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The splice variant Ehm2/1 in breast cancer MCF-7 cells interacted with β-catenin and increased its localization to plasma membrane†

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Ehm2, which belongs to the FERM superfamily, is a metastasis-associated protein. However, its function in cancer metastasis and the associated molecular mechanism is not definitely clear. Alternative splicing is an important biological step during mRNA processing and has been reported to be related with many diseases including cancers. Ehm2 has two transcript variants. Transcript variant 1(Ehm2/1) encodes isoform 1 of 518 amino acids, while transcript variant 2(Ehm2/2) encodes isoform 2 of 913 amino acids. In this study, we found that Ehm2/1 was the main transcript variant in the MCF-7 breast cancer cell line. Forced expression of Ehm2/1 upregulated the total protein amount but had no effect on the mRNA levels of β-catenin. The increased β-catenin was found to be dominantly located at the cell membrane. Meanwhile, knockdown of Ehm2/1 in MCF-7 cells decreased the total protein amount but not the mRNA levels of β-catenin. Further results showed that Ehm2/1 interacted with β-catenin and colocalized with it at the cell membrane. E-cadherin, a partner of β-catenin in cadherin-catenin complexes, was also upregulated by the overexpression of Ehm2/1 and also colocalized with it at the cell membrane. Meanwhile, overexpression of Ehm2/1 inhibited the migration ability of MCF-7 cells. These results suggested that Ehm2/1 may render β-catenin at the cell membrane by interacting with β-catenin and E-cadherin.

Introduction

Ehm2, also called erythrocyte membrane protein band 4.1-like protein 4B (EPB41L4B), is expressed in high metastatic cells and belongs to the FERM (Four.1 protein, ezrin, radixin, moesin) superfamily. Ehm2 has been suggested to be linked with cancer metastasis by regulating interactions between cell surface transmembrane proteins and cytoskeletal proteins.[1,2] However, the exact role of Ehm2 is not well known. Human Ehm2 was shown to be androgen-regulated in a human fibrosarcoma cell line model of steroid-regulated cytoskeletal reorganization.[3] Wang *et al.* reported that the expression of the FGFR-4 Arg388 variant resulted in the increased expression of Ehm2 in prostate epithelial cells.[3,4] They also found that Ehm2 expression was upregulated in prostate cancer cell lines and prostate cancer tissues. The enhanced expression of Ehm2 in prostate cancer may promote disease progression and metastasis.[4] In our previous study, we showed that Ehm2 was highly expressed in breast cancer, and its higher expression was correlated with distant metastasis and poor patient prognosis.[5] We also demonstrated that the knockdown of Ehm2 induced apoptosis potential and decreased the *in vitro* invasive properties of MCF-7 breast cancer cells by regulating the expression and activity of matrix metalloproteinase 9.

Alternative splicing is an important biological step during mRNA biosynthesis in eukaryotes and has been reported to be related to many diseases including cancers.[6–10] It is estimated that 90% of all multi-exon genes are subjected to some form of alternative splicing.[11–13] Alternative splicing of a single gene can give rise to functionally distinct protein isoforms.[14–16] However, very little is known about whether alternate transcripts are a driving force or the result of cancer progression.

Chauhan *et al.* conducted tissue expression analysis of the human Ehm2 gene and found that there were two Ehm2 protein isoforms (isoform 1 and isoform 2).[3,17] Isoform 2, which was translated from Ehm2/2, contains 913 amino acids, whereas isoform 1 exists ubiquitously in the testes, prostate and breast and is produced from Ehm2/1. In comparison with isoform 2, isoform 1 is missing 382 amino acids at the carboxyl terminal region containing recognizable protein motifs.[3] This may suggest that Ehm2 isoform 1 function is a constitutively active

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FERM signaling protein that is controlled by transcriptional regulation rather than by autoregulation involving the intramolecular folding of subdomains.[18,19] As mentioned above, Ehm2 is a metastasis-associated protein in prostate cancer and breast cancer; however, it is difficult to discriminate which transcript variant plays the dominant role in these processes. Thus, understanding the functions of these transcript variants is necessary to determine their potential roles in breast cancer. We analyzed the levels of the transcript variants of Ehm2 in breast cancer cell lines MCF-7 and MDA-MB-231. We found that MCF-7 cells contained primarily the transcript variant 1 of Ehm2, while MDA-MB-231 cells had both variants at relatively high levels. Based on our previous result that the knockdown of Ehm2 induced cell apoptosis and decreased the *in vitro* invasive properties of MCF-7 cells,[5] and that Ehm2/1 was dominantly expressed MCF-7 cells, we speculated that Ehm2/1 may function by regulating β-catenin in MCF-7 cells. In current study, we forced the expression of Ehm2/1 in MCF-7 cells and found that the upregulation of Ehm2/1 may render β-catenin at the cell membrane by interacting with β-catenin and inhibiting the migration ability of MCF-7 cells.

Materials and methods

Cell cultures

MCF-7, MDA MB-231 and A549 cells were purchased from the ATCC. HEK-293A cell lines were gifted by Professor Junqi He from Capital Medical University. Cells were routinely cultured in DMEM with L-glutamine (Thermo Fisher Scientific Inc., Carlsbad, USA) supplemented with streptomycin, penicillin (Ameresco, Solon, USA) and 10% fetal bovine serum (ExCell Bio, Shanghai, China) in an incubator at 37 °C, 5% CO_2 and 95% humidity.

