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Suspension state promotes extravasation of breast tumor cells by increasing integrin β1 expression

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Abstract: Mechanical microenvironment can strongly affect the metastatic efficiency of circulating tumor cells. However, the effect of suspension state on their extravasation and the mechanisms involved are still unclear. To explore the influence of suspension state on extravasation (including adhesion, spreading and transendothelial migration) of breast tumor cells and its relevant molecular mechanism, MDA-MB-231 cells were cultured on poly (2-hydroxyethyl methacrylate) coated 6-well plates to minic the suspension state. Suspension state promoted adhesion, spreading and transendothelial migration of MDA-MB-231 cells to EAhy926 endothelial cells (ECs) monolayer under both the static condition and 0.5 dyne/cm^2 flow shear stress (FSS). The number of cells adhered to ECs monolayer reached 2.15 (static condition, 3 d) and 1.67 (FSS, 3 d) times, and the number of migration reached 10.60 times, respectively, as compared to that in adhesion state. Moreover, MDA-MB-231 cells knockdown of integrin β1 provoked poor adhesion and transendothelial migration, as compared with MDA-MB-231 cells. But it made no difference in cell spreading. Our results implied the increasing expression of integrin β1 induced by suspension culture promoted the adhesion and transendothelial migration of MDA-MB-231 cells, but had no significant influence on their spreading.

Introduction

Major advances in screening and treatment of primary tumors have been made, but metastases are still the main cause of cancer-related deaths (Chen *et al*., 2016). Hematogenous metastasizing of tumor cells is the main way of tumor distant metastasis, which triggers a cascade of multiple events. Because of the hemodynamic shear forces and immunological stress, the majority of circulating tumor cells (CTCs) die within the circulation (Chamber *et al*., 2000; MacDonald *et a*l., 2002) and only a minor part of CTCs survive (Mitchell and King, 2013) and may experience extravasation, which is a key and limiting step for cancer metastasis (Heyder *et al*., 2002).

One of the differences between CTCs and primary tumor cells is their poorly anchored or suspension states, which result in sharp differences in their functional and molecular characteristics (Kim *et al*., 2012; Charpentier *et al*., 2013).

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In the suspension state, tumor cells experience both structural and biochemical changes, which affect their sensitivity to treatment and their whole metastatic efficiency (Matrone *et a*l., 2010). It has been reported that the suspended human breast cancer cell lines produce microtubule-based, dynamic extensions of the plasma membrane, which promotes tumor cell aggregation (Yoon *et al*., 2011) and reattachment to the endothelial layer (Whipple *et al*., 2010). However, the effect of suspension state on extravasation and the mechanisms involved are unclear.

During the extravasation of CTCs, integrin β1 (ITGB1) plays a significant role. A previous study showed that cells expressing low levels of ITGB1 were unable to keep their protrusions into the subendothelial matrix. In addition, attenuation of ITGB1 in a syngeneic tumor model led to a reduced metastatic colonization of the lung (Chen *et al*., 2016). Our transcriptome sequencing data showed that suspension state could induce the increasing expression of many kinds of cell adhesion molecules (CAMs), especially ITGB1 in MDA-MB-231 cells (Zhang *et al*., 2017). Thus, we speculated that the suspended state might promote extravasation of CTCs in an ITGB1 dependent way.

In this study, MDA-MB-231 cells, as the cell model, were cultured in 6-well plates coated with poly (2-hydroxyethyl methacrylate) (poly-HEMA, a nonadhesive material) to mimic the suspension state of CTCs. The effect of suspension state on extravasation of MDA-MB-231 cells and the role of ITGB1 during this process will be discussed. Our results suggest that suspension state of breast tumor cells, inducing high ITGB1 expression, promotes the extravasation of MDA-MB-231 cells.

Materials and Methods

Cell culture

Human breast cancer cells MDA-MB-231 (Chinese Academy of Sciences, China) and EAhy926 ECs (American Type Culture Collection) were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (TBD, China), 100 UI penicillin/mL, 100 UI streptomycin/mL, 2 mM L-glutamine and 26 mM $NaHCO_3$. All cells were cultured in an incubator, with a condition of 37°C and 5% CO_2. When the cells reach 80%-90% confluence, 0.25% trypsinization (trypsin/EDTA/water, 0.25:0.02:1, w/w/v) was used to transfer the generation. In order to mimic suspension state of CTCs, MDA-MB-231 cells were seeded on 6-well plates coated with 100 mg poly-HEMA/mL (Sigma) in anhydrous alcohol. Human umbilical vein endothelial cells (HUVEC) were obtained as described in a preceding study (Chen *et al*., 2016).

