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La-Related Protein 4 as a Suppressor for Motility of Ovarian Cancer Cells

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The La-related proteins (LARPs) are a family of RNA binding proteins that control the degradation and stabilization of RNAs. As emerging research reveals the biology of each LARP, it is evident that LARPs are dysregulated in some types of cancer. Upregulation of cell motility potentiates the metastatic potential of ovarian cancer cells; however, the roles of LARPs in cell motility remain unknown. In the present study, we investigated the roles of LARPs in the progression of ovarian cancer using SKOV3 human ovarian cancer cells and a public database that integrates microarray-based gene expression data and clinical data. To explore the involvement of LARPs in the cell motility, we performed RNA interference screening for LARP mRNAs in SKOV3 cells. The screening identified LARP4 as a potential suppressor of the formation of lamellipodia. Conversely, enforced expression of LARP4 suppressed the formation of lamellipodia. Moreover, cell migration was significantly increased in LARP4-depleted SKOV3 cells. Mechanistically, LARP4 depletion was associated with the decrease in RhoA protein expression. These results suggest that LARP4 may limit RhoA-dependent cell motility. In a mouse xenograft model with SKOV3 cells, LARP4 depletion potentiated peritoneal metastasis. Upon analysis of a public database of patients with ovarian cancer, the LARP4 mRNA-high expression group (n = 166) showed longer overall survival compared with the LARP4 mRNA-low expression group (n = 489), implying a positive correlation of LARP4 mRNA levels in ovarian cancer tissues with patient prognosis. Taken together, we propose that LARP4 could suppress motility and metastatic potential of ovarian cancer cells.

Keywords: cell motility; La-related protein 4; metastasis; ovarian cancer; RhoA
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Introduction

RNA binding proteins (RBPs) are principally involved in the regulation of gene expression by modulating RNA stability and translation (Raineri et al. 2004; Liao et al. 2007; Rajkowitsch et al. 2007; Glisovic et al. 2008). La-related proteins (LARPs) are an ancient family of RBPs characterized by the presence of the La module implicated in direct RNA binding (Bayfield et al. 2010). The family consists of LARP1, LARP3 (SSB/genuine La protein), LARP4, LARP4b considered a paralog of LARP4, LARP6, and LARP7.

Recent studies have demonstrated that post-transcriptional regulation by RBPs including LARPs is involved in tumor development. Sommer et al. (2011b) reported roles of LARP3 in the development of head and neck squamous cell carcinoma. In several types of cancer, LARP1 has been implicated in the tumorigenesis and regulation of cell motility (Burrows et al. 2010; Mura et al. 2015; Hopkins et al. 2016; Ye et al. 2016). LARP7 was reported to be associated with tumor suppression of gastric cancer (Cheng et al. 2012). However, the roles of LARPs in cancer progression remain poorly defined.

Epithelial ovarian cancer is the most lethal gynecologic malignancy (Jelovac and Armstrong 2011). The lethality of ovarian carcinoma primarily stems from the late diagnosis of patients at an advanced stage characterized by metastasis. In the development of metastasis, ovarian cancer cells detach from the primary tumor, spread throughout the peritoneum, and invade organs in the peritoneal cavity (Naora and Montell 2005; Guan 2015; Yeung et al. 2015). Importantly, an increase in cancer cell motility plays a key

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role in the development of metastasis. However, the mechanism regulating cell motility remains poorly understood. In the present study, we thus investigated the roles of LARPs in cell motility and metastatic potential in ovarian cancer cells.

