly increased in invading tumor cells, especially in the leader cells (Fig. 4).

We examined the expression of Netrin-1 in liver cancer cells and found that Netrin-1 was upregulated in SK-Hep1 cells and downregulated in Huh7 cells [17]. To further detect the role and significance of Netrin-1 in liver cancer cell collective migration in the 3D culture model, we stably overexpressed Netrin-1 in Huh7 cells, and we stably knocked down Netrin-1 in SK-Hep1 cells, as confirmed by immunoblotting. We studied the role of Netrin-1 in liver cancer cell collective migration in the 3D cell culture model, as shown in Fig. 5 a, and compared the migration to control cells. The collective migration of Huh7 cells overexpressing Netrin-1 was dramatically enhanced and the distances of migration at the 6th day, 10th day, and 14th day were longer than that of the control cells (Fig. 5b), while the

knockdown of Netrin-1 in SK-Hep1 cells significantly impaired 3D collective migration and presented no evidence of migration beyond the periphery of the primary tumor (Fig. 5c, d). These results suggested that collective migration is a dynamic process and Netrin-1 clearly regulates liver cancer cell collective migration in a 3D cell culture model.

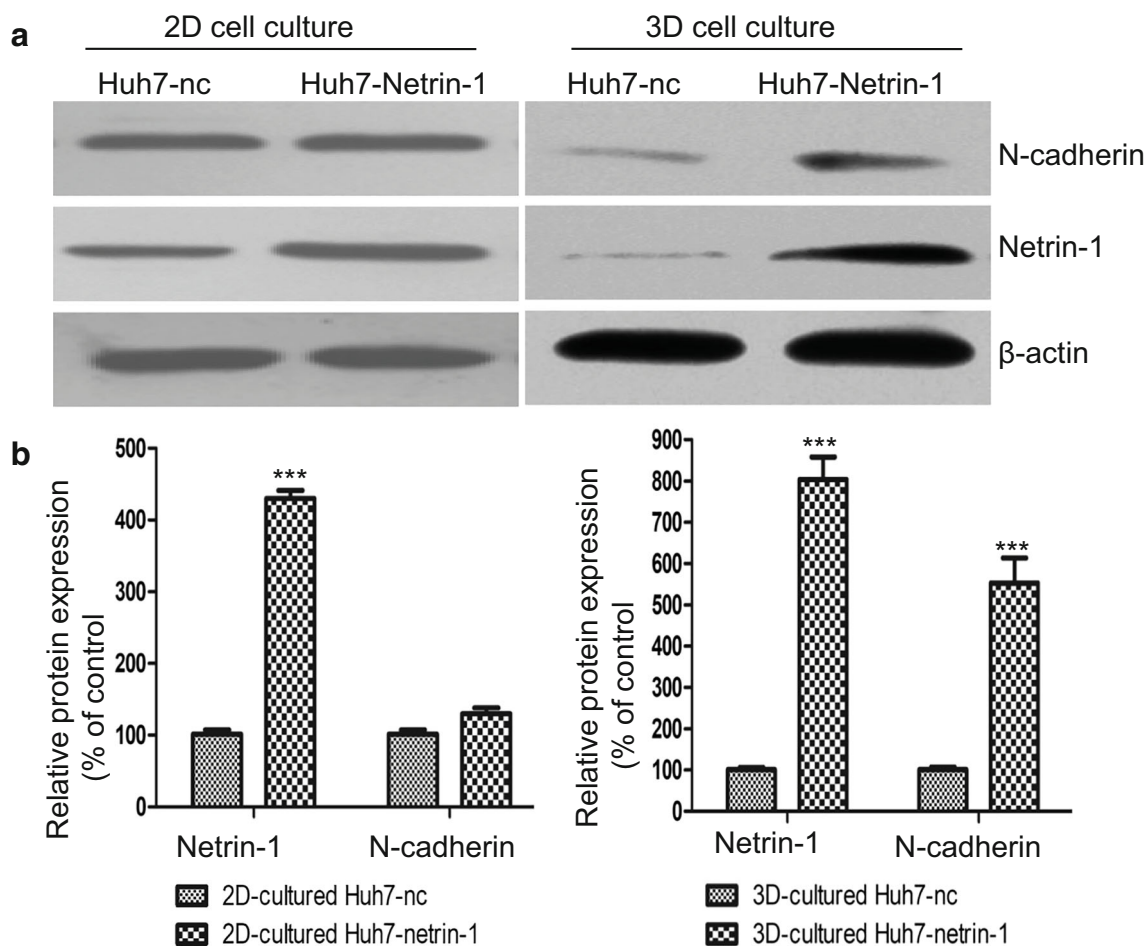
### Netrin-1 regulated collective migration in an N-cadherin-dependent manner