Immunofluorescence

EAhy926 ECs or HUVECs were cultured on glass slide with 1 mL suspension at 1×10^6 cells/mL or 48-well plate with 0.2 mL suspension at 5×10^5 cells/mL or Transwell (24-well Transwell inserts, Corning) upper chamber with 0.2 mL suspension at 5×10^5 cells/mL, respectively. After overnight culture, cells were fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature (20°C-25°C), and then permeabilized with 0.5% (v/v) Triton X-100. Later, the cells were rinsed with PBS and incubated with rhodamine phalloidin (1:200, Invitrogen) for 2 h and DAPI (1:1000, Beyotime, China) for 10 min, under ambient condition. Finally, after the cells were flushed with PBS, an inverted fluorescence microscope (IX71, Olympus) was used to obtain the images.

Small interfering RNA (siRNA) transfection

MDA-MB-231 cells were seeded at 4×10^4/well in 6-well plates and were cultured overnight. The following days, the cells were incubated with a transfection medium including 20 nM siRNA (GenePharma, China) and oligofectamine (Invitrogen) transfection reagent for 6 h-8 h. MDA-MB-231 cells were digested with pancreatin and then seeded into normal 6-well plates or poly-HEMA coated 6-well plates, for 48 h or 72 h. ITGB1 siRNA (si-ITGB1) (5' -GGGCAAGGCCUUGCAGCUC-3'), and the control mock siRNA (si-Ctrl) (5'-AUUGUAUGCGAUCGCAGAC-3') were used.

Real time-PCR

To confirm the inhibition efficiency of the siRNA to ITGB1 at mRNA level, cell samples were collected after being cultured for 48 h. Total RNA was obtained using Total RNA Kit (Tiangen, China) according to the accurate operating process offered by manufacturer. cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis kit (Takara, Japan) on the basis of the operations supplied by the company. The reaction system of PCR includes: pre-denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 10 sec, 57°C for 10 sec, and 72°C for 30 sec. The human ITGB1 primer sequences were 5'-ATGCACGAGGTGGTGAACG-3' (sense) and 5'-GGAGGCAGGTGTCTTGGAAC-3'(antisense), and the human GAPDH primer sequences were 5'-ACCCAGAAGACTGTGGATGG-3' (sense) and 5'-TTCTAGACGGCAGGTCAGGT-3' (antisense).

Western blotting

Cell samples were collected after 72 h treatment, and then rinsed with PBS and lysed with RIPA buffer on ice. The BCA Protein Assay Kit (Beyotime, China) was used to assess the protein concentration of samples. Prepared samples including 40 μg proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford), previously blocked with 5% (w/v) milk. Then membranes were incubated with primary antibodies at 4°C overnight. The next day, corresponding secondary antibodies (1:2000, Beyotime, China) were used to work on the membranes at 37°C for 1 h. These detections were visualized using an enhanced chemiluminescence reagent (ECL, Thermo). The images were got by VersaDoc imaging system and then Quantity One Software (Bio-Rad) was used for densitometric analysis.

Parallel-plate flow chamber adhesion assay

The architecture and operational theory of the parallel-plate flow chamber had been represented exactly (Zhang *et al.*, 2012; Mittal and Dwivedi, 2017) and the schematic of the parallel-plate flow-chamber adhesion assay was referred to the previous report by our group (Zhang *et al.*, 2012). This study explored the adhesion of MDA-MB-231 cells to ECs monolayer both under static condition and 0.5 dyne/cm^2 of flow shear stress (FSS). A glass slide (75 mm×25 mm) with 1 mL EAhy926 ECs suspension at a cell concentration of 1×10^6 cells/mL was incubated for 1 day-2 days. The MDA-MB-231 cells, which were stained with 2 μM calcein-AM (Invitrogen), were harvested and washed twice with PBS. Then the MDA-MB-231 cells were resuspended at 2×10^5 cells/mL in high-glucose DMEM medium with 10% (v/v) FBS. The parallel-plate flow chamber adhesion assay could be carried out after 1 h of incubation at 37°C. Cell-free culture medium was injected into the chamber to balance the ECs and wash the loose ECs away at 0.5 dyne/cm^2 for 20 min. Then the prepared MDA-MB-231 cells suspension was injected into the chamber at 0.5 dyne/cm^2 for 10 min. Finally, the chamber was perfused with PBS to remove non-adherent cancer cells at 0.5 dyne/cm^2 for 5 min. The experiment was repeated under static condition with a cell concentration of 1×10^5 cells/mL for 10 min, following the same protocol. In these assays, the parallel-plate flow chamber system was settled in the incubator always and repeated three times

respectively. The number of adherent cells was quantified with a fluorescence microscope (at least 15 random fields per glass slide at x 20).

