• Basic Research •

Calpastatin participates in the regulation of cell migration in BAP1-deficient uveal melanoma cells

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Abstract

• AIM: To detect how BRCA-associated protein 1 (BAP1) regulates cell migration in uveal melanoma (UM) cells.

• METHODS: Wound healing and transwell assays were performed to detect UM cell migration abilities. Protein chip, immunoprecipitations and surface plasmon resonance analyses were applied to identify BAP1 protein partners. Western blot and calpain activity assays were used to test the expression and function of calpastatin (CAST).

• RESULTS: CAST protein was confirmed as a new BAP1 protein partner, and loss of BAP1 reduced the expression and function of CAST in UM cells. The overexpression of CAST rescued the cell migration phenotype caused by BAP1 loss.

• CONCLUSION: BAP1 interacts with CAST in UM cells, and CAST and its subsequent calpain pathway may mediate BAP1-related cell migration regulation.

• KEYWORDS: uveal melanoma; BRCA-associated protein 1; calpastatin; cell migration

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INTRODUCTION

Uveal melanoma (UM) is the most common intraocular primary malignancy in adults. The estimated incidence of this disease is 5 or 6 cases per million per year[1-2], and almost half of the patients will die from metastases within approximately 10y[3-4]. The most predominant locations for metastases are the liver (89%), followed by the lungs (29%) and bone (17%)[5]. Thus, preventing metastasis at an early stage and discovering the underlying mechanism of micrometastasis are important topics.

In 2010, Harbour *et al*[6] reported that *bap1*, the gene encoding BRCA-associated protein 1 (BAP1), was mutated in approximately 84% of metastatic UMs and indicated that germline *bap1* mutations could cause a new cancer syndrome that is characterized by mesothelioma and UM. Recently, it has been widely proven that mutation of *bap1* in UM strongly indicates poor prognosis[2,6-9]. In our previous study, we summarized the prognosis of patients with UM in our hospital and found that 34% of the 156 patients were BAP1-negative, and their 5-year metastasis-free survival rate was 58% compared to 88% for the BAP1-positive patients (P=0.004)[9]. Additionally, mutated *bap1* is found in many other malignancies such as clear cell renal cell carcinoma, cholangiocarcinoma, colorectal cancer, lung cancers, and serves as a prognostic indicator[10-12].

Bap1 is presumed to be a tumour suppressor gene, is located on chromosome 3p21.1, and usually undergoes an inactive mutation of one copy and deletion of the other copy with the loss of one chromosome 3[13]. Dey *et al*[14] found that deletion of the *bap1* gene in mouse was lethal during embryogenesis, but systemic or haematopoietic-restricted deletion in adults demonstrated features of human myelodysplastic syndrome. At the cellular level, deficiency of BAP1 in UM cells results in a loss of cell differentiation and gain of stem-like properties[15]. Loss of BAP1 also affects cell cycle regulation; BAP1 knockdown can lead to G1 arrest and is accompanied by a decrease in the expression of S phase genes, thus slowing down the cell cycle[16]. In addition, after knockdown of BAP1, UM cells showed decreased cell migration, reduced motility in wound healing assays and reduced cell migration in transwell assays[15-16]. In a nude mouse model with tumour xenografts, BAP1-deficient cells formed fewer metastases in the liver and lungs than control cells[15]. Surprisingly, all these research results seem to have unexpected, paradoxical effects with the phenomenon on patients with *bap1* mutations, suggesting that BAP1 loss may promote tumour growth in a different manner than other well-characterized tumour suppressors.

The BAP1 protein is a member of the ubiquitin C-terminal hydrolase (UCH) subfamily of deubiquitylating enzymes[7] and serves as a regulator in maintaining the balance of the ubiquitination cycle of histone H2A and other proteins. It has been reported to interact with multiple proteins. BAP1 can bind to the BRCA1/BARD1 complex, which serves as a heterodimeric tumour suppressor complex and has important roles in dsDNA repair[6]. BAP1 also binds and de-ubiquitinates the transcriptional regulator host cell factor 1 (HCF-1). In particular, HCF-1 acts as a scaffold connecting histone-modifying enzymes with promoters and thus regulates gene expression by modulating chromatin structure[17]. In addition, BAP1 interacts with ASXL1 and helps to form the polycomb group repressive deubiquitinase complex, which is reported to participate in stem cell pluripotency and deubiquitinates histone H2A[6,18]. BAP1 can also interact with many other molecules, including OGT, YY1, HAT1, PRC1/2 and PHC. Thus, BAP1 may participate in a variety of biological processes, including DNA repair, gene transcription, cell membrane transport, the cell cycle, stress response, cell communication, cell differentiation and apoptosis, tumour occurrence and others[7]. However, how BAP1 regulates cell migration is unclear and needs to be explored.