N-cadherin has been confirmed to promote collective migration in development and in some cancers. We evaluated the expression of N-cadherin after overexpressing or knocking down Netrin-1. As shown in Fig. 6, overexpression of Netrin-1 did not affect the expression of N-cadherin in 2D-cultured liver cancer cells, and the



**Fig. 5** Netrin-1 regulates liver cancer cell collective migration in 3D cell culture. Overexpressing Netrin-1 in Huh7 cells promoted collective cell migration as a dynamic process **a** showed the representative pictures at the 6th and 14th day. **b** The quantification of the distances of migration at

6th day, 10th day, and 14th day,  $p < 0.001$ . **c** Knockdown of Netrin-1 in SK-Hep1 cells decreased collective cell migration at different times. **d** Also presenting as a dynamic process (6th day, 10th day, and 14th day),  $p < 0.001$ . Blue, nuclei; green, N-cadherin



**Fig. 6** The varying role of Netrin-1 overexpression on the expression of N-cadherin in 2D- and 3D-cultured Huh7 cells. **a** Western blotting detected the expression of N-cadherin after overexpression of Netrin-1 in the 2D and 3D cell culture models. **b** Relative protein expression of N-

cadherin shown in figure panel **a**. Protein levels of Netrin-1 and N-cadherin of Huh7-nc cells cultured in 2D and 3D conditions were set to 100%, respectively. Bars represent the means  $\pm$  SEM of three independent experiments,  $n = 5$ , \*\*\*  $p < 0.001$

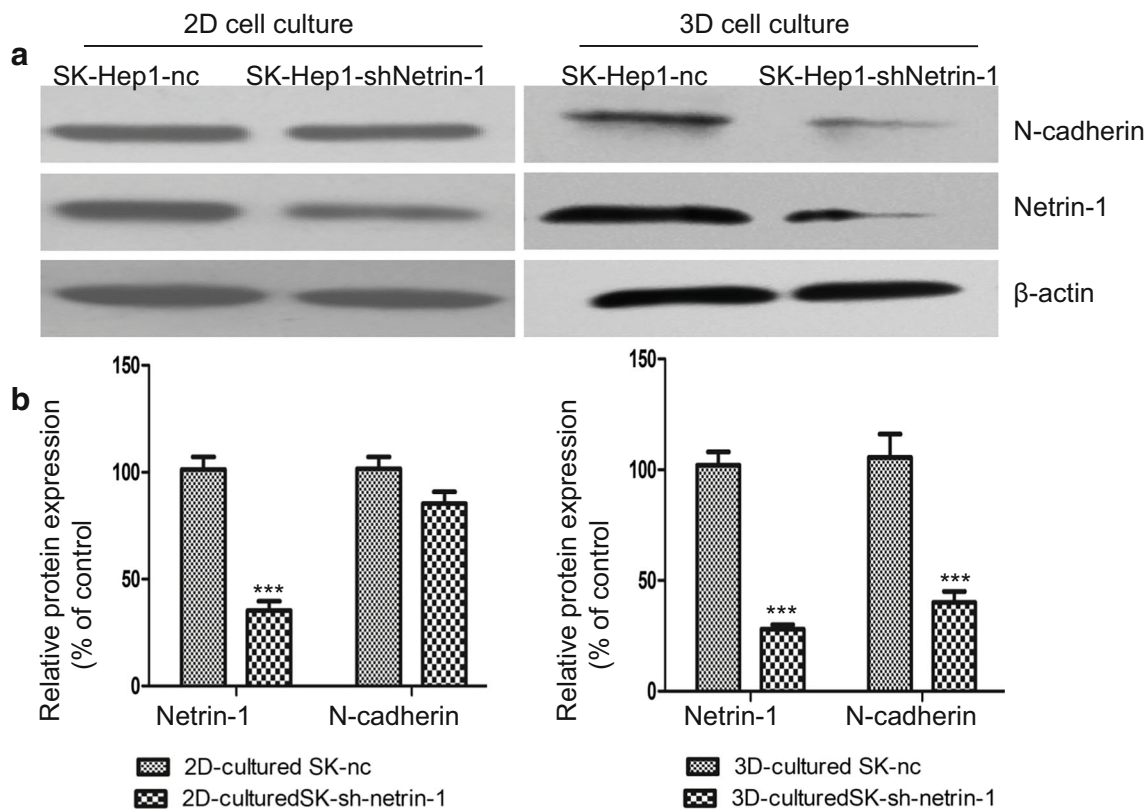
overexpression of Netrin-1 upregulated N-cadherin levels in 3D-cultured Huh7 cells (Fig. 6). Furthermore, knock-down of Netrin-1 in SK-Hep1 cells decreased the N-cadherin expression level in the 3D culture; however, Netrin-1 knockdown did not affect N-cadherin expression in the 2D culture model (Fig. 7). These results suggested that Netrin-1 regulates the expression of N-cadherin in 3D-cultured liver cancer cells, and it seemed to have no effect on 2D-cultured cells.