Spreading assay

0.2 mL EAhy926 ECs suspension at a cell concentration of 5×10^5 cells/mL was seeded into 48-well plates and incubated overnight. The next day, 0.2 mL calcein-AM labeled MDA-MB-231 cells at a cell concentration of 1×10^5 was seeded into 48-well plate and incubated for 15 min, 30 min, 60 min, 360 min. Images were acquired by inverted fluorescence microscope (IX71, Olympus). The morphologies of MDA-MB-231 cells were quantified by ImageJ software (NIH, USA). The data of cell area and circularity were automatically obtained after tracing the cell outlines manually. The circularity is defined as 4π (area/perimeter^2), a circularity value of 1 means a perfect circle. At least 30 cells were selected for analysis.

Transwell assay

Transwells (8 μm pore size) were coated with 10 μg Matrigel (BD Bioscience, Bedford) for 2 h at 37°C. Then 0.2 mL HUVECs suspension at a cell concentration of 5×10^5 cells/mL was seeded into the upper chamber and incubated overnight. The next day, 0.2 mL calcein-AM labeled MDA-MB-231 cells at a cell concentration of 1×10^5 cells/mL were seeded into upper chamber with serum-free medium. Then 600 μL culture medium containing 10% (v/v) FBS was added to the lower chamber as chemoattractant. After 6 h, non-migrated cells were removed with cotton swab. Migrated cells were fixed with 3.7% (w/v) paraformaldehyde. The number of migrated cells was quantified by counting of at least five random fields per Transwell.