Materials and Methods

Antibodies and reagents

Antibodies specific for LARP4 (ab156687), phosphatidylinositol-3-kinase (PI3K) C2β (ab55589), and PTEN (ab32199) were from Abcam (Cambridge, MA, USA). Antibodies specific for JNK (#99252S), phospho-JNK (#4668S), phospho-mTOR (#2971S), p110α (#4249), E-cadherin (#3195P), vimentin (#5741P), slug (#9585S), Rac 1/2/3 (#2465T), CDC42 (#2466T), RhoA (#2117S), Akt (#9272S), and phospho-Akt (#9271S) were from Cell Signaling Technology (Boston, MA, USA). Horseradish-peroxidase-conjugated antibodies for rabbit IgG (sc2004) and mouse IgG (sc2005) were from Santa Cruz Biotechnology (Dallas, TX, USA). RNAiMax, Lipofectamine 2000, control siRNAs, LARP4 siRNAs (LARP4 si1, CCAUGGUACAAGUUGAUGAtt; LARP4 si2, CACCAAAGUUUG ACUUAUUtt; LARP4 si3, GGAUGGUCUCAAUCAGACAtt), and other siRNAs for the LARP family members were from Life Technologies (Carlsbad, CA, USA). Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin and β-actin antibodies (A5441) were from Sigma (St. Louis, MO, USA). Hoechst 33342 was from Dojindo (Kumamoto, Japan). SuperSignal West Dura Extended Duration Substrate and Halt Phosphatase Inhibitor Cocktail were from Thermo Fisher Scientific (Rockford, IL, USA).

Cell culture

Human SKOV3 and CAOV3 ovarian cancer cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Human PC3 prostate cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum. Cells were maintained at < 80% confluence under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, 37°C). No mycoplasma contamination was observed.

Preparation of LARP4 expression vector

Human LARP4 cDNA segments in a cDNA library of SKOV3 cells were amplified by PCR. The PCR products and pcDNA3.1/V5-His empty vector (ThermoFisher Scientific) were digested with BamH1/XhoI and each fragment was ligated, forming pcDNA3.1/V5-His LARP4 vector.

Transfection with siRNAs or LARP4 vector

For siRNA transfection, cells (2×10^4) were grown on 35-mm glass-bottomed dishes and then transfected with 5 nM siRNAs using RNAiMax transfection reagent (Life Technologies) for 48 h. For transfection with plasmid vectors, SKOV3 cells (5×10^4) grown on 35-mm glass-bottomed dishes were transfected with 2 μg of pcDNA3.1/V5-His empty vector or pcDNA3.1/V5-His LARP4 vector for 24 h using Lipofectamine 2000.

Analysis of lamellipodium formation

SKOV3 cells were grown in 35-mm glass-bottomed dishes at a density of 2×10^4 cells/dish. After transfection, cells were washed with PBS twice, fixed with 4% formaldehyde for 10 min, and treated with 0.1% TritonX-100 for 10 min, followed by staining with Hoechst 33342 and TRITC-conjugated phalloidin for 5 min. For the analysis of lamellipodia, samples were examined using confocal microscopy. Lamellipodia are thin and veil-like extensions at the edge of cells that contain a dynamic array of actin filaments and are biologically characterized by the enrichment of F-actin. Cells were counted as having formed lamellipodia if there was an increase in visualized F-actin in the lamellipodia. The percentage of cells forming lamellipodia was determined by counting more than 200 cells.

Immunoblotting analysis

Cells were washed three times with PBS supplemented with Halt Phosphatase Inhibitor Cocktail and then lysed using Laemmli sample buffer (Wako, Tokyo, Japan). After brief centrifugation, the protein content was determined using the BCA protein assay reagent. Then, equal amounts of proteins (5 or 10 μg) were loaded for SDS-PAGE (4%-20% gradient gels). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with PBS/0.1% Tween 20 (PBS-T) containing 5% nonfat dried milk for 30 min, washed with PBS-T, and incubated with primary antibodies for LARP4 (1 in 1,000 dilution), p110α (1 in 1,000 dilution), PI3KC2β (1 in 1,000 dilution), Akt (1 in 1,000 dilution), phospho-Akt (1 in 1,000 dilution), phospho-mTOR (1 in 1,000 dilution), PTEN (1 in 1,000 dilution), ERK1/2 (1 in 1,000 dilution), phospho-ERK1/2 (1 in 1,000 dilution), p38δ (1 in 1,000 dilution), JNK1/2 (1 in 1,000 dilution), phospho-JNK1/2 (1 in 1,000 dilution), E-cadherin (1 in 1,000 dilution), vimentin (1 in 1,000 dilution), Slug (1 in 1,000 dilution), Rac (1 in 1,000 dilution), CDC42 (1 in 1,000 dilution), RhoA (1 in 1,000 dilution), or β-actin (1 in 50,000 dilution). The membranes were washed three times with PBS-T and then incubated with a secondary antibody conjugated with horseradish peroxidase (1:5,000 dilution) in PBS-T containing 5% nonfat dried milk for 1 h. Proteins were visualized using Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Quantification of the chemiluminescent signals was performed with a digital imaging system (ChemiDoc, Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's manual. After reverse transcription of RNA with SuperScript III (Thermo Fisher Scientific), quantitative real-time PCR was performed using the StepOne Plus Real-Time PCR System. TaqMan probes specific for LARP1 (Hs00391726_m1), LARP3 (Hs04187362_g1), LARP4b (Hs00299621_m1), LARP6 (Hs00217969_m1), LARP7 (Hs00277883_m1), and β-actin (4333762F) were employed. β-Actin was used as an internal control.