Next, we speculated whether Netrin-1 could regulate liver cancer cell collective migration in an N-cadherin-dependent manner in the 3D cell culture model. The results showed that N-cadherin knockdown diminished enhanced collective migration in Netrin-1-overexpressing Huh7 cells (Fig. 8a, b), and overexpression of N-cadherin in Netrin-1-knockdown SK-Hep1 cells restored collective migration (Fig. 8c, d). These results confirmed that Netrin-1 regulates liver cancer cell collective migration in an N-cadherin-dependent manner in the 3D cell culture model.

## Discussion

Cancer cell migration is the critical first step of metastasis [7], yet little is known about how cancer cells invade and initiate invasion in a complex extracellular matrix. We have previously described that liver cancer cells could be extruded by neighboring cells to initiate metastasis [19], but the mechanism by which these cells transition from primary tumors to metastases remains unclear.

Epithelial-mesenchymal transition (EMT) was first proposed nearly half of a century ago and has been gradually recognized as an important step in the early metastasis of epithelial carcinomas [1]. Through EMT, tumor cells modify not only their shape but also their attachment to other cells and the extracellular matrix (ECM), eventually promoting cell migration and invasiveness leading to distant metastasis [1, 27]. However, the underlying mechanism of migration and metastasis varies depending on the type of cancer. With the expansion of research on tumor metastasis, collective cell migration



**Fig. 7** The varying role of Netrin-1 knockdown on the expression of N-cadherin in 2D- and 3D-cultured SK-Hep1 cells. **a** Western blotting detected the expression of N-cadherin after knockdown of Netrin-1 in the 2D and 3D cell culture models. **b** Relative protein expression of N-cadherin

shown in figure panel **a**. Protein levels of Netrin-1 and N-cadherin of SK-Hep1-nc cells cultured in 2D and 3D conditions were set to 100%, respectively. Bars represent the means  $\pm$  SEM of three independent experiments,  $n = 5$ , \*\*\*  $p < 0.001$