In this study, we first screened and confirmed a new BAP1 protein partner, calpastatin (CAST), by means of protein chip, immunoprecipitations (IPs) and surface plasmon resonance (SPR) analysis. CAST is an inhibitor of calpain, which plays an important role in cell migration. Thus, we further explored the functional interaction between BAP1 and CAST in cell migration and motility. We demonstrated that CAST might play a key role in BAP1-related cell migration regulation in UM cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture Human UM OCM-1A (Beijing Beina Chuanglian Biotechnology Institute, Beijing, China; No.BNCC100672) and 92.1 cells (gift of Dr Sofie Qiao, Vivace Therapeutics, Inc.), and human cervical cancer HeLa cells (American type culture collection, ACTT, USA; No.CCL-2), which were all *bap1* wild-type, were used in this study. OCM-1A and 92.1 were cultured in RPMI-1640 (Gibco; No. 11875093) supplemented with 10% fatal bovine serun (FBS; Gibco, Carlsbad CA, USA; No.10099141), L-glutamine, and antibiotics (Gibco; No.10378016) with 5% CO_2, and HeLa cells were grown in DMEM (Gibco; No.11965092). In addition, 0.25% trypsin-EDTA (Gibco; No.25200056) was applied when passaging cells.

Transfection and Lentiviral Infection For the knockdown assay, lentiviral-based short hairpin RNA (shRNA; Obio Technology, Shanghai, China) was applied to deplete BAP1 or CAST. Lentiviral pLKD-eGFP shRNA vectors expressing the shRNA sequence against BAP1 (NM_004656.2, target sequence: CGTCCGTGATTGATGATGATA), CAST (NM_001042440, target sequence: GCTCGACCTCCGC TCAATTAA) and control (target sequence: TTCTCCGAA CGTGTCACGT) were constructed. In the overexpression experiments, CAST (pLenti-EF1a-EGFP-P2A-Puro-CMV-CAST-3Flag, NM_001042440) and control (pLenti-EF1a-EGFP-P2A-Puro-MCS-3Flag) were also constructed by Obio Technology. Viral production and infections were carried out according to an established protocol (Broad Institute). Cells in the control and knockdown/overexpression groups were harvested 72h post-infection to perform the following experiments with the blank group (without infection).

Cell Migration Assays Wound healing assays were carried out in these cell lines by plating 4×10^5 cells in 6-well plates overnight. Before scratching with a 200 μL tip, culture media was replaced with serum-free media. A live cell imaging system with a Leica microscope (Leica Microsystems, DMZ6000B, Germany) was applied to capture images (200×) every hour for 24 or 72h, and the scratch was measured using ImageJ.

In the transwell assay, suspended in serum-free medium, 150 μL of cells (1.0×10^5 cells/mL) was added to the upper chamber, and 600 μL of medium with 20% FBS was added to the lower chamber. After 24h, the cells from the upper side of the chamber were removed, and the chambers were soaked in crystal violet solution to stain the cells on the lower side for 20min. Subsequently, the chambers were washed three times with ddH_2O. When the chambers were dried, photos were taken of five random fields of view for every group using a microscope (Leica DMI3000B, Germany). Finally, the cell numbers were counted using ImageJ. Experiments were independently repeated three times.

Tracking the Migration of Tumour Cells With minor modification from a previously described protocol[19], eGFP images of tumour cells were acquired in a live cell imaging system. After being placed in 6-well plates, 5×10^4 cells were cultured overnight at 37℃. In every group, 3-4 random fields of view with at least 5 separate and recognizable eGFP+ cells were observed. Photos of every field were taken every 15min for 48h, and cell tracks were drawn and measured using an ImageJ plugin ("Manual Tracking" Rasband, W.S., ImageJ, U.S).

Antibodies and Proteins The primary antibodies used were mouse anti-human BAP1 antibody (Santa Cruz, Texas, USA; sc-28383), mouse anti-human calpastatin antibody (CAST, Santa Cruz; sc-20779), rabbit anti-human vinculin antibody (Santa Cruz; sc-5573) and rabbit anti-human calpain antibody (Santa Cruz; sc-30064). The secondary antibodies included goat anti-mouse antibody (Santa Cruz; sc-2005) and goat anti-rabbit antibody (Santa Cruz; sc-2004). The human full-

length proteins of BAP1 (Abnova, Taipei, Taiwan, China, H00008314-P01; Abcam, Cambridge, UK, ab188681) and calpastatin (Abcam, ab112256) were used in microarrays or SPR.