Cell viability assay

Cell viability was assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Cells were seeded on 96-well plates at a density of 1×10^3 cells/well. After incubation for the indicated periods, the cell viability was determined in accordance with the manufacturer's protocol.

Cell migration assay

For SKOV3 and CAOV3 cells, cell migration was determined by a Transwell migration assay, as described by Zhang et al. (2018), while for PC3 prostate cancer cells the incubation time was extended up to 24 h, as described by Seetharaman et al. (2016).

Invasion assay

Cells (5×10^4 cells/well) in serum-free medium were seeded onto the upper chamber of the Transwell with 8-mm pores that were coated with 5% Matrigel. The lower chamber was filled with 10% FBS-containing DMEM. After 18 h of incubation, noninvading cells at the top of the membrane filter were removed using a cotton swab. Invading cells attached to the bottom of the membrane filter were fixed in 100% methanol and then stained with 1% toluidine blue for 5 min. The cell invasiveness was determined by counting invading cells in optical microscopy images (at least ten fields for each determination).

Xenograft model with human ovarian cancer cells

SKOV3 cells (5×10^6 cells/mouse) were transfected with 5 nM of control si2 or LARP4 si2 for 24 h and then inoculated into the peritoneal cavity of female nude mice (BALB/c; Charles River Japan, Yokohama, Japan) to determine peritoneal metastatic potential. The mice were sacrificed at 4 weeks after inoculation, and the number of overt metastases (> 1 mm) was quantified. When the inoculation of cells into mouse peritoneum failed, the mice were euthanized and excluded from the analysis. All animal studies were approved by the Institutional Animal Care and Use Committee of Tohoku University, Japan.

Kaplan-Meier Plotter analysis

The prognostic value of LARP mRNA expression in ovarian cancer was analyzed using an online database, Kaplan-Meier Plotter (http://www.kmplot.com). Online analysis of the database that integrates microarray-based gene expression data and clinical data was performed, leading to the creation of Kaplan-Meier survival plots. Patients were split into two groups (with low and high gene expression) by an automatically selected optimal cut-off. All possible cut-off values between the lower and upper quartiles were computed and the best performing threshold was used.

Statistical analysis

Data in bar graphs are presented as the mean ± standard error (SE) of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 6.0. All P values less than 0.05 were considered significant.

Results

Identification of LARPs that regulate cell motility

Cell migration involves reorganization of the F-actin cytoskeleton, leading to the formation of protrusions, such as filopodia and lamellipodia (Vignjevic and Montagnac 2008; Parsons et al. 2010; Krause and Gautreau 2014). Lamellipodia are critical for directional cell migration and are responsible for cell motility, which contributes to metastatic potential in cancer cells (Machesky 2008; Bisi et al. 2013). To identify LARPs responsible for modulating the formation of lamellipodia, we used siRNAs specific for six LARPs (LARP1 si, LARP3 si, LARP4 si2, LARP4b si, LARP6 si, and LARP7 si). The effectiveness of siRNAs for individual LARPs was confirmed in SKOV3 ovarian cancer cells (data not shown). As shown in Fig. 1A and B, knockdown of LARP4 or LARP4b had stimulatory effects on the formation of lamellipodia. LARP4 appeared to have particular potential to suppress the formation of lamellipodia. In contrast, LARP3 knockdown had inhibitory effects on their formation, while knockdown of other LARPs (LARP1, LARP6, and LARP7) had no significant effects.

