

# Caldesmon suppresses cancer cell invasion by regulating podosome/invadopodium formation

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**Abstract** The podosome and invadopodium are dynamic cell-adhesion structures that degrade the extracellular matrix (ECM) and promote cell invasion. We recently reported that the actin-binding protein caldesmon is a pivotal regulator of podosome formation. Here, we analyzed the caldesmon's involvement in podosome/invadopodium-mediated invasion by transformed and cancer cells. The ectopic expression of caldesmon reduced the number of podosomes/invadopodia and decreased the ECM degradation activity, resulting in the suppression of cell invasion. Conversely, the depletion of caldesmon facilitated the formation of podosomes/invadopodia and cell invasion. Taken together, our results indicate that caldesmon acts as a potent repressor of cancer cell invasion.

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**Keywords:** Caldesmon; Podosome; Invadopodium; Invasion

## 1. Introduction

Cell invasion, one of the hallmarks of cancer progression, is mediated, at least in part, by podosomes and invadopodia. These protrusions from the ventral surface of the plasma membrane are highly dynamic cell-adhesion structures that degrade the extracellular matrix (ECM) by secreting matrix metalloproteases. Thus, podosomes/invadopodia permit cells to cross the basement membrane and invade tissues [1–4]. These adhesive structures are referred to as “podosomes” in monocyte-derived cells, osteoclasts, smooth muscle cells, and Src- or Rous sarcoma virus (RSV)-transformed cells and as “invadopodia” in cancer cells. Because podosomes and invadopodia appear to have similar morphologies, functions, and molecular compositions, they are considered related structures with different cellular contexts. Accumulating evidence suggests that actin and its associated proteins are important for the formation and dynamics of podosomes/invadopodia [3,4]. It was recently reported by three independent groups that the actin-binding protein caldesmon (CaD) [5] plays a critical role in regulating the formation and dynamics of podosomes in smooth muscle cells

and RSV-transformed fibroblasts [6–8]. We demonstrated that CaD negatively regulates podosome formation in RSV-transformed fibroblasts by competing with the Arp2/3 complex, and that the phosphorylation of CaD by p21-activated kinase 1/2 further enhances CaD's action [8]. Moreover, CaD is down-regulated in several transformed and cancer cells [9–11]. The significance of the reduced expression of CaD in cancer progression is, however, unclear. Here we demonstrate that CaD acts as a potent repressor of cancer cell invasion.

## 2. Materials and methods

### 2.1. Cell culture and antibodies

BY1 is a clonal cell line of Rous sarcoma virus (RSV)-transformed 3Y1 cells derived from Fisher rat embryos [12]. The human breast cancer (MB435s) and human colon carcinoma (HCA7) cells were purchased from ATCC and ECACC, respectively. The rat breast cancer (MTC) and murine melanoma (B16F10) cells are kind gifts from Dr. T. Irimura (Tokyo University) and Dr. S. Taniguchi (Shinshu University). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Gibco).

Antibodies were purchased as follows: anti- $\alpha$ -tubulin (DM 1A Sigma), anti-HA (3F10 Roche), anti-caldesmon [10], anti-p-34 arc (Upstate).

### 2.2. Transfection and clone isolation

Cells were transfected with a pcDNA3.1(+)-HA-CaD vector [8] using the Optifect transfection reagent (Invitrogen). To isolate stable BY1 cell lines expressing HA-CaD (BY1-CaD), BY1 cells were transfected with the pcDNA3.1(+)-HA-CaD vector, cultured with 100  $\mu$ g/ml G418, and the drug-resistant clones were isolated.

### 2.3. Immunofluorescence microscopy

Cells were plated on coverslips, fixed using 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. The cells were incubated with primary antibody, followed by the appropriate secondary antibody. To visualize actin filaments, Alexa 568-conjugated phalloidin (Molecular Probes) was added.

### 2.4. ECM degradation assay

Coverslips were coated with fluorescein isothiocyanate (FITC)-conjugated gelatin [13]. The cells were cultured on the FITC-gelatin-coated coverslips for 20–24 h, and then fixed in 4% paraformaldehyde. To visualize F-actin, the cells were stained with Alexa-568-conjugated phalloidin.