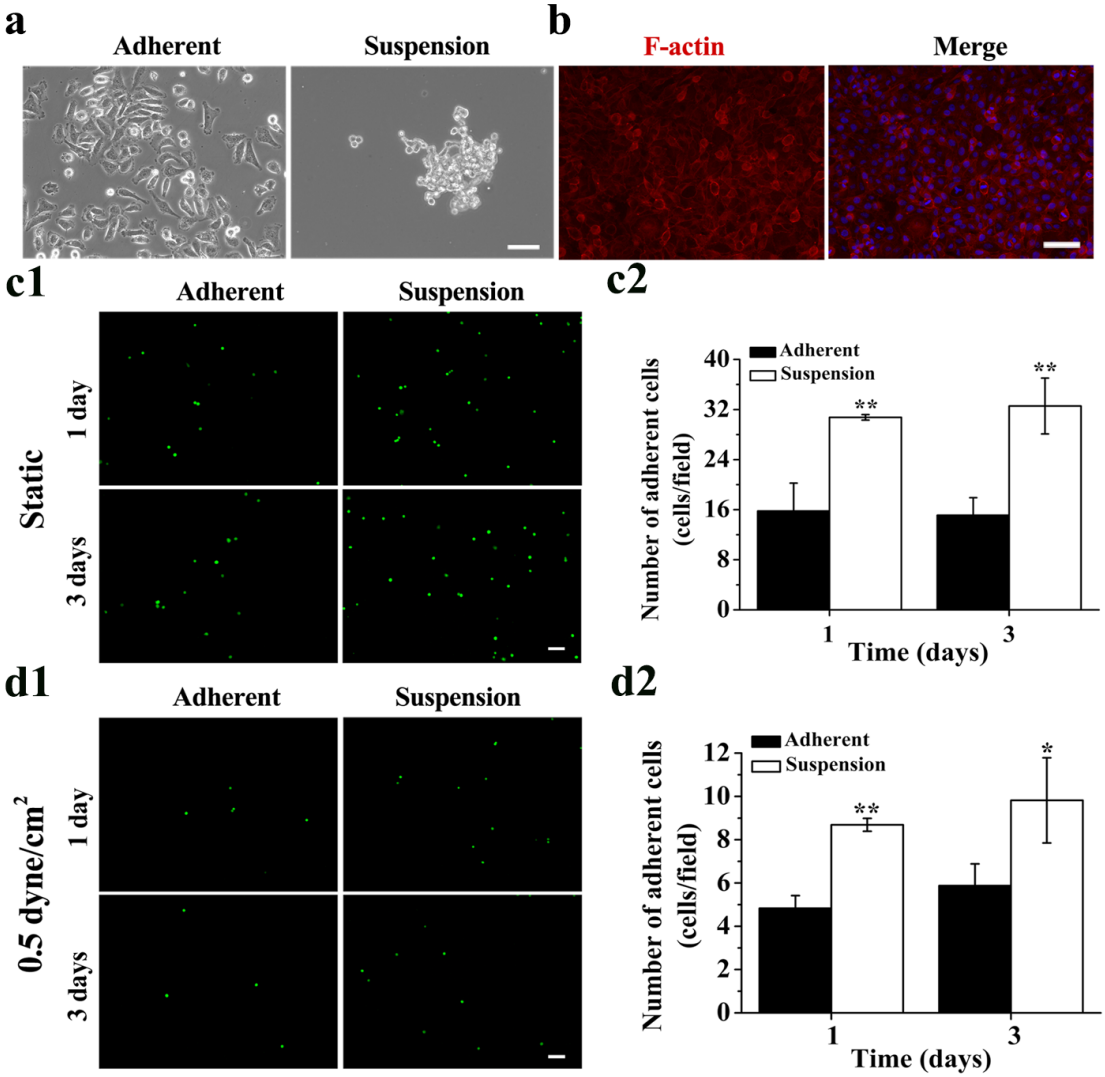

FIGURE 1. Suspension state promotes adhesion of MDA-MB-231 cells on EAhy926 ECs monolayer. (a) The morphology of adherent and suspended MDA-MB-231 cells. (b) The F-actin of EAhy926 ECs was stained with rhodamine-phalloidin (red). Cellular nuclei were stained with DAPI (blue). Scale bar = 100 μm. The adherent and suspended MDA-MB-231 cells were adhered to the EAhy926 ECs monolayer under static condition (c) or 0.5 dyne/cm^2 shear stress (d). Data was presented as mean ± SD, n = 3. * P<0.05. ** P<0.01 vs. adherent group.