Human Proteome Microarray To screen for proteins interacting with BAP1, we used the HuProt™ microarray (CDI Laboratories, Inc., Mayaguez, Puerto Rico), which is composed of approximately 20 000 human full-length proteins with N-terminal glutathione S-transferase (GST) tags. The HuProt™ microarray was performed according to the following procedure, as previously described[20]. The human full-length BAP1 protein (Abnova) was first concentrated with an Amicon® Ultra 3kD super filter (Millipore), and the buffer solution was replaced with labelling buffer (Full Moon protein-labelling kit) to remove tris. Next, biotin-labelled human BAP1 recombinant protein was prepared for testing. The microarray was blocked for 1.5h in blocking buffer [5% bovine serum albumin (BSA) in 1×phosphate buffered saline with 0.1% Tween 20 (PBST), pH 7.2] and then incubated with BAP1 protein sample at a final concentration of 1 μg/mL in blocking buffer overnight at 4℃. After washing three times with 1×PBST and twice with ddH2O, the slide was incubated with Cy5-conjugated streptavidin (1:1000, Invitrogen, Camarillo, CA, USA) in the dark for 20min. After washing three times with 1×PBST and then three times with ddH2O, the microarray was spun to dryness. The slide was finally scanned with a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA), and the binding signals were acquired using GenePix Pro v6.0 software. The probes were considered detectable when the signal-to-noise ratios (SNRs) for both duplicates were greater than 0.3.

Immunoprecipitations and Western Blots To verify the interactions between BAP1 and selected proteins suggested by the proteome microarray in cells under physiological conditions, IP was performed using an anti-BAP1 antibody. OCM-1A cells (10^6) were lysed in lysis buffer (50 mmol/L Tris, pH 8.0; 150 mmol/L NaCl; 1% NP-40). Samples were then incubated on ice for 10min before a 10s low-power sonication. After removing cellular debris by centrifugation for 30min at 4℃, extracts of the cell lysates were collected and precleared by incubating with protein A/G sepharose beads (A10001, Abmart) and a blank IgG at 4℃ for 60min with gentle agitation. Pre-cleared cell lysates were then incubated with protein A/G beads and either the anti-BAP1 antibody or the IgG (control) overnight at 4℃ with gentle mixing. The beads were collected and washed 3 times with lysis buffer, and the immunoprecipitated proteins were eluted by 1×SDS-sample buffer (50 mmol/L Tris-HCl pH 6.8, 50 mmol/L DTT, 2% SDS). Subsequently, the IP samples and cleared lysates were subjected to SDS-PAGE followed by Western blotting with the indicated antibodies.

For Western blots, cells were lysed and centrifuged to obtain the extracts as previously described. For each sample, 30 μg of protein was loaded and subjected to SDS-PAGE for 2h. Next, the samples were transferred to a PVDF membrane and blocked in TBST (20 mmol/L Tris, pH 7.5. 150 mmol/L NaCl, 0.1% Tween 20) with 5% skim milk for 1h. The membranes were incubated with primary antibodies overnight at 4℃ with gentle mixing. After washing 3 times with 1×TBST, membranes were incubated with the corresponding secondary antibodies in the dark for 2h. After washing 3 times with 1×TBST, membranes were developed and photographed. Densitometry was performed on Western blots using ImageJ software.

Surface Plasmon Resonance Analysis To further determine the real-time data on affinity and interaction kinetics between two proteins (BAP1 and CAST), SPR analysis was performed using a ProteOn™ XPR36 protein interaction array system (Bio-Rad, Hercules, California, USA). According to the manufacturer's instructions, channel surfaces of the chip (BIO-RAD, ProteOn™ Sensor Chip, GLC 176-5011) were activated by injection of the amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 100 mmol/L) and N-hydroxysulfosuccinimide (sulfo-NHS, 25 mmol/L) (components of the ProteOn amine coupling kit). The human full-length CAST protein was immobilized onto the 1600RU channel after being diluted to 50 ng/μL in 10 mmol/L NaAc solution (pH 4.5). To deactivate the remaining carboxyl groups in the CAST and blank channels, 1 mol/L ethanolamine HCl, pH 8.5 (ProteOn amine coupling kit), was then injected at a flow rate of 30 μL/min for 300s. The channels were washed twice with running buffer (10 mmol/L PIPES, 150 mmol/L NaCl, 0.005% Tween 20, pH 6.0). Serial dilutions of BAP1 (Abcam) samples were prepared at 160, 64, 25.6, 10.24, 4.096, and 1.638 nmol/L in PIPES solution. Samples (400 μL) of each concentration were injected into the analyte channels orthogonal to the CAST and blank channels at a flow rate of 50 μL/min. The binding kinetics for the interactions of CAST and BAP1 were then rapidly and accurately obtained.

Calpain Activity To assess the activity of calpain in intact cells, we used a cell-permeable synthetic fluorogenic substrate for calpain, N-Succinyl-Leu-Leu-Val-Tyr 7-amido-4-methylcoumarin (suc-LLVY-AMC, Sigma, Missouri, USA; S6510)[21]. The intact substrate exhibits little fluorescence at 445 nm upon excitation at 345 nm. However, specific proteolysis of the substrate by calpain emits the fluorescent AMC group, leading to an increase in its fluorescence. After infection, the cells were washed with Hank's balanced salt solutions (HBSS) followed by digestion and suspension at 1×10^5/mL in HBSS. A 90 μL cell suspension was plated in 96-well plates and kept on ice until the assay was performed.