To further evaluate the potential of LARP4 as a suppressor of cell motility, we used an additional siRNA sequence (LARP4 si1) and also constructed a human LARP4-overexpressing vector (pcDNA3.1/V5-His LARP4). Transfection of SKOV3 cells with either LARP4 si1 or LARP4 si2 was confirmed to suppress LARP4 proteins (Fig. 1C) and significantly increased the formation of lamellipodia (about 1.5- to 2-fold compared with that in the control; LARP4 si2 shown in Fig. 1B, LARP4 si1 not shown). Reciprocally, LARP4 overexpression by transfection with pcDNA3.1/V5-His LARP4 vector suppressed the formation of lamellipodia to 0.4-fold compared with that of pcDNA3.1/V5-His empty vector (Fig. 1D-F). Knockdown of RhoA partly attenuated the formation of lamellipodia in SKOV3 cells (Fig. 1G). These results suggest that LARP4 suppresses the RhoA-dependent formation of lamellipodia.

In addition, the effects of LARP4 knockdown on the Transwell migration of SKOV3 cells were assessed. Similar to the results on the formation of lamellipodia, LARP4 knockdown with individual sequences (LARP4 si1 and LARP4 si2) significantly increased cell migration by 1.35- and 1.85-fold compared with that upon the control knockdown, respectively (Fig. 2A and B). Similar results were obtained in CAOV3 ovarian cancer cells (Fig. 3). Thus, LARP4 may serve as a suppressor of the motility of ovarian cancer cells.

On the other hand, LARP4 knockdown had no significant effects on the invasiveness of SKOV3 cells (Fig. 2C). We next tested the effects of LARP4 siRNA on the migratory and invasive activities of PC3 prostate cancer cells. LARP4 knockdown promoted both cell migratory and invasive activities (Fig. 2D-F), consistent with a previous report (Seetharaman et al. 2016). These results suggest a difference in the suppressive effect of LARP4 on the invasiveness between ovarian cancer and prostate cancer.

LARP4 suppresses RhoA expression

Class II PI3K β isoform (PI3K C2β) has been implicated in the formation of lamellipodia in SKOV3 cells (Bai et al. 2015; Kitatani et al. 2016). To determine the molecular mechanisms by which LARP4 suppresses cell motility, we evaluated the effects of LARP4 knockdown on PI3K C2β and its associated signaling pathways in SKOV3 cells (Fig. 4A). The PI3K-Akt-mTOR pathway was unlikely to be involved in the regulatory mechanisms of LARP4-controlled cell motility. Moreover, the involvement of MAPK and epithelial-mesenchymal transition (EMT) pathways (Dhillon et al. 2007; Heerboth et al. 2015) was assessed. No changes in the expression of those pathway-