### 2.5. Invasion and migration assays

The invasion activity was measured by Boyden-chamber assay using BD BioCoat Matrigel Invasion Chambers (BD Biosciences), as described previously [8]. The lower chambers were filled with DMEM with 10% FBS, and cells were placed in the insert chambers, which

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**Abbreviations:** ECM, extracellular matrix; RSV, Rous sarcoma virus; CaD, caldesmon; FITC, fluorescein isothiocyanate

contained DMEM without FBS. After 24 h of incubation, cells that had invaded through the Matrigel were fixed using 4% paraformaldehyde, stained with hematoxylin-eosin, and counted.

The migration activity was measured by Matrigel-uncoated Boyden-chamber assay using BD BioCoat Control Inserts (BD Biosciences) as in the invasion assay except for a shorter incubation time (12–24 h).

### 2.6. RNA interference

Cells were transfected with indicated siRNAs using HiPerFect Transfection Reagent (Qiagen) and cultured for 3 days before analysis. The sequences of siRNA were as follows: rat CaD ATCGAGCTTAA-GATTGTCCAA (Qiagen), human CaD CAGATAGGTATCAA-TATGTTT (Qiagen), mouse CaD ACCAACCACATCGGAAGTCAA (Qiagen). As a control, scrambled siRNA (Santa Cruz) was used.

## 3. Results and discussion

### 3.1. CaD is a potent repressor of podosome/invadopodium formation

We first analyzed the relationship between the expression levels of CaD and podosome/invadopodium formation in rat fibroblast (3Y1), RSV-transformed 3Y1 (BY1), human colon carcinoma (HCA7), murine melanoma (B16F10), human breast cancer (MB435s), and rat breast cancer (MTC) cell lines. The CaD expression level in BY1 cells was markedly lower than in 3Y1 cells. In BY1 cells, podosomes containing dot-shaped F-actin clusters were predominantly observed in place of stress fibers, as previously reported (Fig. 1a and b) [8,10]. Both HCA7 and B16F10 cells expressed extremely low levels of CaD, whereas MB435s and MTC cells expressed higher amounts of it, similar to the level in 3Y1 cells (Fig. 1a). HCA7 and B16F10 cells formed invadopodia, while MB435s and MTC cells exhibited fully extended stress fibers without invadopodia (Fig. 1b), indicating that the expression level of CaD may be inversely correlated with invadopodium formation in cancer cell lines. To test this possibility, we expressed exogenous HA-tagged CaD (HA-CaD) in BY1, HCA7, and B16F10 cells. The ectopic expression of HA-CaD in these cell lines reduced the number of podosomes/invadopodia and led to the formation of new cortical actin bundles with thin stress fibers (Fig. 1c). These results suggest that when CaD is expressed at low levels, podosome/invadopodium formation increases in transformed and cancer cells, and that higher levels of CaD inhibit their formation.

### 3.2. CaD overexpression suppresses podosome formation, ECM degradation and invasion

To examine whether CaD's regulation of podosome/invadopodium formation is involved in cell invasion and migration, we isolated stable BY1 cell lines expressing HA-CaD (BY1-CaD cells). The total amount of exogenous and endogenous CaD in three BY1-CaD clones (C4, C5, and C11) was nearly equivalent to that of endogenous CaD in the parental 3Y1 cells (Fig. 2a). Consistent with the morphological changes in BY1 cells transiently transfected with HA-CaD (Fig. 1c), these three clones exhibited a reduced number of podosomes, a flattened cell morphology, and stress fiber formation (Fig. 2b).

Next, we tested the ECM degradation activity of podosomes using FITC-conjugated gelatin-coated coverslips (Fig. 2c). The 3Y1 cells lacking podosomes did not degrade the FITC-gelatin. In contrast, BY1 cells exhibited efficient degradation activity, as shown by dark spots in the FITC-gelatin (Fig. 2c). None of three BY1-CaD clones, which had reduced numbers of