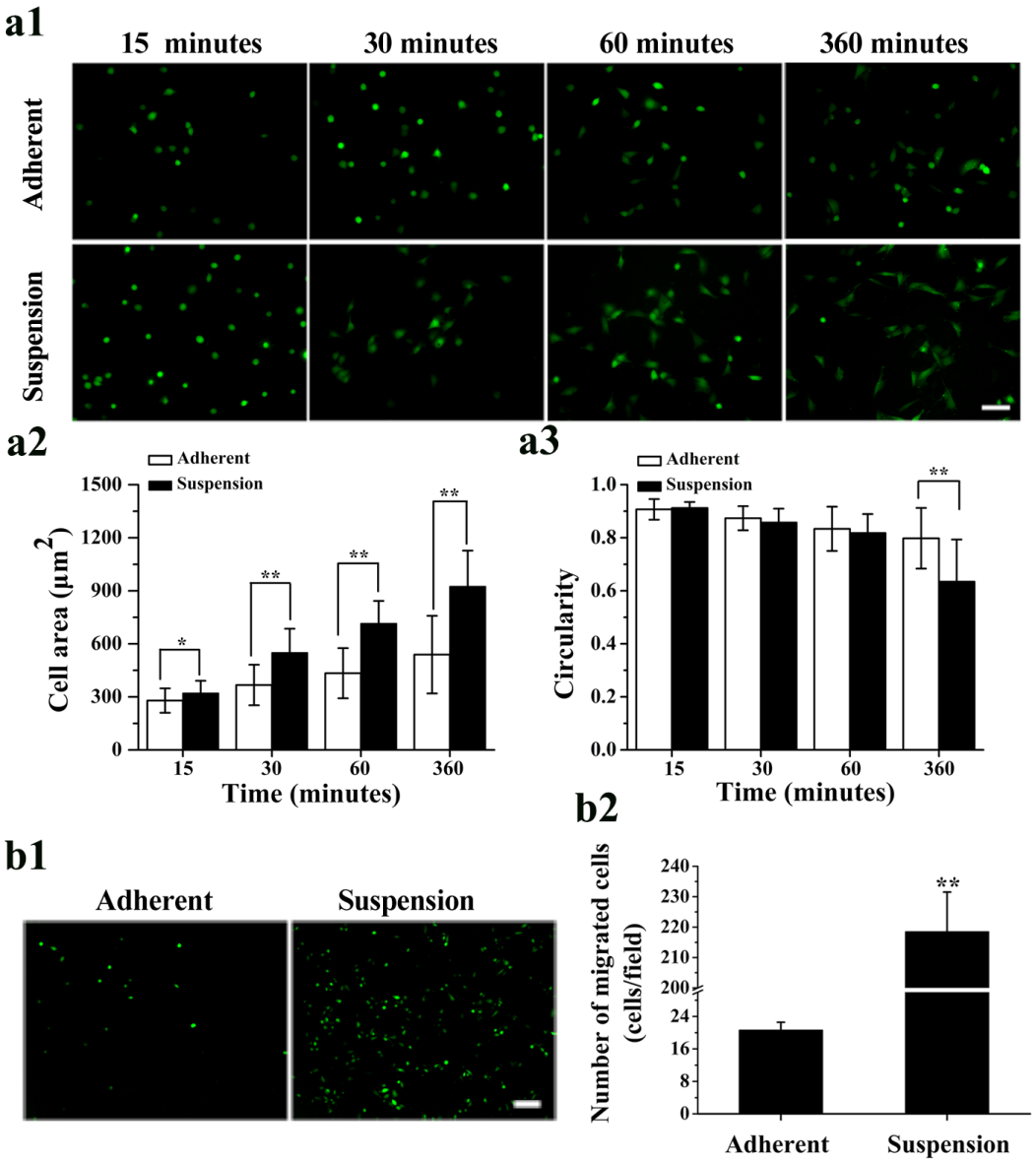

FIGURE 2. Suspension state promotes spreading and transendothelial migration of MDA-MB-231 cells. (a) The representative pictures of calcein-AM labeled adherent and suspended MDA-MB-231 cells spreading on EAhy926 ECs monolayer. Scale bar: 100 μm. Data was presented as mean ± SD, n = 30. b The representative pictures of calcein-AM labeled the migrated MDA-MB-231 cells. Scale bar: 100 μm. Data was presented as mean ± SD, n = 3. * P<0.05. ** P<0.01 vs. adherent group.

Statistical analysis

Each experiment was repeated 3 times or more. All data were expressed as means ± standard deviation (SD). The significant difference of the experimental data was evaluated by one way analysis of variance (ANOVA). $P < 0.05$ indicated a significant difference between the two sets of data, and the $P < 0.01$ indicated an extremely significant difference.

Results

Suspension state promoted adhesion of breast tumor cells to ECs monolayer

Suspension state caused significant morphological changes of MDA-MB-231 cells compared with adherent conditions (Fig. 1a). In order to obtain 100% confluent ECs monolayer, high density EAhy926 ECs or HUVECs were cultured on glass slides or 48-well plates or Transwell upper chambers overnight. More detailed analyses of cytoskeletal distribution and topography of ECs monolayer were performed by confocal imaging of nuclei and F-actin (Fig. 1b). The results showed that the connection of ECs was tight, which could mimic the intravital vascular endothelial barrier. Parallel-flow chamber assays were performed to examine the adhesion of MDA-MB-231 cells to EAhy926 ECs monolayer both under static condition (Fig. 1c) and 0.5 dyne/cm^2 of FSS (Fig. 1d). MDA-MB-231 cells were labeled with calcein-AM to distinguish the MDA-MB-231 cells from the ECs. The results showed that suspension state, maintained for 24 h or 72 h, increased significantly the number of adherent MDA-MB-231 cells to EAhy926 ECs monolayer both under static station and 0.5 dyne/cm^2 of FSS. There was no significant

difference between the adherent cells at 24 h and 72 h.

Suspension state promoted spreading and transendothelial migration of breast tumor cells

As shown in Fig. 2a, at the initial stage of spreading, MDA-MB-231 cells appeared round and homogeneous. Suspended MDA-MB-231 cells had bigger areas than adherent ones because of halo. After 30 min, the spreading ability of suspended MDA-MB-231 cells on EAhy926 ECs monolayer apparently was stronger than adherent ones. With the extension of time, most of suspended MDA-MB-231 cells began to spread obviously and form filaments at the edge of the cells, while the shape of adherent ones did not obviously change. Transendothelial migration assay of MDA-MB-231 cells showed that more suspended MDA-MB-231 cells migrated into the lower chamber compared with the adherent MDA-MB-231 cells (P<0.01) (Fig. 2b). These results showed that the suspended MDA-MB-231 cells had better spreading and transendothelial migration, as compared with adherent cells.

Effects of ITGB1 on the adhesion of breast tumor cells

ITGB1 silencing was verified via qPCR (Fig. 3a) and western blotting (Fig. 3b). Integrin β1's mRNA expression level of suspended MDA-MB-231 cells is 2.35±0.58 times of that of adherent cells (P<0.05). Knocking-down of ITGB1 significantly decreased ITGB1's mRNA expression level of adherent MDA-MB-231 cells from 1 to 0.38 (±0.10, S.D., n=3) (P<0.01), and decreased the ITGB1's mRNA expression level of suspended MDA-MB-231 cells from 2.35 (±0.58, S.D., n=3) to 0.61 (±0.04, S.D., n= 3) (P<0.01) (Fig. 3a). The western blot results showed that integrin β1's protein expression level of suspended MDA-MB-231 cells is 2.17 ± 0.40 times of that of adherent cells (P<0.01). Knock-down of ITGB1 significantly decreased the integrin β1's protein expression levels of adherent MDA-MB-231 cells from 1 to 0.61 (±0.07, S.D., n=3) (P<0.01), and decreased the integrin β1's expression levels of suspended MDA-MB-231 cells from 2.17 (±0.40, S.D., n=3) to 0.61 (±0.07, S.D., n=3) (P<0.01) (Fig. 3b).