Gene knockdown and overexpression

Knockdown of Ehm2 in MCF-7 cells was performed using transfection with anti-human Ehm2 hammerhead ribozyme, which was designed based on the secondary structure of Ehm2 transcript variant 1 generated using Zuker's RNA mFold program[20] and cloned into a mammalian expression pEF6/V5-His-TOPO plasmid vector (Thermo Fisher Scientific Inc, Carlsbad, USA). The stable clones were established by treatment with blasticidine.

Express plasmid of Ehm2/1 (NM_018424.2) tagged with FLAG was purchased from OriGene Technologies (Cat. RC223085, Beijing, China). For overexpression of Ehm2/1, cells were transfected with pCMV-entry and pCMV-Ehm2/1-FLAG. Stable transfectants were selected with medium containing 500 μg ml^{-1} G418.

For transfection, 1 μg of express vectors or ribozyme vectors were mixed with 4 μl of Lipofectamine® 2000 Reagent (Thermo Fisher Scientific Inc, Carlsbad, USA). After 20 min of complex formation, the liposomes were given to the cells plated in six-well plates for the analysis of gene expression.

RNA isolation and reverse transcription PCR

Total RNA was extracted from the cell lines with TRIzol Reagent (Thermo Fisher Scientific Inc, Carlsbad, USA). Extracted RNA was reverse transcribed into first-strand cDNA using an IScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, USA). PCR was carried out using a REDTaq™ ReadyMix PCR reaction mix according to the manufacturer's instructions. The specific primers were: Ehm2/1 forward, 5′-CACTTTGAGAGACTGAAGCATCTC-3′ and reverse, 5′-CAACTTCTACGACAGGAATATATGC-3′; Ehm2/2 forward, 5′-CCTGTTGCGGATCATGTGAAGTG-3′ and reverse, 5′-TATCAGGAAACGGGTTCATTGTATC-3′; β-catenin forward, 5′-AGGGATTTTCTCAGTCCTTC-3′ and reverse, 5′-GAACCAAGCATTTTCACCAG-3′; CyclinD1 forward, 5′-GAACAGAAGTGCGAGGAGGAG-3′ and reverse, 5′-AGGCGGTAGTAGGACAGGAAG-3′; GAPDH forward, 5′-GGCTGCTTTTAACTCTGGTA-3′ and reverse, 5′-GACTGTGGTCATGAGTCCTT-3′. Cycling conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s. This was followed by a final 10 min extension period at 72 °C. The products were visualized on 1.2% agarose gel stained with ethidium bromide.

Immunoblotting and isolation of nuclear, cytosol and membrane proteins

Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA and 10% glycerol) containing complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) for 30 min before clarification at 13 000 × *g* for 20 min. Protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific Inc, Carlsbad, USA) and an ELx800 spectrophotometer (BioTek Instruments Inc, Burlington, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose sheets. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated overnight with the desired primary antibody. The membranes were then treated with the appropriate HRP-conjugated secondary antibody (115-035-044 and 111-035-003, Jackson ImmunoResearch Inc, West Grove, USA). Protein bands were visualized using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc, Carlsbad, USA) and photographed using an LAS-3000 imager (FujiFilm, Tokyo, Japan).

The primary antibodies used to target β-catenin (ab22656), E-cadherin (ab40772), Snail (ab167609) and LaminA/C (ab108595) were from Abcam Ltd, (Cambridge, UK). Anti-β-actin (SC-130301) and anti-Ehm2 antibodies (SC-14234) were from Santa Cruz (San Diego, California, USA). Anti-FLAG antibody (F-1804) was from Sigma-Aldrich Ltd. (Poole, UK).

Nuclear, cytosol and membrane proteins analyzed by immunoblotting were isolated using the Nucl-Cyto-Mem Preparation Kit (APPLYGEN, Beijing, China) according to the manufacturer's instructions.

Immunoprecipitation and GST pull-down assay

For immunoprecipitation, the cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4 containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA and 10% glycerol) containing complete protease inhibitor cocktail. Lysates (200 μg of total protein) were incubated with 2 μg of Anti-FLAG antibody

(F-1804) overnight and then with 20 μl of protein A-agarose beads (GE Healthcare, Chicago, USA) for 4 h at 4 °C. β-Catenin, E-cadherin and FLAG were detected by incubating the blots with specific antibodies.

For GST pull-down assays, 293A cells transiently transfected with pCMV-Ehm2/1-FLAG were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4 containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA and 10% glycerol) containing complete protease inhibitor cocktail. β-Catenin-GST fusion proteins and GST proteins (a gift from Dr He Junqi, Capital Medical University) and glutathione-Sepharose 4B beads (GE Healthcare, Chicago, USA) were added to the lysate. After 4 h of incubation at 4 °C, the beads were washed five times in lysis buffer. The obtained samples were analyzed by western blotting.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature (RT). The fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 2% BSA in PBS for 30 min at RT. Thereafter, the cells were incubated with the appropriate primary antibodies in 1% BSA in PBS with 0.2% Triton X-100 for 1.5 h at 37 °C. The primary antibody used to stain β-catenin (9581) was from Cell Signaling Technology (Beverly, Massachusetts, USA). The primary antibodies used to stain E-cadherin (ab40772) and Ehm2/1 (ab77484) were from Abcam Ltd. (Cambridge, UK). Anti-FLAG antibody (F-1804) was from Sigma-Aldrich Ltd. (Poole, UK). Next, the cells were washed three times with PBS and incubated with Alexa Fluor 488 donkey anti-mouse IgG (1423052) or Alexa Fluor 594 donkey anti-rabbit IgG (1454437; Thermo Fisher Scientific Inc., Carlsbad, USA) in 1% BSA in PBS with 0.2% Triton X-100 for 1 h at RT. After three washes with PBS, coverslips were incubated with DAPI for 5 min at RT. After three washes with PBS and then rinsing in water (Milli-Q; Millipore, Billerica, USA), the coverslips were mounted with DAKO Fluorescent Mounting Medium (DAKO North America, Inc., Carpinteria, USA). Spectral image acquisition was performed using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Cell migration assay