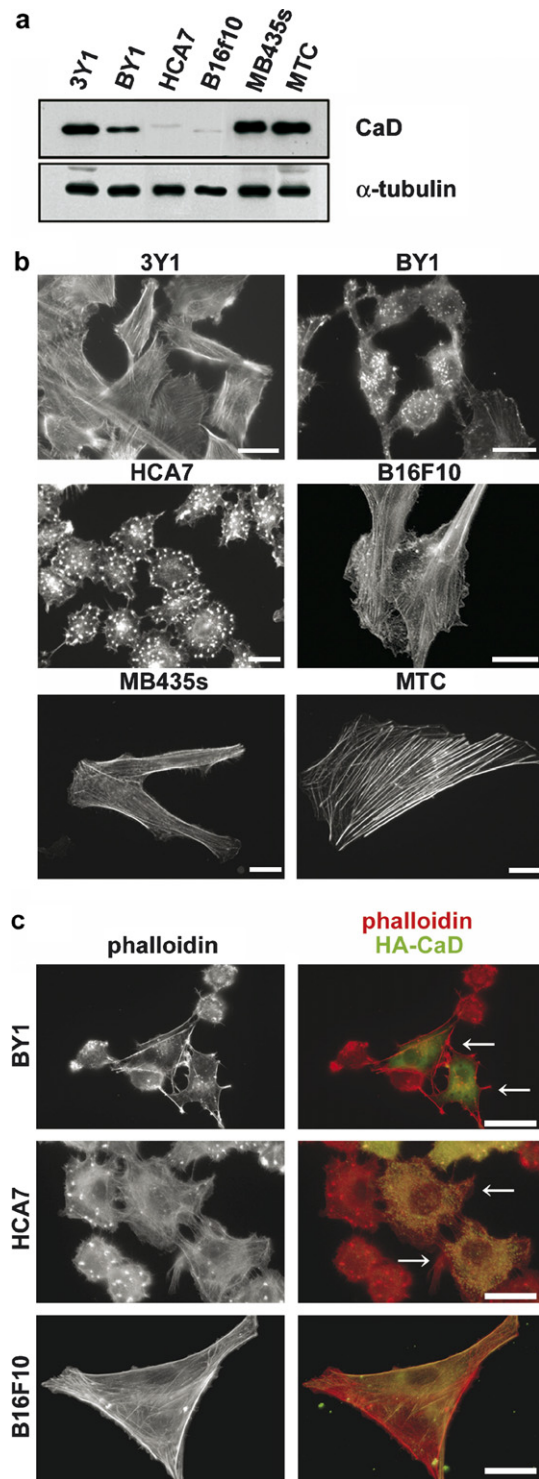


Fig. 1. Relationship between the expression levels of CaD and podosome/invadopodium formation. (a) Total proteins were extracted from 3Y1, RSV-transformed 3Y1 (BY1), MB435s, HCA7, MTC, and B16F10 cell lines. The expression levels of CaD were compared by Western blot with anti-CaD and anti- $\alpha$ -tubulin antibodies. (b) Cells were stained with phalloidin to label the F-actin. Scale bar is 20  $\mu$ m. (c) BY1, HCA7, and B16F10 cells were transiently transfected with a pcDNA3.1(+)-HA-CaD vector (HA-CaD). The cells were stained with an anti-HA antibody (green) to detect the exogenous expression of HA-CaD and with phalloidin (red). Arrows show the HA-CaD-transfected cells. As the B16F10 cells exhibited high transfection efficiency, almost all cells were transfected with HA-CaD. Scale bar is 20  $\mu$ m.