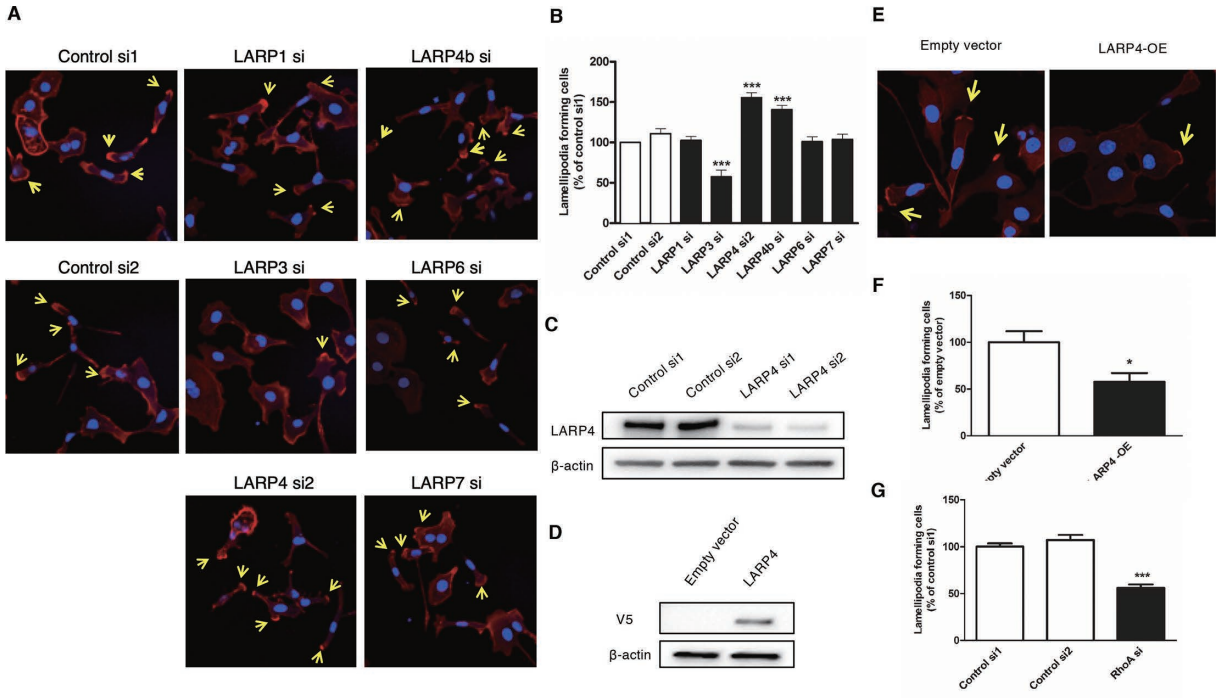

Fig. 1. Identification of LARPs that regulate the formation of lamellipodia in SKOV3 cells.
(A and B) SKOV3 cells (2×10^4) grown on glass-bottomed dishes were transfected with 5 nM siRNAs for 48 h. Cells were fixed followed by staining with TRITC-conjugated phalloidin (red) and Hoechst 33342 (blue). Representative images of confocal microscopy are shown; yellow arrows point to cells forming lamellipodia (A). The data shown (mean ± SE, n = 4) are the percentages of cells forming lamellipodia (B). (C) SKOV3 cells (2×10^4) grown on glass-bottomed dishes were transfected with 5 nM siRNAs for 48 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for LARP4 or *β*-actin. Equal amounts of protein were loaded in each lane, from which representative images are shown. (D-F) Cells (5×10^4) grown on glass-bottomed dishes were transfected with pcDNA3.1/V5-His empty vector or pcDNA3.1/V5-His LARP4 vector (LARP4-OE) for 24 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for V5 or *β*-actin and equal amounts of proteins were loaded in each lane (D). Transfected cells were fixed followed by staining with TRITC-conjugated phalloidin (red) and Hoechst 33342 (blue). Representative images of confocal microscopy are shown; yellow arrows point to cells forming lamellipodia (E). Data shown (mean ± SE, n = 4) are the percentages of cells forming lamellipodia (F). (G) Cells transfected with 5 nM siRNAs were fixed followed by staining with TRITC-conjugated phalloidin (red) and Hoechst 33342 (blue). Representative images of confocal microscopy are shown; yellow arrows point to cells forming lamellipodia.
*P < 0.05, ***P < 0.00011.

associated proteins were observed in LARP4-depleted SKOV3 cells (Fig. 4A). Tumor suppressor genes such as P53 and PTEN are associated with ovarian cancer progression (Lee and Park 2009). We thus tested the possible involvement of LARP4 in regulating the expression of these tumor suppressor proteins. As shown in Fig. 4A, LARP4 knockdown had no noticeable effects.

Rho GTPases are intracellular signal transducers implicated in the control of actin cytoskeleton organization, cell migration, and invasion (Schmitz et al. 2000; Ridley 2015). We investigated the expression of Rho GTPases in LARP4-knocked-down SKOV3 cells. LARP4 knockdown resulted in a significant increase in RhoA protein expression (Fig. 4B and C), while the expression of Rac and CDC42 did not change (Fig. 4B, D, and E). In addition, we used a third sequence of LARP4 siRNA (LARP4 si3) and confirmed the significant upregulation of RhoA protein expression by the knockdown (data not shown). These results suggest that LARP4 suppresses RhoA expression.