Cell migration was assessed by wound-healing assay; 500 000 cells were seeded into each well of a six-well plate and allowed to reach near confluence. The layer of cells was then scraped with a fine-gauge needle and washed once with phosphate buffered saline. The movement of cells to close the wound was recorded over time using an Olympus phase-contrast microscope (Olympus, Tokyo, Japan). Cell migration was evaluated with Image J.

Statistical analysis

Experimental procedures were repeated independently at least three times. Data were expressed as mean ± s.d., and statistical comparisons were made using analysis of variance. Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Student's *t*-test.

Results

Ehm2 transcript variants were expressed differently in different breast cancer cells, and Ehm2/1 was distributed at the cell membrane

To investigate the potential functions of Ehm2 transcript variants in breast cancer, we first analyzed the expression patterns of the two Ehm2 transcript variants in two breast cancer cell lines. Ehm2/1 was expressed at moderate levels in both cell lines, although its expression was much higher in MDA-MB-231 cells; in contrast, the expression of Ehm2/2 was high in MDA-MB-231 cells but very low in MCF-7 cells (Fig. 1A). This result indicates that both transcript variants of Ehm2 are expressed differently and may have distinct roles in breast cancer progression. The western blot results showed that only Ehm2/1 (about 58 kDa) was detected in MCF-7 cells (data not shown). Cell immunofluorescence using goat-anti-Ehm2 antibody was carried out to detect the subcellular localization of endogenous Ehm2/1; the results showed that endogenous Ehm2/1 was mainly distributed at the cell membrane (Fig. 1B, ESI Fig. S1†), although this antibody is poor for immunofluorescence assay.

Changing the expression of Ehm2/1 in MCF-7 altered the protein levels but had no effect on the mRNA levels of β-catenin

Considering that Ehm2 transcript 1 was moderately expressed in MCF-7 cells, whereas the expression of Ehm2 transcript variant 2 was very low, we focused on the role of Ehm2/1 in MCF-7 cells. To study the role of Ehm2/1 in MCF-7 cells, we developed an Ehm2/1-overexpressed MCF-7 cell line by transfecting MCF-7 cells with pCMV-Ehm2/1-FLAG (MCF-7-Ehm2/1^{ex}) and Ehm2/1 knockdown MCF-7 cell lines using anti-Ehm2

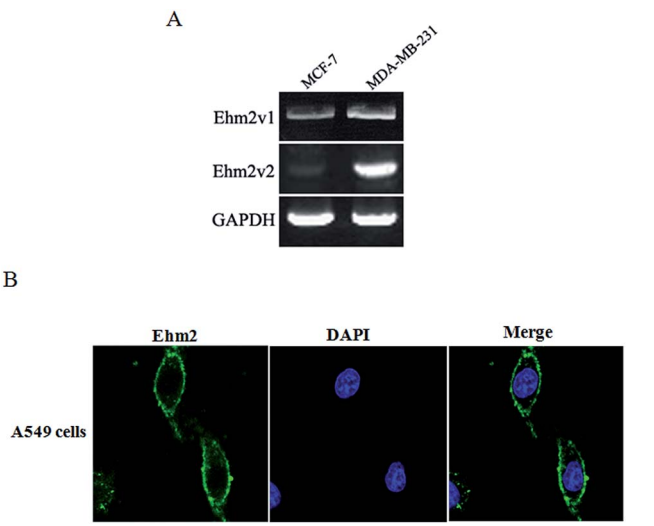

Fig. 1 Expression of endogenous Ehm2 and its subcellular distribution. (A) Ehm2 transcript variants in breast cancer cell lines were differently expressed. RNA was extracted from cell lines. Reverse transcription PCR was carried out using primers specific to Ehm2 transcript variants 1 and 2, and GAPDH was used as internal control. (B) Endogenous Ehm2/1 (green) was mainly distributed along the cell membrane.

hammerhead ribozyme transgenes (MCF-7-ΔEhm2/1). We verified the overexpression of Ehm2/1 using ordinary reverse-transcription PCR and western blotting (Fig. 2A and C, ESI Fig. S2†). The antibody used to detect Ehm2 was raised against a peptide mapping within the internal region of Ehm2 so that it could recognize Ehm2/1 and Ehm2/2. The results showed that only Ehm2/1 was detected. We also confirmed that Ehm2 ribozyme transgenes successfully knocked down the expression of Ehm2/1 within the MCF-7 cells (Fig. 2B and D).