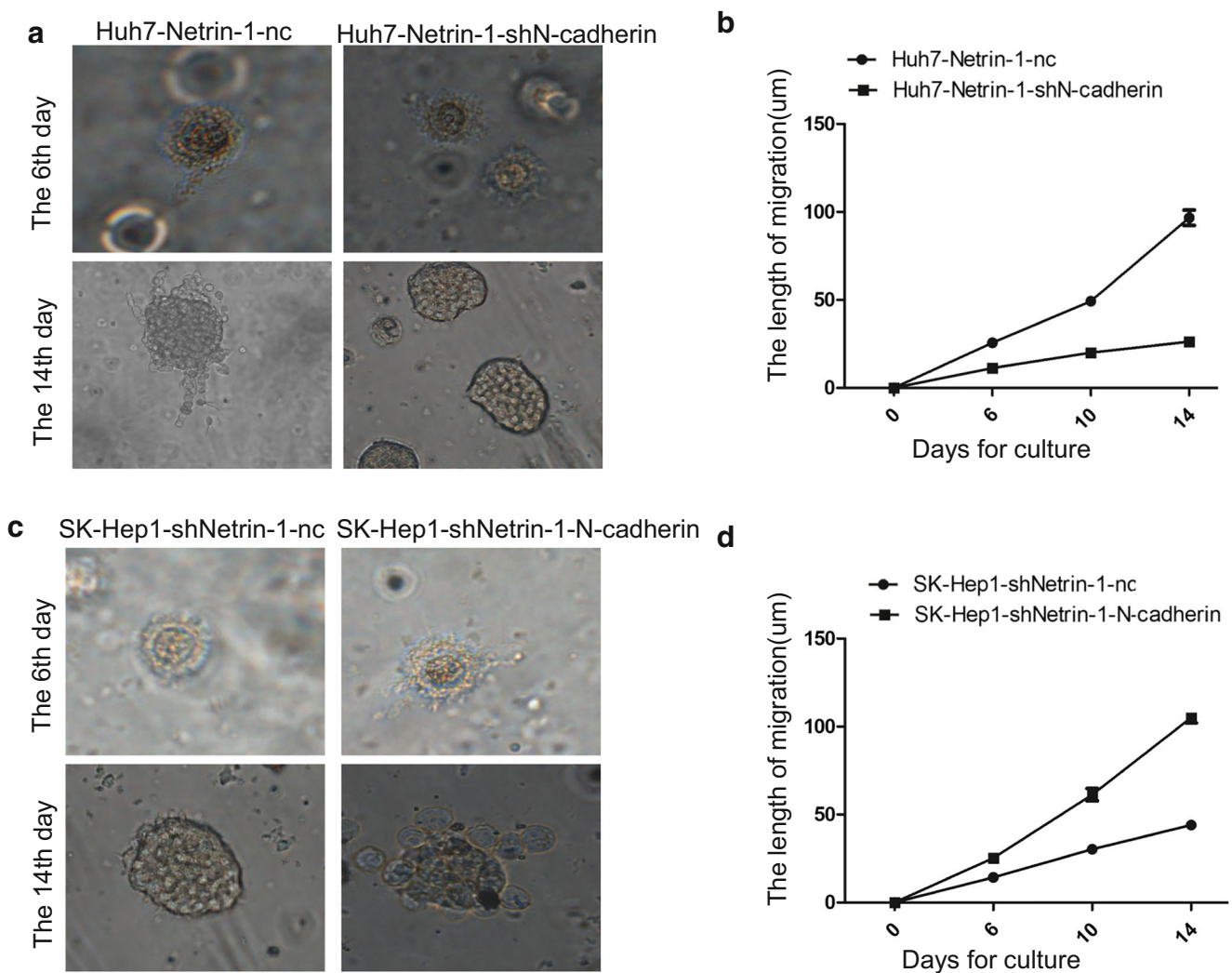
has been found to be a new mode of tumor metastasis [16, 30]. In this model, certain cancer cells invade in front of collectively migrating cells as leaders, whereas other cells follow the leader cells; furthermore, collective migration retains cell-cell adhesions, and cells migrate in the same direction at a similar speed and affect one another while migrating [21]. In some carcinomas, such as breast [20], colon [6], prostate [7], and thyroid gland tumors [23], cancer cells invade cohesively with features of collective migration. Recently, the concept of EMT has been challenged because pathologists have not observed direct evidence of EMT in tissues until recently [11, 33]. Some scholars have proposed different views that collective cell migration versus single-cell migration and epithelial versus mesenchymal characteristics might combine to varying degrees, not only in different migratory events but also at different times in a single migratory process [3]. However, the mechanism that promotes liver cancer cell collective migration remains unclear.