Anti-metastatic potential of LARP4 in a mouse xenograft model of ovarian cancer

To assess the involvement of LARP4 in the metastasis, we employed a xenograft model of SKOV3 ovarian cancer cells (Kitatani et al. 2016) and evaluated the effects of LARP4 knockdown on metastasis. LARP4 siRNA was confirmed to persistently achieve knockdown for up to 96 h (Fig. 5A). SKOV3 cells transfected with control or LARP4 siRNA for 24 h were inoculated into the peritoneal cavity of nude mice. Four weeks later, the mice were sacrificed, and the number of visible metastatic nodules was counted. LARP4 knockdown caused a significant increase in the number of peritoneal metastatic nodules compared with that upon control knockdown (Fig. 5B). LARP4 was, therefore, suggested to suppress the metastatic potential of ovarian cancer cells.

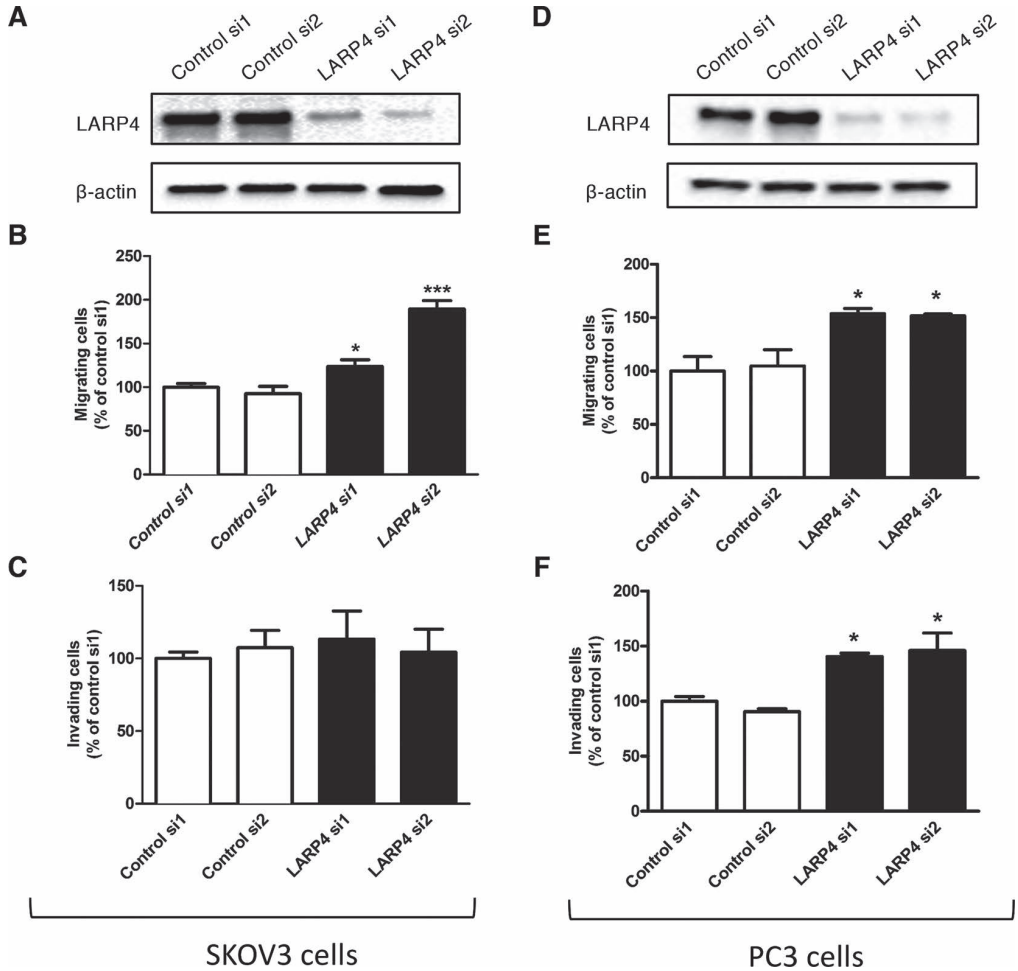

Fig. 2. LARP4 knockdown specifically promotes ovarian cancer cell migration, rather than invasion.