In our previous study, we found that the knockdown of Ehm2 had inhibitory effects on the *in vitro* growth and invasion of MCF-7 cells and significantly decreased the mRNA and protein levels of MMP9 as well as its enzymatic activities.[5] We hypothesized that Ehm2 may cause these effects by interacting with β-catenin, a co-transcription factor in Wnt signaling.[21,22] Therefore, we assessed the expression of β-catenin in MCF-7-Ehm2/1^{ex} and MCF-7-ΔEhm2/1 cells. The overexpression of Ehm2/1 in MCF-7 cells significantly increased the protein levels of β-catenin ($p < 0.01$, Fig. 2A and ESI Fig. S3†) but had no effect on its mRNA levels (Fig. 2C and ESI Fig. S4†). In contrast, the knockdown of Ehm2/1 significantly decreased the protein levels of β-catenin ($p < 0.05$, Fig. 2B and ESI Fig. 3†) and also had no effect on its mRNA levels (Fig. 2D and ESI Fig. S4†). These results showed that Ehm2/1 regulates the protein levels of β-catenin through a mechanism other than transcription regulation.

Ehm2/1 interacted with β-catenin

To understand the molecular mechanism by which Ehm2/1 upregulates the protein levels of β-catenin, we identified the interaction of Ehm2/1 with β-catenin. First, the interaction between FLAG-tagged Ehm2/1 and endogenous β-catenin was detected by the overexpression of Ehm2/1 in MCF-7 cells and co-immunoprecipitation assay (Fig. 3A). The results showed that FLAG coprecipitated with β-catenin and verified the upregulation of endogenously expressed β-catenin in the total lysate of Ehm2/1 overexpressed MCF-7 cells. We confirmed the interaction of Ehm2/1 with β-catenin by GST pull-down assays using GST-fused β-catenin in HEK-293A cells transiently transfected with pCMV-Ehm2/1-FLAG (Fig. 3B). Cell immunofluorescence showed that Ehm2/1 detected using tagged-FLAG antibody was mainly localized at the cell membrane and colocalized with β-catenin (Fig. 3C). These results indicated that Ehm2/1 can interact with β-catenin at the cell membrane.

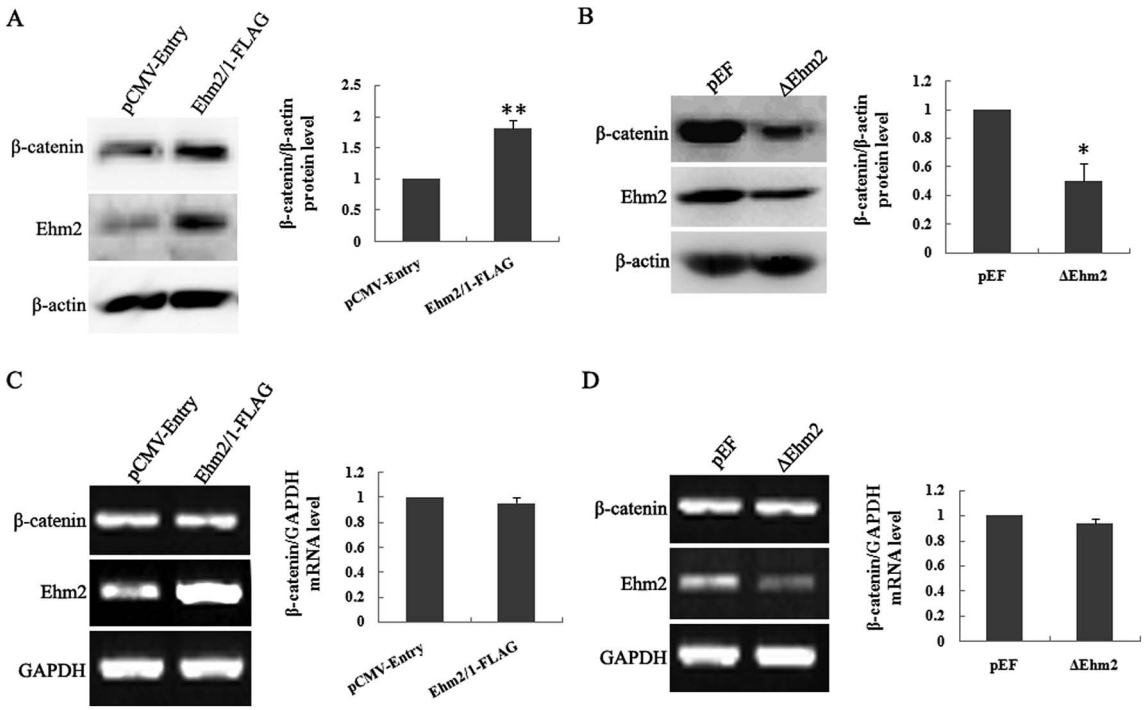

Fig. 2 Effect of overexpression and knockdown of Ehm2/1 on endogenous β-catenin. (A) Overexpression of Ehm2/1 increased the endogenously expressed β-catenin protein levels. MCF-7 cells stably transfected with pCMV-entry and pCMV-Ehm2/1-FLAG were harvested and subjected to western blotting using anti-Ehm2 and anti-β-catenin antibodies (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged. Data shown are relative to the pCMV-entry control (set to 1, right panel). (B) Knockdown of Ehm2/1 decreased the endogenously expressed β-catenin protein levels. Cells stably transfected with pEF and Ehm2 ribozyme constructs were harvested and subjected to western blotting using anti-Ehm2 and anti-β-catenin antibodies (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged. Data shown are relative to the pEF control (set to 1, right panel). (C, D) mRNA levels of β-catenin were unaffected by altering the expression of Ehm2/1. RNA was extracted from the same cell lines as in A and B. Reverse transcription PCR was carried out using primers specific to Ehm2/1 and β-catenin (left panel), and the intensities were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the corresponding control (set to 1, right panel). Note: $n = 3$; statistical significance was assessed by paired *t*-test in comparison to control; error bars indicate SD; * indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$.