Netrin-1, a diffusible laminin-related protein, has been observed to play a major role in the control of neuronal navigation during the development of the nervous system [28]. A recent study has suggested that guidance signaling regulates leading-edge behavior during the collective cell migration of

cardiac cells in *Drosophila*, and Slit- and Netrin-mediated signals maintain the balance between epithelial and mesenchymal characteristics of migrating cardioblasts [25]. An increasing number of studies have found that Netrin-1 is involved in the development and metastasis of many types of cancers, such as colorectal cancer [29], metastatic breast cancers [12], lung cancer [9], and neuroblastoma [10], and Netrin-1 overexpression has been shown to be a selective advantage for tumor progression. We previously demonstrated that Netrin-1 induces EMT and promotes liver cell invasiveness in a hypoxic environment [32]. The effect of Netrin-1 on the collective migration of cancer cells has not been studied.

In this study, we first observed the phenomenon of collective cell migration in liver cancer tissues, and Netrin-1 was upregulated in cancer cells at the interface front of the tumor. To study cancer cells migrating in a more physiologically relevant environment, we established a 3D cell culture model using a hydrogel. Interestingly, we clearly observed collective cell migration in 3D-cultured liver cancer cells, demonstrating that certain liver cells invaded the Matrigel as leader cells, forming a canal, and cancer cells followed along the canal.

Cadherin-mediated adherens junctions are important for collective cell migration [15], and both E-cadherin and N-



**Fig. 8** Netrin-1 regulates 3D liver cancer cell collective migration in an N-cadherin-dependent manner. **a** Dynamic analysis showed that knockdown of N-cadherin diminished the enhanced collective migration of Huh7-Netrin-1 cells; pictures show the representative images at the 6th and 14th day. **b** The quantification of the distances of migration in the 3D

culture at 6th day, 10th day, and 14th day,  $p < 0.001$ . **c** Analysis of the cell migratory behavior suggested that overexpression of N-cadherin restored collective cell migration in SK-Hep1-shNetrin-1 cells; the dynamic migration was also quantified in **(d)**,  $p < 0.001$ . The assay shown is representative of three experiments with similar results

cadherin have been shown to regulate cancer cell collective migration. We have shown that Netrin-1 decreases E-cadherin in liver cancer cells [32]; therefore, we speculated that Netrin-1 could regulate collective cell migration in an N-cadherin-dependent manner. Using immunofluorescence, we found that collectively invading liver cancer cells maintained adherens junctions and expression of N-cadherin, suggesting that liver cancer cells could invade through an N-cadherin-dependent collective cell migration mechanism. Then, we stably overexpressed or knocked down Netrin-1 expression in liver cancer cells. The overexpression or knockdown of Netrin-1 did not affect the expression of N-cadherin in 2D-cultured liver cancer cells. However, Netrin-1 did regulate N-cadherin expression in the 3D cell culture model. Furthermore, N-cadherin knockdown diminished Netrin-1-

enhanced collective cell migration, and overexpression of N-cadherin in Netrin-1-knockdown SK-Hep1 cells restored collective migration, suggesting that Netrin-1 regulates liver cancer collective migration in an N-cadherin-dependent manner in the 3D cell culture model.

Overall, increased Netrin-1 expression in liver cancer cells increased the likelihood of collective cell migration and was associated with distant metastasis. Adhesion molecules play important roles in cancer metastasis, and they seem to be closely related to collective migration [13]. Although we demonstrated N-cadherin-mediated collective cell migration in this study, whether other adhesion molecules, such as BVES (blood vessel epicardial substance), which we have indicated to be regulated in liver cancer cells [17], are involved in the collective migration of liver cancer cells deserves further study.



**Funding information** This study was supported by the National Natural Science Foundation of China (No. 81472311, No. 81572419, and No. 81702396).

## Compliance with ethical standards

This study was performed according to the guidelines of the Ethics Committee of the Tongji Hospital and approved in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

**Conflict of interest** The authors declare that they have no conflict of interest.

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