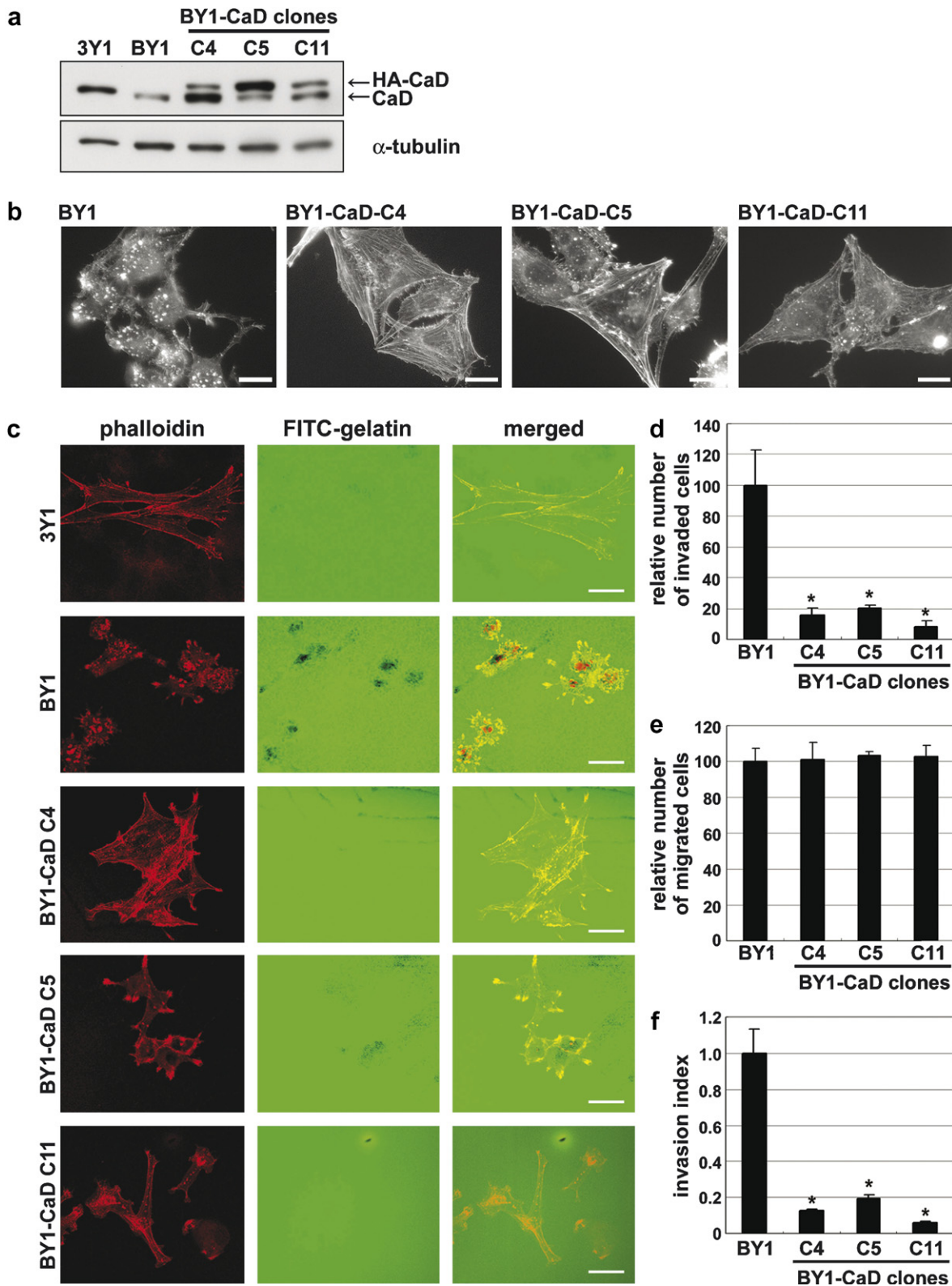


Fig. 2. ECM-degradation, cell invasion, and migration activities in BY1-CaD cells. (a) Total proteins were extracted from the BY1-CaD and their parental BY1 cells, and the expression levels of CaD were compared by Western blot using anti-CaD and anti- $\alpha$ -tubulin antibodies. The exogenous HA-CaD and endogenous CaD were distinguished by the differences in their molecular weights. (b) BY1-CaD cells (clones C4, C5, and C11) were stained with phalloidin. Scale bar is 20  $\mu$ m. (c) Coverslips were coated with FITC-conjugated gelatin (green). The cells were cultured on the FITC-gelatin-coated coverslips for 20–24 h. To visualize F-actin, the cells were stained with phalloidin (red). Scale bar is 30  $\mu$ m. (d) The invasion activity of BY1-CaD clones and parental BY1 cells was measured using Matrigel-coated Boyden-chamber assay. Statistical analysis was carried out for three independent experiments. Asterisks indicate significant differences from parental BY1 cells. \*  $P < 0.05$ . (e) The migration activities of BY1 cells and BY1-CaD clones were measured by Matrigel-uncoated Boyden-chamber assay. (f) The invasion index was calculated as the ratio of the number of cells invading through the Matrigel-coated chambers to the number of cells migrating through chambers without Matrigel coating, as assayed in d and e. Asterisks indicate a significant difference from parental BY1 cells. \*  $P < 0.05$ .