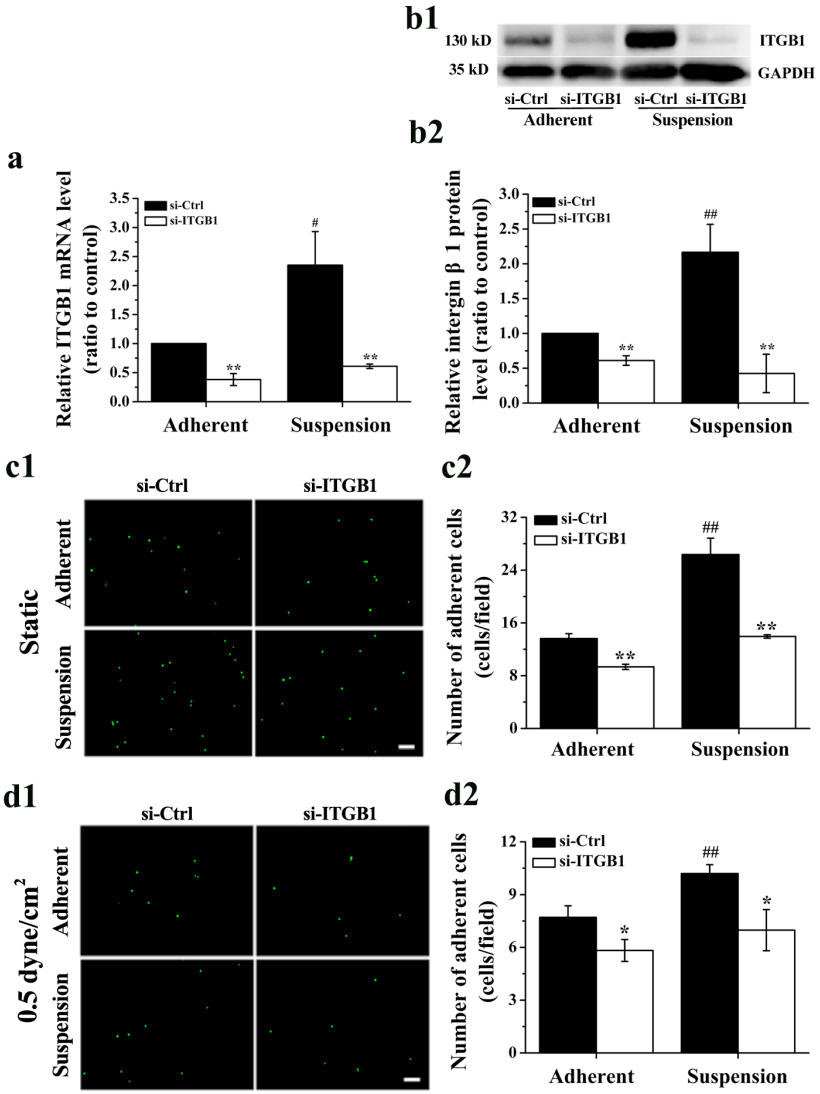

FIGURE 3. Effect of ITGB1 on the adhesion of MDA-MB-231 cells. The expression level of ITGB1 in MDA-MB-231 cells was detected by qPCR (a) and western blot (b). Data was presented as mean ±SD, n=3. The adherent and suspended MDA-MB-231 cells were adhered to the EAhy926 ECs monolayer under static condition (c) or 0.5 dyne/cm2 shear stress (d). Scale bar: 100 μm. Data was presented as mean ±SD, n=3. * P<0.05, ** P<0.01 *vs.* si-Ctrl group. # P<0.05, ## P<0.01 vs. Adherent-Ctrl group.

Under static condition, 13.62 (±0.74, S.D., n=3) cells/field adherent cells adhered to the ECs monolayer (Fig. 3c). Knock-down of ITGB1 significantly decreased the number of adherent MDA-MB-231 cells to 9.33 (±0.39, S.D., n=3) cells/field (P<0.01). 26.35 (±2.48, S.D., n=3) cells/field suspended cells (cultured for 72 h) adhered to the ECs monolayer. Knock-down of ITGB1 significantly decreased the number of adherent MDA-MB-231 cells 13.94 (±0.26, S.D., n=3) cells/field (P<0.01).