SKOV3 cells (1×10^4) or PC3 prostate cancer cells (5×10^4) were transfected with 5 nM siRNAs for 48 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for LARP4 or β-actin. Equal amounts of protein were loaded in each lane, for which representative images are shown (A and D). Cell migration was assessed using a Transwell assay. Data shown (mean ± SE, n = 7-19 for SKOV3 cells, n = 4 for PC3 cells) are the percentages of migrating cells (B and E). Invasive activity was assessed by a Matrigel-coated Transwell assay, as described in Materials and Methods. Data shown (mean ± SE, n = 6 for SKOV3 cells, n = 4 for PC3 cells) are the percentages of invading cells (C and F).
*P < 0.05, ***P < 0.0001.

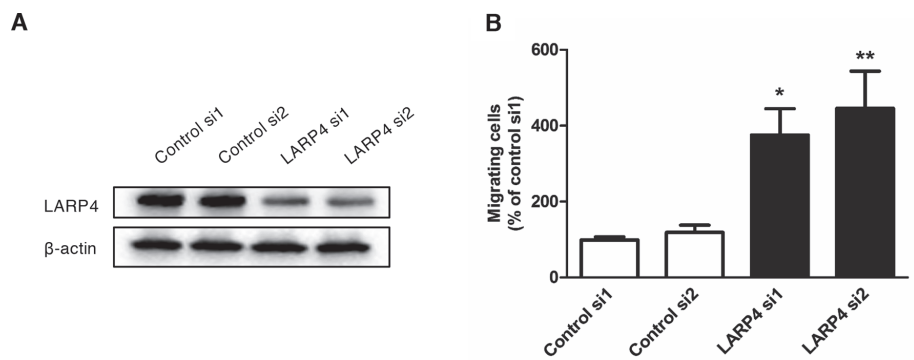

Fig. 3. LARP4 knockdown promotes the migration of CAOV3 ovarian cancer cells.
(A and B) CAOV3 cells (5×10^4) were transfected with 5 nM control si1, control si2, LARP4 si1, or LARP4 si2 for 48 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for LARP4. Equal amounts of protein were loaded in each lane, for which representative images are shown (A). Cell migration was determined using a Transwell assay. Data shown (mean ± SE, n = 5) are the percentages of migrating cells (B).
*P < 0.05, **P < 0.01.