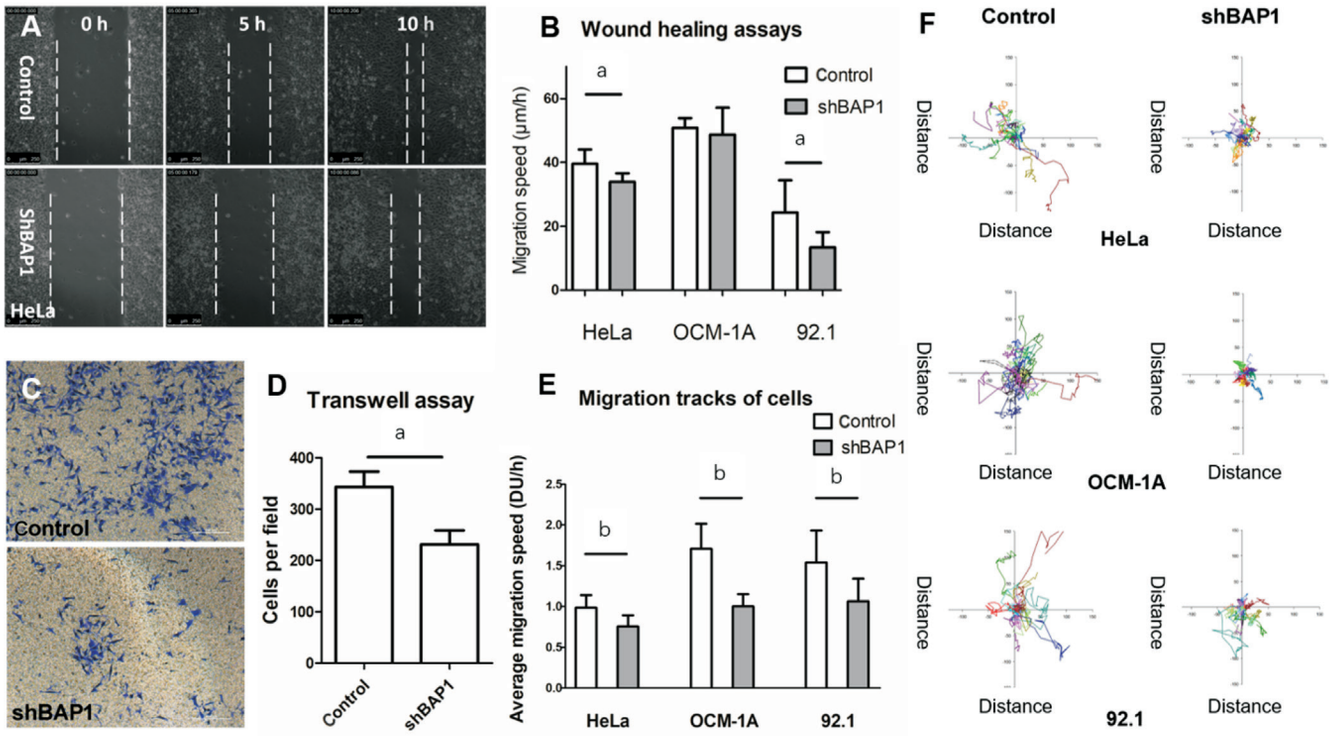

Figure 1 Loss of BAP1 reduces cell migration A, B: Representative images and quantification of wounding healing assays of control and shBAP1 groups in three cell lines (HeLa: human cervical cancer cell, OCM-1A and 92.1: human uveal melanoma cells); C, D: Transwell assay between control and shBAP1 groups in OCM-1A cells; E, F: Migration track comparisons between control and shBAP1 groups in three cell lines, and every line indicate the track of a single cell over 48h. n=3. Data are presented as the mean±SEM. Differences between groups were assessed by unpaired t-test. $^aP<0.05$, $^bP<0.01$. Scale bars, 200 μm.

The substrate suc-LLVY-AMC was kept in dimethyl sulfoxide (DMSO) (5 mg/mL) at -20℃ and dilated to 100 μmol/L in HBSS before the assay. The cells were rewarmed for 10min, then at t=-1min, 10 μL ionomycin in DMSO (2.5 μmol/L final concentration, ionomycin group) or DMSO (solvent control group) was added to the cells. At t=0min, 10 μL suc-LLVY-AMC was added to cells of both groups (1 μmol/L final concentration). At t=5, the fluorescence [in relative fluorescence units (RFU)] was read by a spectrofluorometer (BioTek, SynergyH1 Hybrid Reader) with an excitation wavelength of 345 nm and an emission wavelength of 445 nm. Subsequently, measurements were taken at t=30, 60, and 90min. This assay measured the initial activity of Ca^{2+}-dependent substrate (calpain) cleavage, determined by subtracting the ionomycin-independent RFU (solvent control group) from the ionomycin-dependent RFU (ionomycin group).