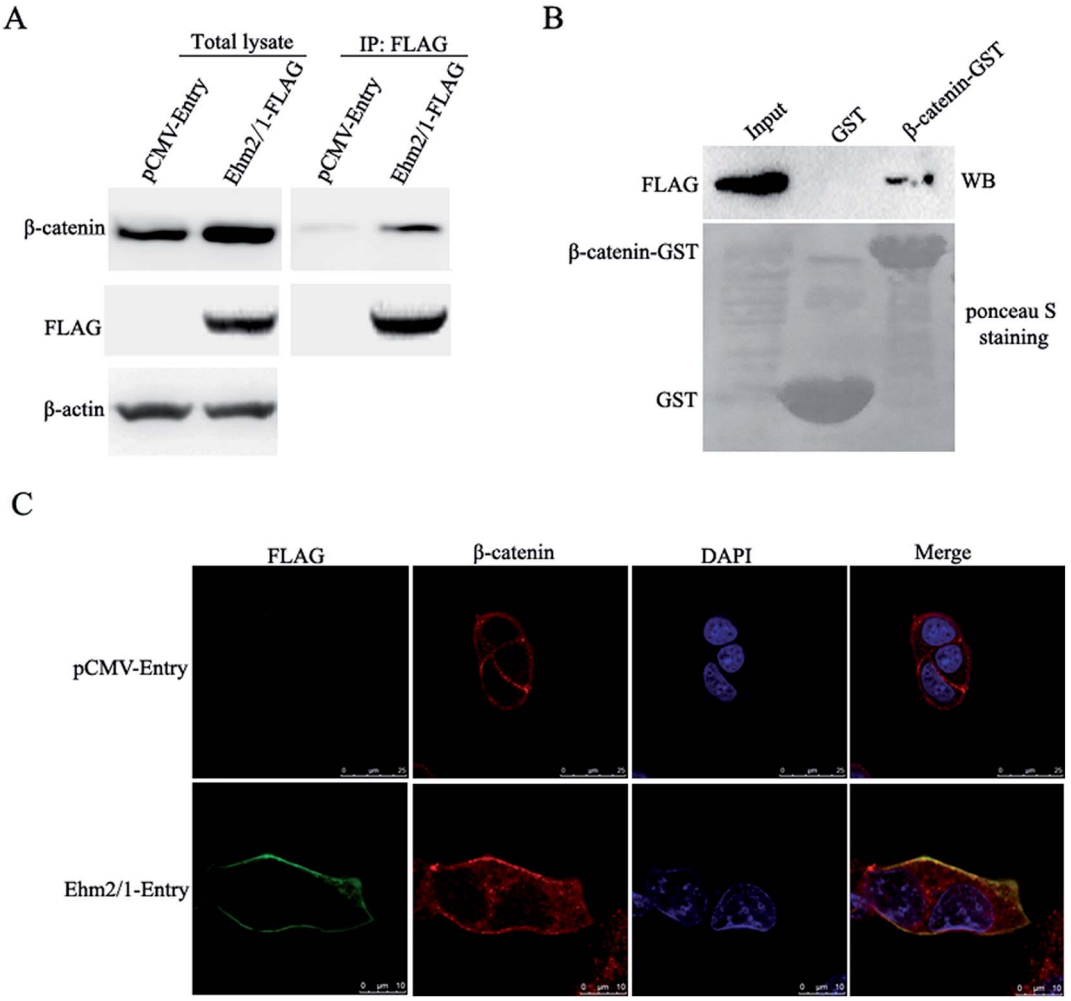

Fig. 3 Ehm2 interacted with β-catenin. (A) Lysates of MCF-7 cells stably transfected with pCMV-entry or pCMV-Ehm2/1-FLAG constructs were immunoprecipitated (IP) with anti-FLAG antibody. (top) Total lysate and coprecipitated β-catenin were detected by immunoblotting with anti-β-catenin antibody. (middle) Total lysate and IP FLAG were detected with immunoblotting. (bottom) Comparable amounts of β-actin were expressed as loading control. (B) Lysates of HEK-293A cells transfected with pCMV-Ehm2/1-FLAG were examined for GST pull-down assays using GST or β-catenin-GST. Ehm2/1 was detected by immunoblotting with anti-FLAG antibody. Comparable amounts of GST and β-catenin-GST beads were used by staining the membrane using ponceau S. (C) In MCF-7 cells expressing Ehm2/1-FLAG, the Ehm2/1-FLAG staining (green) significantly overlaps with that of β-catenin (red) along the cell membrane.