Under 0.5 dyne/cm^2 of FSS, knock-down of ITGB1 significantly decreased the number of adherent MDA-MB-231 cells from 7.71 (±0.66, S.D., n=3) to 5.82 (±0.62, S.D., n=3) cells/field (P<0.01) (Fig. 3d). 10.19 (±0.51, S.D., n=3) cells/field suspended cells adhered to ECs monolayer. Knock-down of ITGB1 significantly decreased the number of suspended cells to 6.98 (±1.17, S.D., n=3) cells/field (P< 0.01).

Influence of ITGB1 on the spreading and transendothelial migration of breast tumor cells

To further assess the role of ITGB1 in spreading and transendothelial migration of MDA-MB-231 cells on EAhy926 ECs monolayer, spreading assay of MDA-MB-231 cells on EAhy926 ECs monolayer was carried out. Fig. 4a showed that ITGB1 depletion MDA-MB-231 cells (Ad-ITGB1 and Susp-ITGB1) have similar spreading areas and perimeters on ECs monolayer compared with those on the control group (Ad-Ctrl and Susp-Ctrl). Thus, knockdown of ITGB1 had no significant effects on the spreading of MDA-MB-231 cells on ECs monolayer.

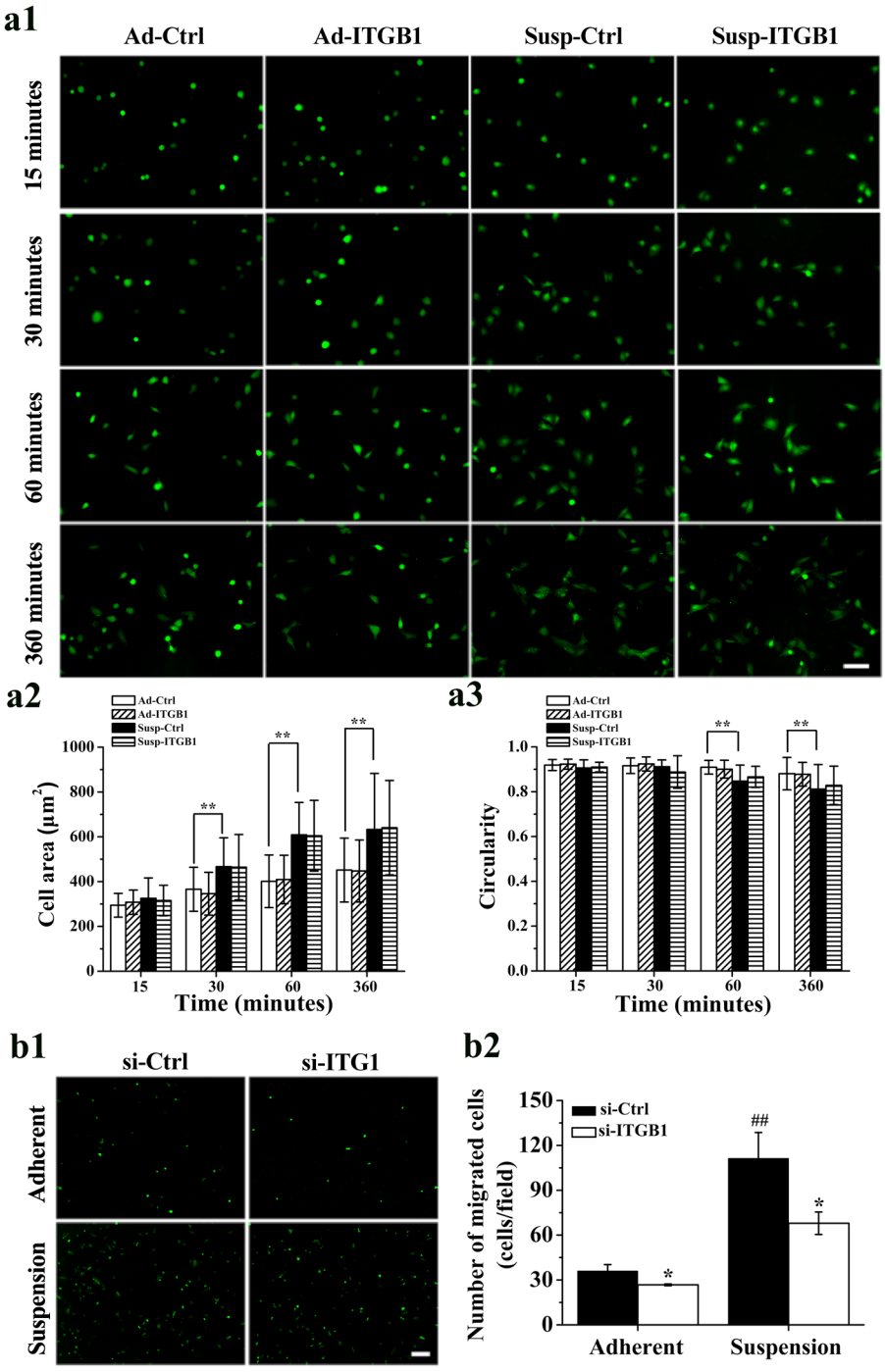

FIGURE 4. Influence of ITGB1 on the spreading and transendothelial migration of MDA-MB-231 cells on EAhy926 ECs monolayer. (a) The pictures of calcein-AM labeled adherent and suspended MDA-MB-231 cells spreading on EAhy926 ECs monolayer. Scale bar: 100 μm. Data was presented as mean ±SD, n=30. ** P<0.01 *vs.* si-Ctrl group. (b) Calcein-AM labeled the migrated MDA-MB-231 cells. Scale bar: 100 μm. Data was presented as mean ±SD, n=3. * P<0.05, ** P<0.01 vs. si-Ctrl group. ## P<0.01 vs. si-Ctrl group in adherent cells. Ad: adhesion, Susp: suspension.

Furthermore, transendothelial migration assay of MDA-MB-231 cells was performed by Transwell. Fig. 4b showed that 36.07 (±4.21, S.D., n=3) cells/field adherent MDA-MB-231 cells migrated into the lower chamber. Knock-down of ITGB1 significantly decreased the number of migrated MDA-MB-231 cells to 26.73 (±0.55, S.D., n=3) cells/field ($P<0.05$). For suspended cells, knock-down of ITGB1 significantly decreased the number of migrated MDA-MB-231 cells from 111.37 (±17.29, S.D., n=3) to 67.93 (±7.51, S.D., n=3) cells/field ($P<0.01$) (Fig. 4b). But, suspended MDA-MB-231 cells still had more excellent transendothelial migration characteristic compared with adherent cells ($P<0.01$).

Discussion

During metastasis, the mechanical and biochemical microenvironment, which CTCs are facing, are different from those of solid tumor. A series of novelcytoskeletal, functional and molecular performances would be undertaken by tumor cells to overcome the alterations of microenvironment. Investigating the changes of CTC's characteristics may provide a new way to diagnose and treat cancer. It also deserves a further exploration whether these responses of tumor cells to the microenvironmental change could impact the metastatic potency of CTCs (Whipple *et al*., 2007; Xie *et al*., 2017).