RESULTS

Loss of BAP1 Reduces Cell Migration To study the effects of BAP1 loss in cell lines, we used shRNA to knockdown the expression of BAP1. In the wound healing assays, of the three cell lines, HeLa and 92.1 cells in the shBAP1 group were less motile than control cells (Figure 1A, 1B). In the transwell assay, BAP1-deficient OCM-1A cells showed reduced cell migration (Figure 1C, 1D). To observe cell migration more directly, we used a live cell imaging system to track the migration of every single tumour cell. Unsurprisingly, all three cell lines displayed reduced motility and lower migration speed in the shBAP1 groups (Figure 1E, 1F).

Identification of Proteins Interacting with BAP1 Using the Human Proteome Microarray The HuProt™ microarray examines 47616 probes including interior labels, positive controls, blanks and 19841 proteins, and every protein has two duplicate probes in case of false positives. In our study, due to slight residual tris in the buffer, the background fluorescence of the chip was slightly high. However, the following 5 proteins were found to have an SNR>0.3 and interact with BAP1 protein: vinculin (VCL, GenBank: BC039174), CAST (NM_173060.2), phytanoyl-CoA hydroxylase-interacting protein-like (PHYHIPL, NM_032439.1), galectin-9 (LGALS9, NM_002308.3) and von Willebrand factor A domain-containing protein 5A (LOH11CR2A, NM_014622.4) (Figure 2A). Through a literature review, we found that VCL, CAST and LGALS9 may have functions in cell adhesion and migration, and the SNRs of VCL and CAST were both greater than 0.5. Thus, we chose VCL and CAST to perform IPs in UM cells.

BAP1 Interacts with Calpastatin To verify the interactions between BAP1 and VCL or CAST identified by proteome microarray under physiological conditions, IP in OCM-1A cells

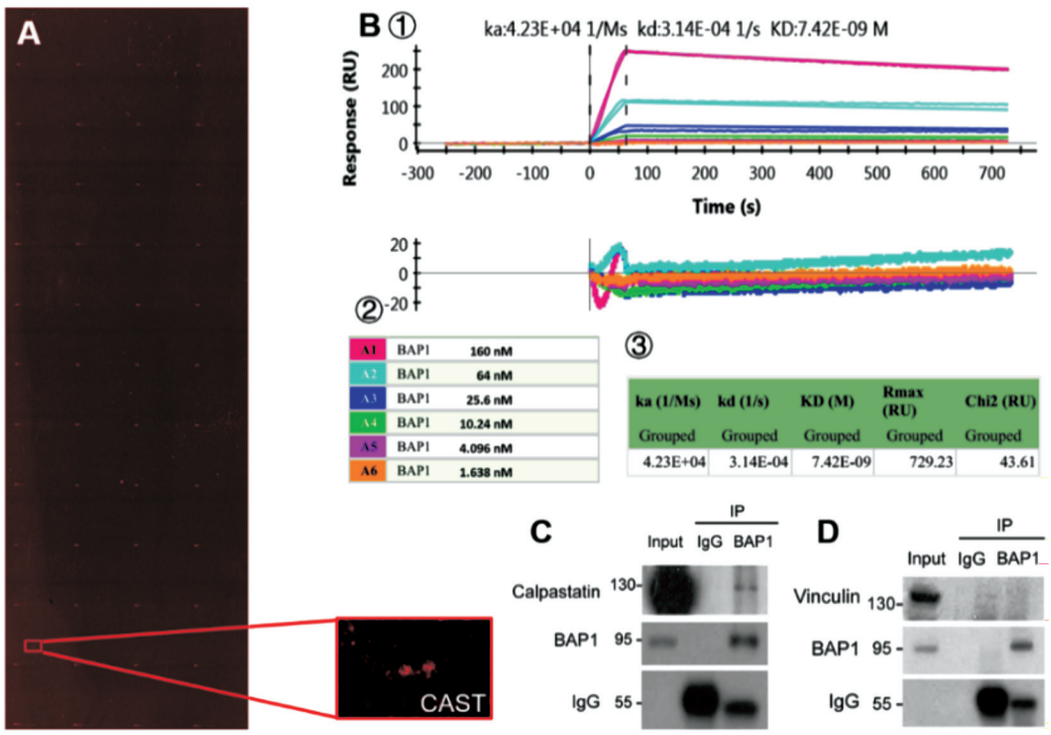

Figure 2 CAST interacts with BAP1 protein A: Scannogram of the human proteome microarray (HuProt™ microarray) detecting interacting proteins with BAP1 and a partially enlarged lattice indicating the positive signal of CAST; B: Interactions between BAP1 and CAST proteins by surface plasmon resonance analysis: ① the original graph (upper) and the weighted graph (lower) showing the binding kinetics for the interaction between CAST and BAP1 over time; ② legend showing dilutions of BAP1 protein; ③ table listing the dissociation constant (KD value, 7.42×10^{-9}) between these two proteins; C, D: IPs in OCM-1A cells to detect the protein interaction between BAP1 and CAST, as well as BAP1 and vinculin.

was performed. As shown in Figure 2C and 2D, BAP1 and CAST interacted, while interactions between BAP1 and VCL were not detected. To further determine the force and dynamics of the interaction between BAP1 and CAST, SPR analysis was performed, and the results are shown in Figure 2B. The dissociation constant (KD value) between the two proteins was 7.42×10^{-9}, which is relatively small, indicating a high affinity. The two proteins combined quickly in the early stage and dissociated slowly.