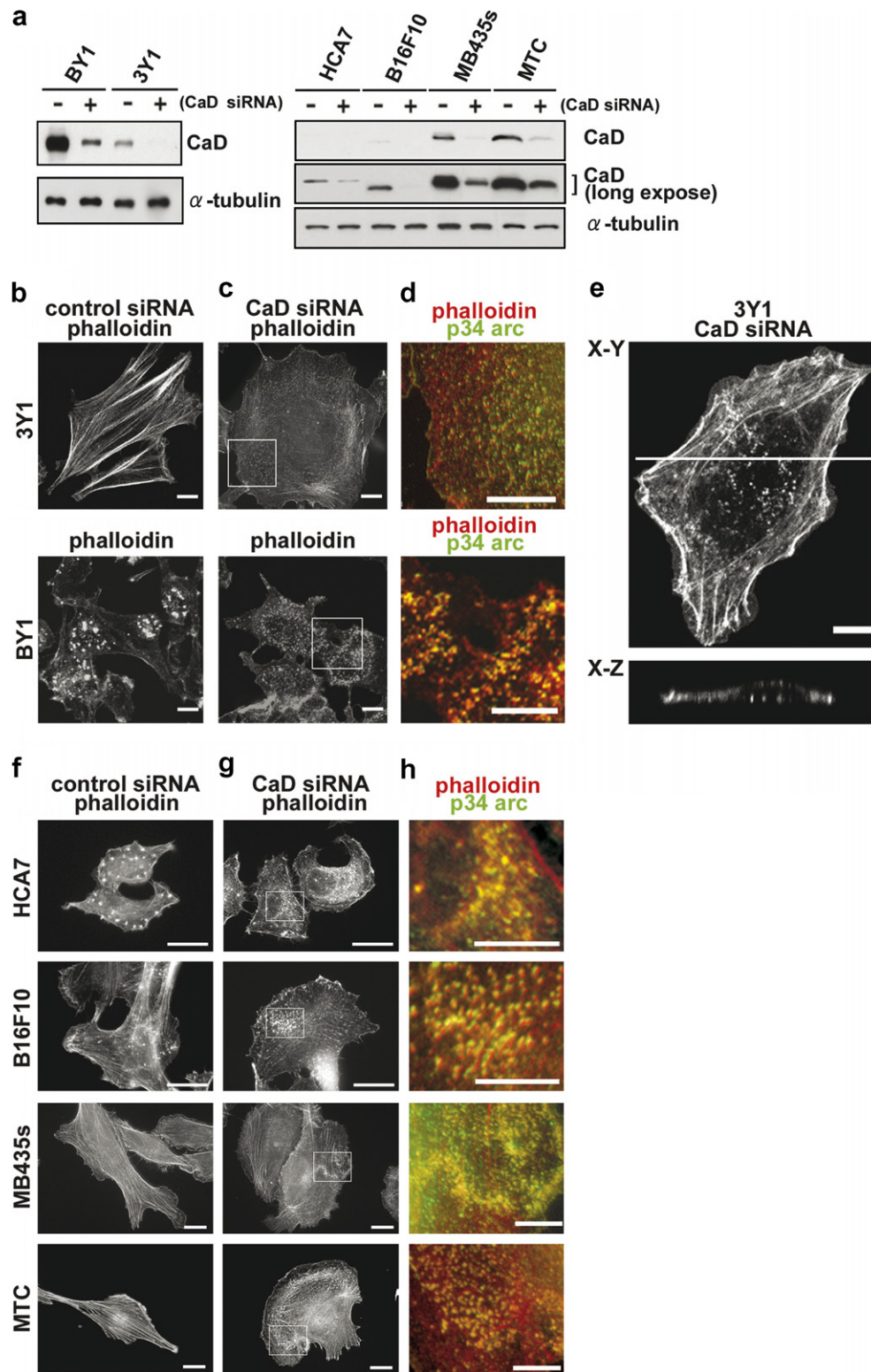


Fig. 3. Effect of CaD depletion on podosome/invadopodium formation. (a) 3Y1, BY1, HCA7, B16F10, MB435s, and MTC cells were transfected with control siRNA (–) or CaD siRNA (+). The total proteins were extracted from the cells, and the expression levels of CaD were measured by Western blot using an anti-CaD antibody. (b–d) 3Y1 and BY1 cells were transfected with control siRNA (b) or CaD siRNA (c). Three days after transfection, the cells were stained with phalloidin (b, c, and red in d) and an anti-p34 arc antibody (green in d). Boxed areas in c are shown at higher magnification in d. Scale bars are 20  $\mu$ m (b, c) and 10  $\mu$ m (d). (e) CaD-depleted 3Y1 cells were stained with phalloidin. Bottom image is the Z projection of the region indicated by the white line in the upper image. (f–h) HCA7, B16F10, MB435s, and MTC cells were transfected with control siRNA (f) or CaD siRNA (g). Three days after transfection, the cells were stained with phalloidin (f, g, and red in h) and the anti-p34 arc antibody (green in h). Boxed areas in g are shown at higher magnification in h. Scale bars are 20  $\mu$ m (f, g) and 10  $\mu$ m (h).

podosomes, degraded the gelatin. We also measured the invasion activity of three clones using a Matrigel-coated Boyden-chamber (Fig. 2d). In this assay, cells degraded the Matrigel and migrated through it. Compared with parental BY1 cells, the invasion activity of three BY1-CaD clones was markedly suppressed.

Since this assay measures both Matrigel degradation and cell migration, we also evaluated cell migration alone using a Boyden-chamber without Matrigel coating. In contrast to the results of Matrigel invasion assay, the cell migration activities of three BY1-CaD clones and parental BY1 cells were nearly equivalent (Fig. 2e). We expressed the Matrigel degradation activity as the invasion index, which was defined as the ratio of the invasion activity to the migration activity (Fig. 2f). The invasion index of BY1-CaD clones was significantly lower than that of parental BY1 cells (Fig. 2f), consistent with the results of ECM degradation assay (Fig. 2c). These data indicated that CaD's suppression of podosome formation decreases the invasion activity of cells.