As an important CAMs receptor, integrin is widely distributed on the surface of cells and closely related with cell proliferation, adhesion and migration (Arnaout *et al*., 2007; Luo *et al*., 2007). The β1 subunit has been the focus of the current study. ITGB1 is widely expressed on various cell surfaces, forming a complete structure by combining with diverse α subunits. The expression level or spatial redistribution of ITGB1 significantly affects the adhesion between cells and with the extracellular matrix (ECM) (Hynes 2002). Morini *et al*. (2000) found that ITGB1 is upregulated in highly invasive breast carcinoma cells *in vivo* and Grzesiak *et al*. (2011), Lahlou *et al*. (2011) and Zhou *et al*. (2014) showed that it has important effects on primary tumor progression and metastasis, in cancers of breast, ovary, pancreas and skin. *In vitro* studies have found that ITGB1 knock-down significantly inhibits the proliferation and metastasis of tumor cells (Grzesiak *et al*., 2011). Integrin could affect the dynamic of the cytoskeleton reorganization and maturation of the initial adhesion points. Overall, these researches indicate that ITGB1 plays an important part in mediating growth and migration of primary tumor cells. In this study, much more attention was paid on the role of ITGB1 in the extravasation of breast tumor cells under suspension condition.

MDA-MB-231 cells were cultured on poly-HEMA coated 6-well plates to mimic the suspension state of CTCs. The adhesion, spreading and migration of MDA-MB-231 cells on ECs monolayer were also examined. The results showed that the suspension state could significantly promote the high expression of ITGB1 and suspended MDA-MB-231 cells had better adhesion, spreading and transendothelial migration characteristics. Knockdown of ITGB1 by siRNA significantly inhibited the adhesion and transendothelial migration of MDA-MB-231 cells, but had no significant effects on spreading. The suspended MDA-MB-231 cells with ITGB1 knock-down had similar adherent ability when compared with adherent ones, indicating that ITGB1 plays a primary role in the adhesion of MDA-MB-231 cells to ECs monolayer. The suspended MDA-MB-231 cells with ITGB1 knock-down showed a stronger ability for transendothelial migration, when compared with adherent ones, indicating that ITGB1 just plays a partial role in this process. The spreading assay results indicated that other adhesion molecules, such as intercellular cell adhesion molecule-1 or fibronectin, rather than integrin β1, likely mediates spreading of tumor cells on ECs monolayer.

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