Loss of BAP1 Reduces the Expression and the Function of Calpastatin Since BAP1 is a deubiquitinase, which is usually involved in regulating the protein level of downstream targets, we tested whether BAP1 regulates the CAST protein level. By using immunoblotting, we found that knockdown of BAP1 significantly decreased the protein level of CAST in two UM cell lines (Figure 3A, 3B). CAST acts as an inhibitor of calpain protein, and its activity can be measured by the calpain activity assay using a fluorogenic substrate of calpain[22-24]. Thus, we next examined whether BAP1 deficiency could influence the function of CAST by monitoring the activity of calpain. In accordance with the Western blot data, the activity of calpain was significantly enhanced in the BAP1-deficient melanoma cells when compared to control cells (Figure 3C, 3D), indicating that the function of CAST was reduced in these cells. Thus, our data suggest that BAP1 is necessary for maintaining the CAST protein level, whereas defects in BAP1 cause reduced CAST function.

Calpastatin Rescues the Cell Migration Phenotype Caused by BAP1 Loss To directly test our hypothesis that CAST is a downstream target of BAP1 mediating its ability to regulate cell migration, we performed a rescue experiment by overexpressing CAST in 92.1 cells with BAP1 knockdown and then analysed the cell migration ability by wound healing and transwell assays. In the wound healing assay, consistent with our previous results, the migration ability of 92.1 UM cells was significantly reduced by knockdown of BAP1 (Figure 4A). We also noticed that knockdown of CAST alone slightly reduced cell migration but did not reach statistical significance, even with a more significant decrease in protein level compared with BAP1 knockdown (Figure 4E). This suggests that loss of CAST is not the only factor contributing to the BAP1-related migration phenotype (Figure 4A). However, after overexpressing CAST in the 92.1 cells with BAP1 knockdown, the migration ability was comparable to the control cells, suggesting a complete rescue by CAST (Figure 4A). Similarly, we observed that the overexpression of CAST also fully rescued the BAP1-related cell invasion defect in the transwell assay (Figure 4B, 4C). These two experiments indicate that