### 3.3. CaD depletion facilitates podosome/invadopodium formation and cell invasion

We further examined the effect of CaD depletion on podosome/invadopodium formation and cell invasion using short interfering RNA against CaD (CaD siRNA). Western blots of 3Y1, BY1, HCA7, B16F10, MB435s, and MTC cells

showed that the endogenous expression of CaD was efficiently suppressed by CaD siRNA (Fig. 3a). Consistent with our recent report [8], the CaD-depleted BY1 cells had numerous, small podosomes (Fig. 3c). Interestingly, the CaD-depleted 3Y1 cells also showed a reduced number of stress fibers, and numerous small podosome-like structures at the ventral cell surface (Fig. 3c and e). The Arp2/3 subunit p34 arc, which is one of the initiator components of podosomes/invadopodia [8,14], was localized to these dot-shaped structures, as observed in podosomes of CaD-depleted BY1 cells (Fig. 3d). In HCA7 and B16F10 cells, CaD depletion also markedly increased the number of small invadopodia (Fig. 3g), as observed in CaD-depleted BY1 cells. The MTC and MB435s cells never formed invadopodia under control conditions, but CaD-depleted MTC and MB435s cells formed numerous small invadopodia, to which p34 arc was localized (Fig. 3h). These data indicated that the suppression of CaD expression permits invadopodium formation in some cancer cell lines.

We further examined the effect of CaD depletion on the invasion activity. As shown in Fig. 4a and b, both CaD-depleted 3Y1 and BY1 cells exhibited significantly higher invasion activity than their control siRNA-transfected cells, while their migration activities were not affected by CaD depletion. Importantly, in all the cancer cell lines examined, the invasion activity was markedly facilitated by CaD depletion, whereas the migration activity was not distinguishable from that of