Overexpression of Ehm2/1 increased the fraction of membraneous β-catenin but not cellular and nuclear β-catenin

β-Catenin is a critical component of cadherin-based adhesion junctions and also a regulatory node of the Wnt signaling pathway.[23] The result that Ehm2/1 interacted with β-catenin at the cell membrane prompted us to investigate the subcellular localization of β-catenin upon Ehm2/1 upregulation. We isolated the cell-membrane, cytosolic and nuclear fractions and detected the level of β-catenin in each fraction. The results showed that Ehm2/1 upregulation significantly increased the levels of β-catenin in the cell-membrane fraction ($p < 0.05$) and had no obvious effects on the cytosolic and nuclear β-catenin fractions (Fig. 4A), which promotes the transcription of target genes including cyclin D1 and c-myc.[24–26] In fact, the overexpression of Ehm2/1 in MCF-7 did not increase the mRNA levels of cyclin D1, a downstream target gene of β-catenin (Fig. 4B), indicating that the nuclear localization of β-catenin was not affected by the overexpression of Ehm2/1. This result indicated that Ehm2/1 may render β-catenin at the cell membrane.

Overexpression of Ehm2/1 increased the protein levels of E-cadherin and inhibited cell migration

Cell membrane-localized β-catenin is a member of the E-cadherin/catenin complex, which forms adhesion junctions. To determine if Ehm2/1 regulates cadherin-catenin complexation, we analyzed the protein levels of E-cadherin and the colocalization of Ehm2/1 with E-cadherin in MCF-7-Ehm2/1^{ex} cells. E-cadherin was significantly upregulated in MCF-7-Ehm2/1^{ex} cells compared to in control cells ($p < 0.05$, Fig. 5A). Cell immunofluorescence showed that Ehm2/1 mainly distributed

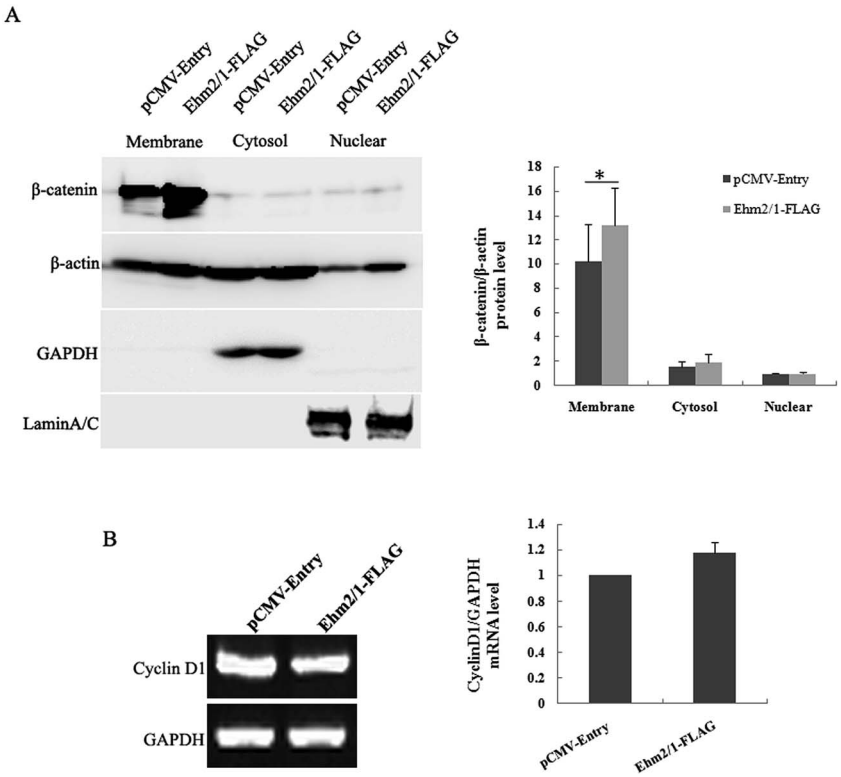

Fig. 4 Overexpression of Ehm2/1 elevated cell-membrane localization of β-catenin. (A) Overexpression of Ehm2/1 increased β-catenin levels in the cell-membrane fraction. Cells stably transfected with pCMV-entry and pCMV-Ehm2/1-FLAG were harvested and fractioned with a kit to isolate cell-membrane, cytosolic and nuclear proteins, and β-catenin was then analyzed using western blotting. GAPDH was the loading control of cytoplasmic proteins, and LaminA/C was used as a loading control for nuclear proteins. Along with GAPDH and LaminA/C, β-actin, a loading control for entire cell proteins, was also used as a loading control for cell membrane (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged (right panel). (B) Cyclin D1, a target gene of β-catenin, was unaffected at the transcription level by the overexpression of Ehm2/1. RNA isolated from cells stably transfected with pCMV-entry, and pCMV-Ehm2/1-FLAG was reverse transcripted into cDNA. The mRNA levels of cyclin D1 were then detected using ordinary RT-PCR (left panel), and the intensities were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the pCMV-entry control (set to 1, right panel). Note: $n = 3$; statistical significance was assessed by paired t-test in comparison to the control; error bars indicate SD; * indicates significance at $p < 0.05$.

at the cell membrane and colocalized with E-cadherin (Fig. 5B). Adhesion junctions were associated with cell migration; therefore, we analyzed the migration ability of MCF-7-Ehm2/1^{ex} cells using wounding assay. The results showed that the upregulation of Ehm2/1 caused an obvious decrease in cell migration, although the tendency was not significant (Fig. 5C, $p > 0.05$). This result was in line with the increasing distribution of β-catenin at the cell membrane. In fact, the upregulation of Ehm2/1 not only decreased cell migration, but also reduced the invasion ability of MCF-7 cells ($p < 0.05$, ESI Fig. S5†).