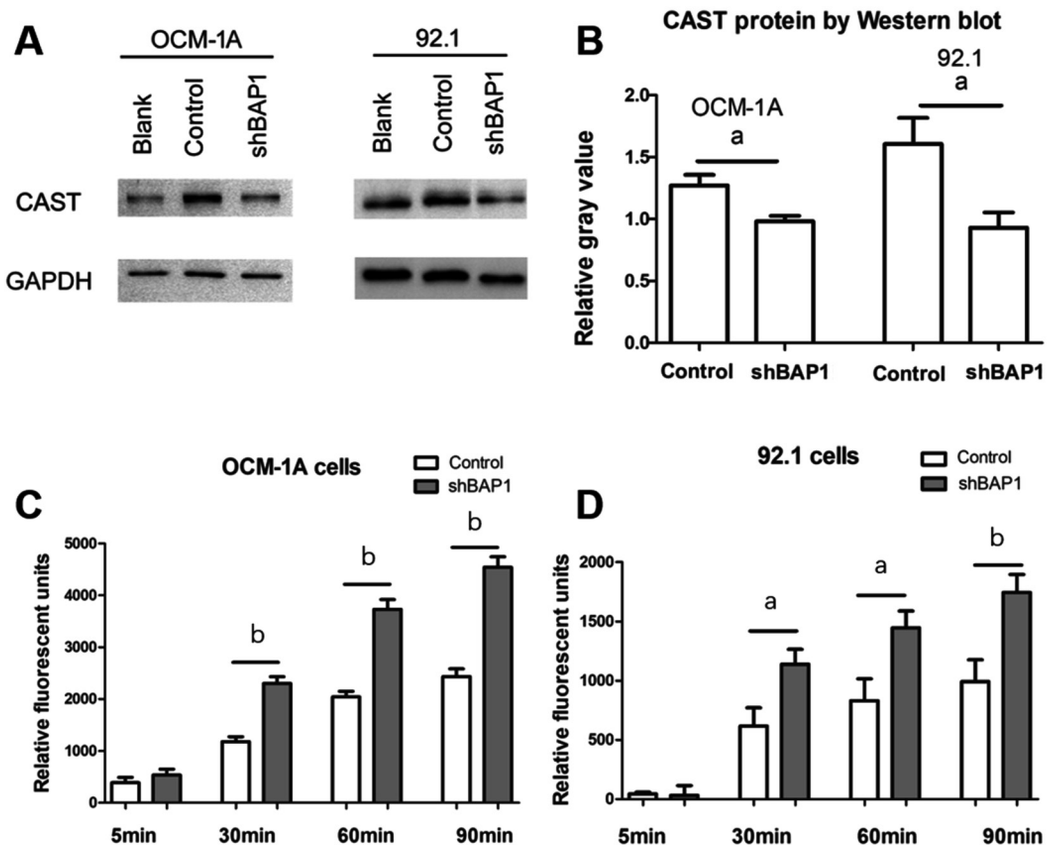

Figure 3 Loss of BAP1 decreased the expression and function of CAST A, B: CAST expression by Western blot between control and shBAP1 groups in OCM-1A and 92.1 cells; C, D: Calpain activity comparisons between control and shBAP1 groups in OCM-1A and 92.1 cells assessed by the calpain activity assay with Suc-LLVY-AMC. Larger fluorescence signal indicates higher calpain activity. Data are presented as the mean±SEM. Differences between groups were assessed by unpaired *t*-test. $^{a}P<0.05$, $^{b}P<0.01$.

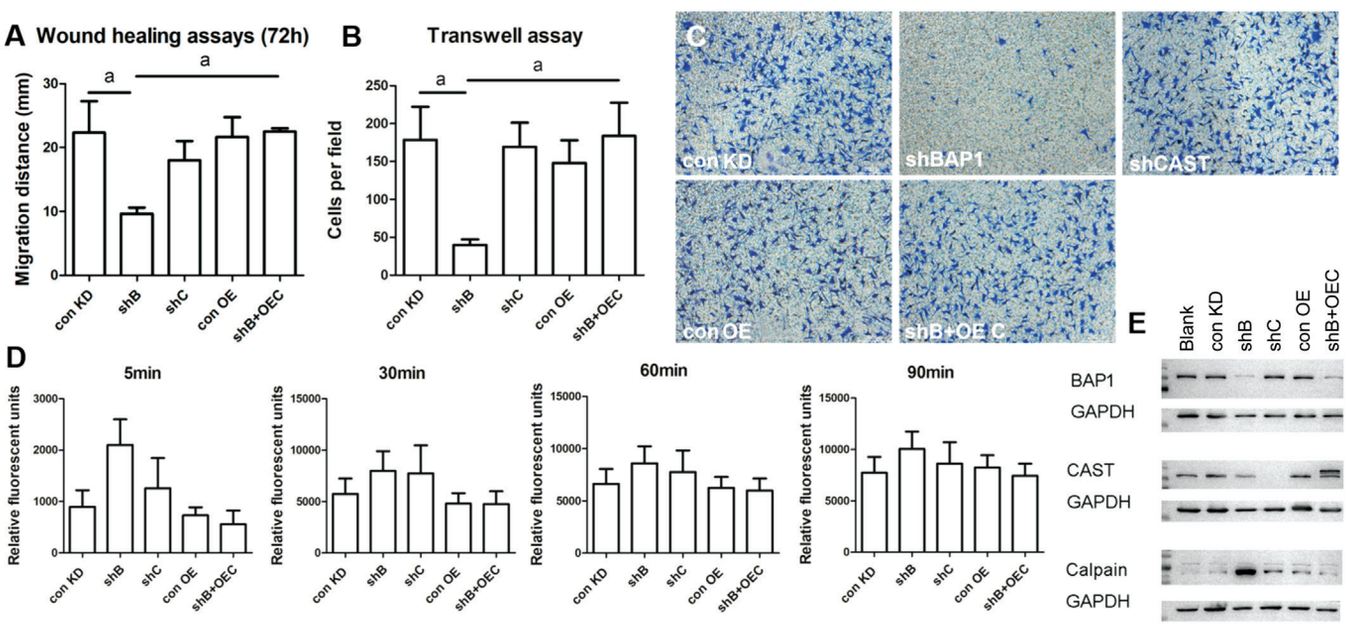

Figure 4 CAST overexpression in BAP1-deficient cells enhances cell migration A: Wounding-healing assay of indicated groups of 92.1 cells; B, C: Transwell assay of indicated groups of 92.1 cells with statistical results and representative images; D: The calpain activities of indicated groups by the calpain activity assay with Suc-LLVY-AMC; E: Protein levels of BAP1, CAST and calpain in 92.1 cells transduced with the indicated shRNAs were analysed by Western blot. con KD: Control for knockdown; shB: shBAP1; shC: shCAST; con OE: Control for overexpression; shB+OEC: shBAP1+overexpressing CAST. *n*=3. Data are presented as the mean±SEM. Differences between groups were assessed by unpaired *t*-test with/without Welch's correction. $^{a}P<0.05$.

when CAST was overexpressed in BAP1-deficient cells, cell migration in UM cells was rescued, which indicates that CAST may serve as a downstream target of BAP1.