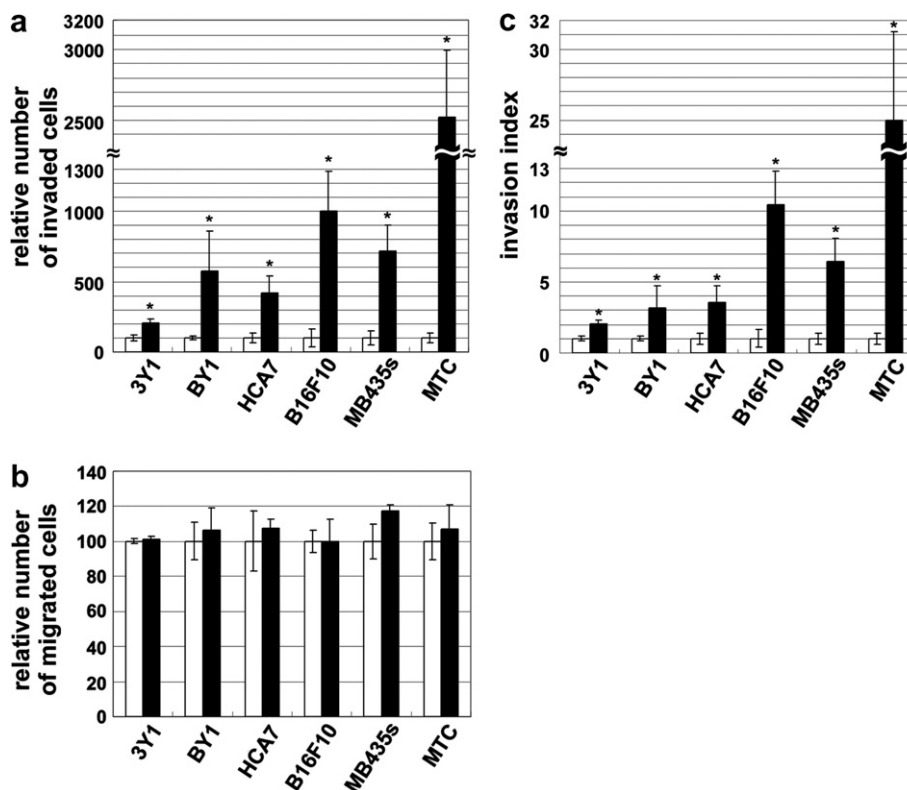


Fig. 4. Effect of CaD depletion on cell invasion and migration. (a) 3Y1, BY1, HCA7, B16F10, MB4352, and MTC cells were transfected with control siRNA (white bars) or CaD siRNA (black bars). Two days after transfection, the cells' invasion activities were measured. (b) 3Y1, BY1, HCA7, B16F10, MB4352, and MTC cells were transfected with control siRNA (white bars) or CaD siRNA (black bars). Two days after transfection, the cells' migration activities were measured. Statistical analysis was carried out for three independent experiments. Asterisks indicate significant differences from control siRNA-transfected cells. \* $P < 0.05$ . (c) The invasion index was calculated as the ratio of the number of invading cells to the number of migrating cells, as assayed in a and b. The white bars indicate the invasion index of control siRNA-transfected cells and the black bars indicate that of the CaD siRNA-transfected cells. Asterisks indicate a significant difference from the parental 3Y1 cells. \* $P < 0.05$ .

control cells (Fig. 4a and b). The invasion index of CaD-depleted cancer cells was higher than that of control cells, suggesting that CaD-depleted cancer cells had a higher degradation activity than control cells (Fig. 4c). These results indicated that CaD-depleted cancer cells, in which podosome/invadopodium formation is facilitated, have a higher invasion activity, reflecting a higher ECM degradation activity.

In summary, we demonstrated that CaD acts as a potent suppressor of cell invasion by regulating the podosome/invadopodium formation of transformed and cancer cells. The up-regulation of CaD expression suppressed the invasion activity and vice versa. Thus, the expression levels of CaD are inversely associated with cell invasiveness. The ECM degradation activity was also dependent on the regulation of podosome/invadopodium formation. Thus, CaD depletion facilitated podosome/invadopodium formation and ECM degradation activity, resulting in the increased invasion activity. Although further study is required to investigate the relationship between the expression levels of CaD and the malignancy of cancer cells *in vivo*, our present results provide a new insight supporting CaD as a plausible therapeutic target in cancer cell invasion and metastasis.

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