Discussion

Ehm2/1 is generated by transcript variant 1, which lacks several exons and includes an alternate 3′ terminal exon compared to variant 2. Thus, Ehm2/1 is shorter and has a distinct C-terminus compared to Ehm2/2. The biological functions of Ehm2/1 and Ehm2/2 are not definitively known. In the present study, we found that MCF-7 cells mainly express Ehm2/1, which is a membrane-associated protein. One of the main findings of this study is that Ehm2/1 interacts with β-catenin and retards it at the cell membrane. Membranous β-catenin is a critical component of cadherin-based cell–cell adhesion. Our results also confirmed that Ehm2/1 interacts with E-cadherin at the cell membrane and upregulates its protein levels.

Ehm2 is a FERM domain-containing protein and was originally identified as a metastasis-promoting protein in murine melanoma cells.[2] FERM domain-containing proteins have conserved FERM domains, which mediate protein–protein interactions.[27–31] FERM domains can mediate intermolecular interactions, usually by interacting with the cytoplasmic tails of transmembrane proteins.[18,32,33] For example, the ERM proteins bind *via* their FERM domains to the cytoplasmic domains of transmembrane proteins such as CD44.[18] Murine Ehm2, also called Lulu2, is a potent activator of cortical myosin II contractile forces in epithelial cells.[34] Murine Ehm2 can interact with p114RhoGEF through its FERM domain and be recruited at cell–cell boundaries.[35] The *Drosopholia* orthologue of Ehm2, called Yurt, was reported to be a negative regulator of apical membrane size in epithelial cells.[36] Yurt was recruited to the