To further confirm the rescue effect of CAST, we analysed its activity by calpain activity. Consistent with our previous data, knockdown of BAP1 increased calpain activity, suggesting that CAST is dysfunctional. However, overexpression of CAST in these cells fully rescued the calpain activity to the control level. Since CAST also inhibits calpain at the expression level, we further analysed the calpain expression level by Western blot. Consistent with a previous study[22-23], knockdown of CAST increased the calpain expression level (Figure 4E). We also found that the calpain expression level was significantly increased in the BAP1-deficient cells (Figure 4E), which suggests a reduction in CAST function and is consistent with our hypothesis that BAP1 is necessary for CAST function. Interestingly, however, the calpain level upon knockdown of CAST was significantly lower than that upon BAP1 knockdown (Figure 4E), suggesting that additional pathways contribute to BAP1-regulated calpain expression. After overexpression of CAST in these cells, the calpain expression level again dropped to the control level (Figure 4E), suggesting that CAST rescued the enhanced calpain activity in the BAP1-deficient cells.

Taken together, our data suggest that CAST and its subsequent calpain pathway may mediate BAP1-regulated cell migration.

DISCUSSION

The *bap1* mutation has been recognized as an indicator of poor prognosis in UM[6-7,13]. However, knockdown of BAP1 in UM cells leads to decreased cell migration[15-16], as shown in our study. These results seem paradoxical. Currently, there has not been an exact explanation for this phenomenon. We infer that there might be some possibilities. 1) Unlike the 95% of GNAQ and GNA11 mutations in UM that affect codon 209[25], there are no *bap1* mutation hotspots[26]. Multiple types and regions of mutation of *bap1* may lead to different functional consequences[17]. For example, mutations in the UCH region of *bap1* result in abrogation of its deubiquitination activity, while mutations in the NLS regions hinder its nuclear localization[17,27]. Thus, it is difficult to simulate all *bap1* mutations concurrently. On the other hand, knockdown of BAP1 using RNA interference cannot perfectly mimic the genetic background of patients. 2) Functions of BAP1 protein are extensive and include intracellular biological processes, such as DNA repair, gene transcription, cell cycle, cell differentiation and apoptosis[7], as well as extracellular activities, such as cell communication, cell membrane transport[7], cell adhesion[28] and transendothelial migration[29]. Most recent studies have focused on the intracellular processes, yet whether the extracellular function, which is affected in *bap1*-mutated cells, functions in tumour metastasis requires further study. Taken together, the results suggest that BAP1, as a significant high-risk indicator, needs to be studied more extensively.

In this study, we employed a knockdown model of BAP1 to detect its possible function and underlying mechanism in regulating cell migration. Based on the protein chip, IP and SPR analysis, we found that BAP1 can interact with CAST in UM cells. As an inhibitor of calpain, CAST suppresses the expression and activity of calpain[21-23]. Calpain is a calcium-dependent, soluble, neutral, protease that promotes cell motility with hydrolysis of specific substrates in integrin activation, adhesion complex turnover and membrane protrusion dynamics[23]. Calpain-mediated migration and invasion mechanisms include focal adhesion turnover, reinforcing the expression and activity of matrix metalloproteinases (MMP)1, MMP2 and urokinase plasminogen-type activators (uPAs), protein tyrosine phosphatase 1B (PTP1B)-SRC-mediated invadopodia formation, cortactin-mediated actin reorganization, as well as lamellipodia and pseudopodia stabilization at the leading edge of the cell[24]. Under calpain-mediated regulation of the cytoskeleton, cells can stretch and migrate similar to amoeba. Thus, we hypothesized that CAST might participate in cellular migration of *bap1*-mutated UM cells. First, we found the biological protein binding effects between BAP1 and CAST by means of proteome microarray and SPR and further verified the interactions under physiological conditions in UM cells using IP (Figure 2). Next, when we knocked down BAP1, CAST expression decreased, and the activity of calpain increased (Figure 3). Lastly, we overexpressed CAST in cells after BAP1 knockdown and found that the cell migration capacity in this group was significantly enhanced compared to the shBAP1-only group and restored fully to the control level. Our data suggest that BAP1 mediates cell migration by downregulating the CAST protein level and function. We also noticed that knockdown of CAST alone did not increase the cell migration ability. These data suggest that reductions in CAST protein level and function in BAP1-deficient cells alone may not be sufficient to alter cell migration behaviour and additional pathways that also change upon BAP1 loss are required to amplify such defects. However, overexpression of CAST fully restored the cell migration defect induced by loss of BAP1, suggesting that CAST dysregulation may serve as an initial but key step in a sophisticated cascade upon loss of BAP1 that eventually leads to altered cell migration. Consistent with this hypothesis, we noticed that BAP1 knockdown alone significantly increased the calpain level beyond the CAST knockdown level, suggesting that additional factors related to BAP1 contribute to this pathway, which needs to be further characterized.

In summary, we confirmed that the loss of BAP1 could inhibit cellular migration capacity in UM cell lines. Moreover, for the first time, we identified that CAST is a strong BAP1 interacting partner. We also found that CAST plays a key role in BAP1-related cell migration and that the overexpression of CAST fully rescued BAP1-induced cell motility defects. Thus, our data supports a novel mechanism underlying the cellular function of BAP1 and may shed light on the pathological role of BAP1 in related cancers/diseases.

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