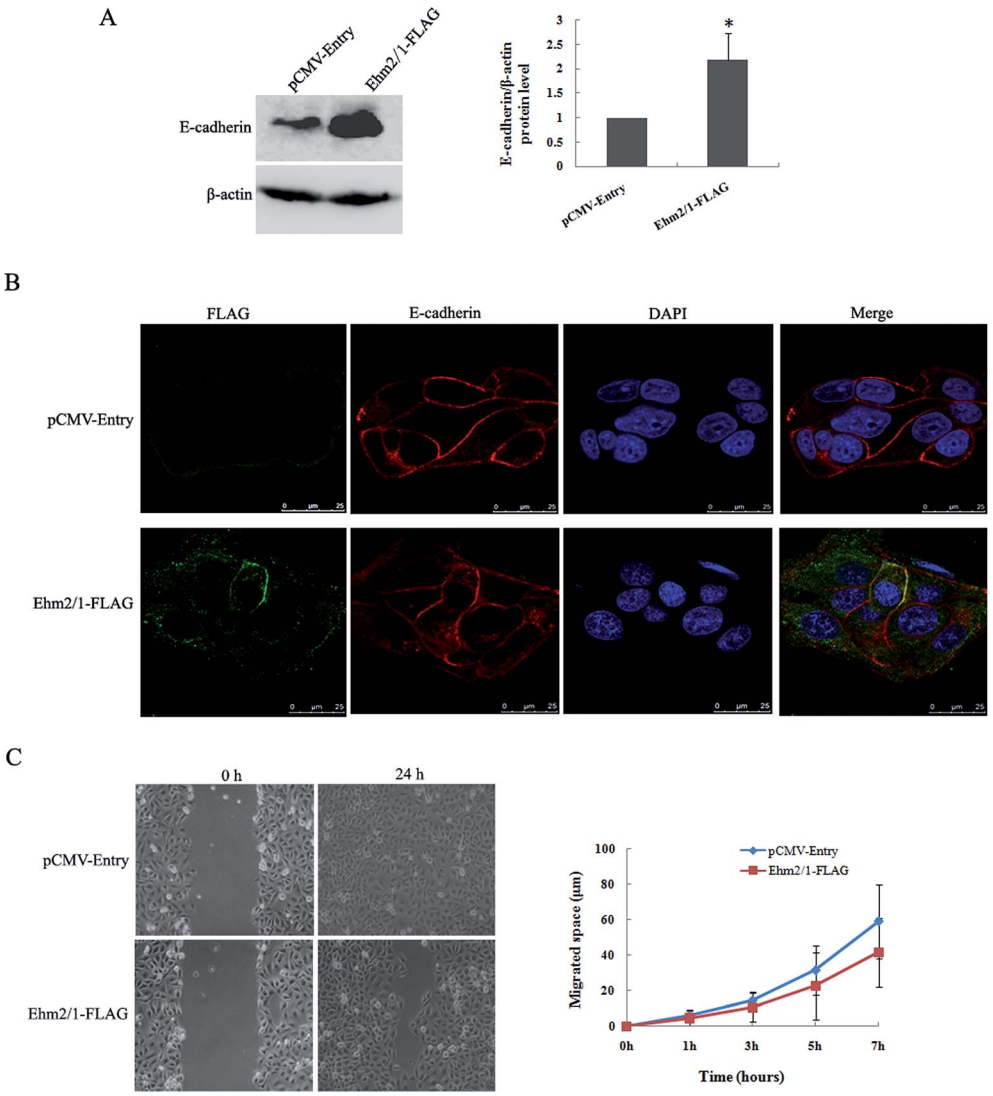

Fig. 5 Effects of Ehm2/1 upregulation on endogenous E-cadherin and cell migration. (A) Overexpression of Ehm2/1 increased the endogenously expressed E-cadherin protein levels. Cells stably transfected with pCMV-entry and pCMV-Ehm2/1-FLAG were harvested and subjected to western blotting using anti-E-cadherin antibodies (left panel), and the intensities of E-cadhesion bands were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the pCMV-entry control (set to 1, right panel). Note: $n = 3$; statistical significance was assessed by paired t-test in comparison to the control; error bars indicate SD; * indicates significance at $p < 0.05$. (B) In MCF-7 cells expressing Ehm2/1-FLAG, the Ehm2/1-FLAG staining (green) significantly overlaps with that of E-cadherin (red) along the cell membrane. (C) Ehm2/1 upregulation reduced cell migration compared with the pCMV-entry group, although the difference was not significant. Shown are representative results of three independent experiments of wounding assay. Quantification of migrated space is shown in the right graph ($n = 3$; error bars indicate SD).

apical membrane by Crb and can bind directly to the FDB site in the cytoplasmic tail of Crb through its FERM domain.[37] Zebrafish Moe, the sole Ehm2 molecule in the species, participates in the layering of the retina and inflation of the brain ventricles as well as restricting the photoreceptor apical domain.[38] Moe also interacts with and negatively regulates Crumbs, thereby restricting apical membrane size in epithelial structures.[39] Mammalian EPB41L5, a FERM protein very similar to Ehm2 (EPB41L4B), associates with the intracellular domains of Crumbs through its FERM domain and is involved in maintaining cell polarity.[40] Like its orthologues in other species and homology in mammals, human endogenous Ehm2 was distributed at the cell membrane in this study. Considering that Ehm2/1 is not highly expressed in MCF-7 cells, and Ehm2 antibody is not quite right for cell immunofluorescence, human lung adenocarcinoma epithelial A549 cells were used for endogenous distribution assay. Exogenous overexpressed Ehm2 was also distributed at the cell membrane.

β-Catenin is a critical component of cadherin-based cell–cell adhesion and also a regulatory node of the Wnt signaling pathway.[41] It links cadherins indirectly to the actin cytoskeleton.[42,43] It also interacts with the LEF/TCF family members of transcriptional activators as a critical intermediate in Wnt signal transduction pathways.[24,44–47] In this study, we found that

β-catenin can be regulated by Ehm2/1 and retained at the cell membrane *via* interacting with Ehm2/1. The supporting evidence was as follows: (i) in extracts of transfected MCF-7 cells, FLAG-tagged Ehm2/1 co-immunoprecipitated β-catenin; (ii) β-catenin pulled down FLAG-tagged Ehm2/1 in extracts of the transfected 293A cells; and (iii) FLAG-tagged Ehm2/1 co-localized with β-catenin at the cell membrane. These results were consistent with the role of 4.1R, the prototypical member of the protein 4.1 superfamily, in linking the cadherin/catenin complex to the cytoskeleton through its direct interaction with β-catenin.[48,49] Therefore, we speculated that the FERM domain of Ehm2/1 behaves similarly to those of the 4.1 and ERM proteins. However, we cannot be sure whether the interaction of Ehm2/1 with β-catenin is direct or indirect. EPB41L5 can bind to the C-terminal armadillo repeat region of p120ctn through its N-terminal FERM domain.[50] In future work, we will analyze the structural basis of the interaction between Ehm2/1 and β-catenin to verify the binding of the N-terminal FERM domain of Ehm2/1 to the armadillo repeat region of β-catenin. Furthermore, we cannot exclude the possibility of indirect interaction between Ehm2/1 and β-catenin. In fact, Ehm2/1 also co-localized with E-cadherin at cell–cell contacts. Ehm2/1 has the potential to indirectly interact with β-catenin through binding to E-cadherin. Ezrin, forming a subfamily of conserved proteins in the band 4.1 superfamily with radixin, moesin and merlin, regulates cell–cell and cell-matrix adhesion by interacting with the cell-adhesion molecules E-cadherin and β-catenin.[51] Cadherin–catenin complexes at adherens junctions can decrease cell migration and cell invasion. In our study, we found that MCF-7 cells overexpressing Ehm2/1 showed decreased cell migration, although the difference was not statistically significant. We speculate that the reason for the lack of statistical significant was that the basal E-cadherin and β-catenin levels were quite high in MCF-7 cells. Our results showed that the upregulation of Ehm2/1 indeed reduced the invasion ability of MCF-7 cells significantly ($p < 0.05$). Considering the important role of Ehm2/1 in regulating cell migration and invasion through the β-catenin-E cadherin axis, we speculated that Ehm2/1 has negative regulation in EMT. Our finding that the overexpression of Ehm2/1 decreased the protein levels of the EMT marker Snail further confirmed our hypothesis (ESI Fig. S6†). However, how Ehm2/1 is connected to E-cadherin at the molecular level and how Ehm2/1 regulates EMT remain to be elucidated.

In summary, we demonstrated that Ehm2/1 is the principal Ehm2 protein in MCF-7 cells and is a regulator of the cadherin–catenin complexes. Elucidating the more detailed mechanisms of the Ehm2/1-cadherin–catenin system is a future important challenge to understand the role of Ehm2.

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