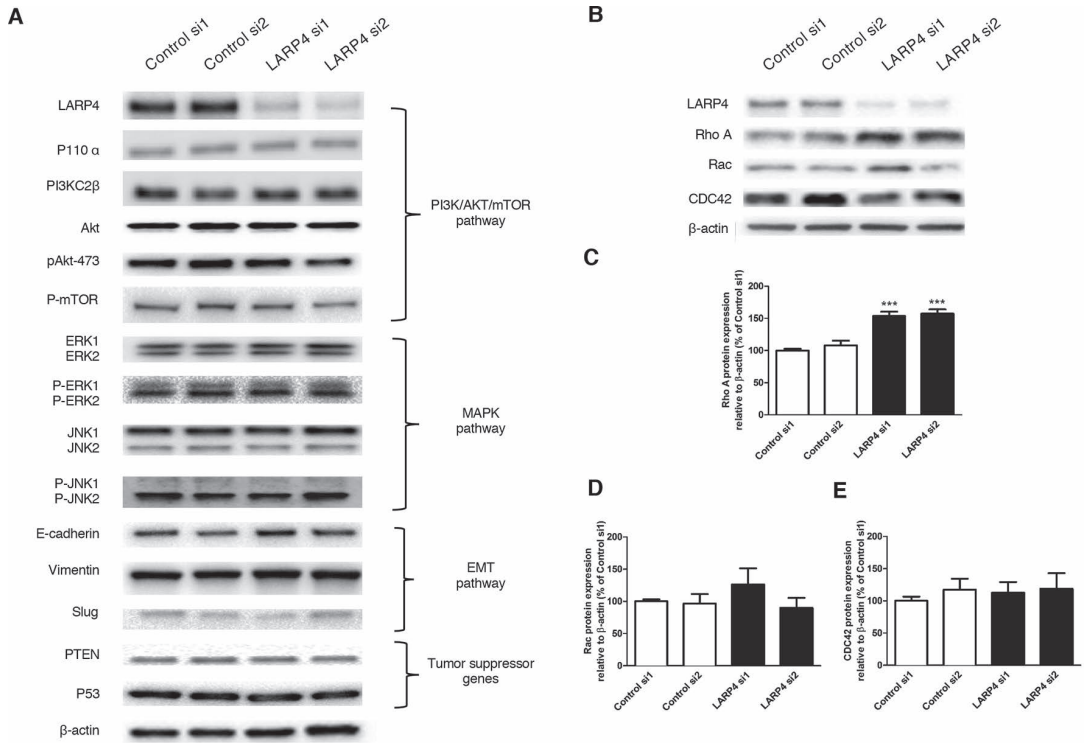

Fig. 4. LARP4 suppresses RhoA protein expression in SKOV3 cells.
(A and B) SKOV3 cells (1×10^4) were transfected with 5 nM control si1, control si2, LARP4 si1, or LARP4 si2 for 48 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for LARP4, PI3K/Akt/mTOR pathway proteins, MAPK pathway proteins, EMT proteins, tumor suppressor proteins, Rho GTPase family proteins, or *β*-actin. Equal amounts of protein were loaded in each lane, for which representative images are shown. (C-E) Protein bands were quantified and the ratio values of individual protein expression relative to *β*-actin are shown (mean ± SE, n = 6 or 8). All statistical analyses were performed using unpaired Student's t-test.
***P < 0.0001.

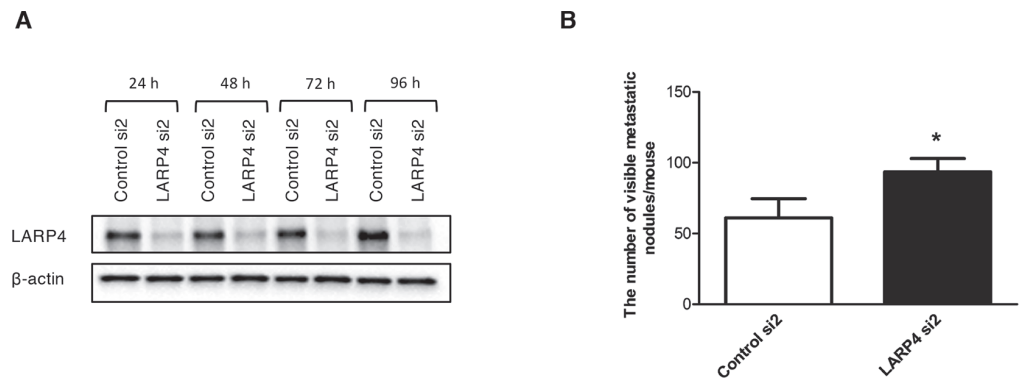

Fig. 5. The anti-metastatic potential of LARP4 in an ovarian cancer xenograft model.
(A) SKOV3 cells (1×10^4) were transfected with 5 nM control si2 or LARP4 si2 for up to 96 h. Extracted proteins were subjected to immunoblot analysis using antibodies specific for LARP4 or *β*-actin. Representative images are shown. (B) SKOV3 cells (5×10^6 cells/mouse) transfected with 5 nM control si2 or LARP4 si2 for 24 h were inoculated into the peritoneal cavity of nude mice (n = 6 or 9). Four weeks later, mice were sacrificed and the number of metastatic nodules in the peritoneum was determined. All statistical analyses were performed by one-tailed *t*-test.
*P < 0.05.

Correlation of LARP mRNA expression with survival of patients with ovarian cancer

To assess the relevance of LARP expression to the prognosis of patients with ovarian cancer, we performed an online analysis using ovarian cancer microarray datasets (Gyorffy et al. 2012). Patients were divided into two groups, with high and low LARP expression. Overall survival periods were plotted to generate Kaplan-Meier curves. As shown in Fig. 6, the LARP4-high patient group showed longer overall survival than the LARP4-low group, suggest-

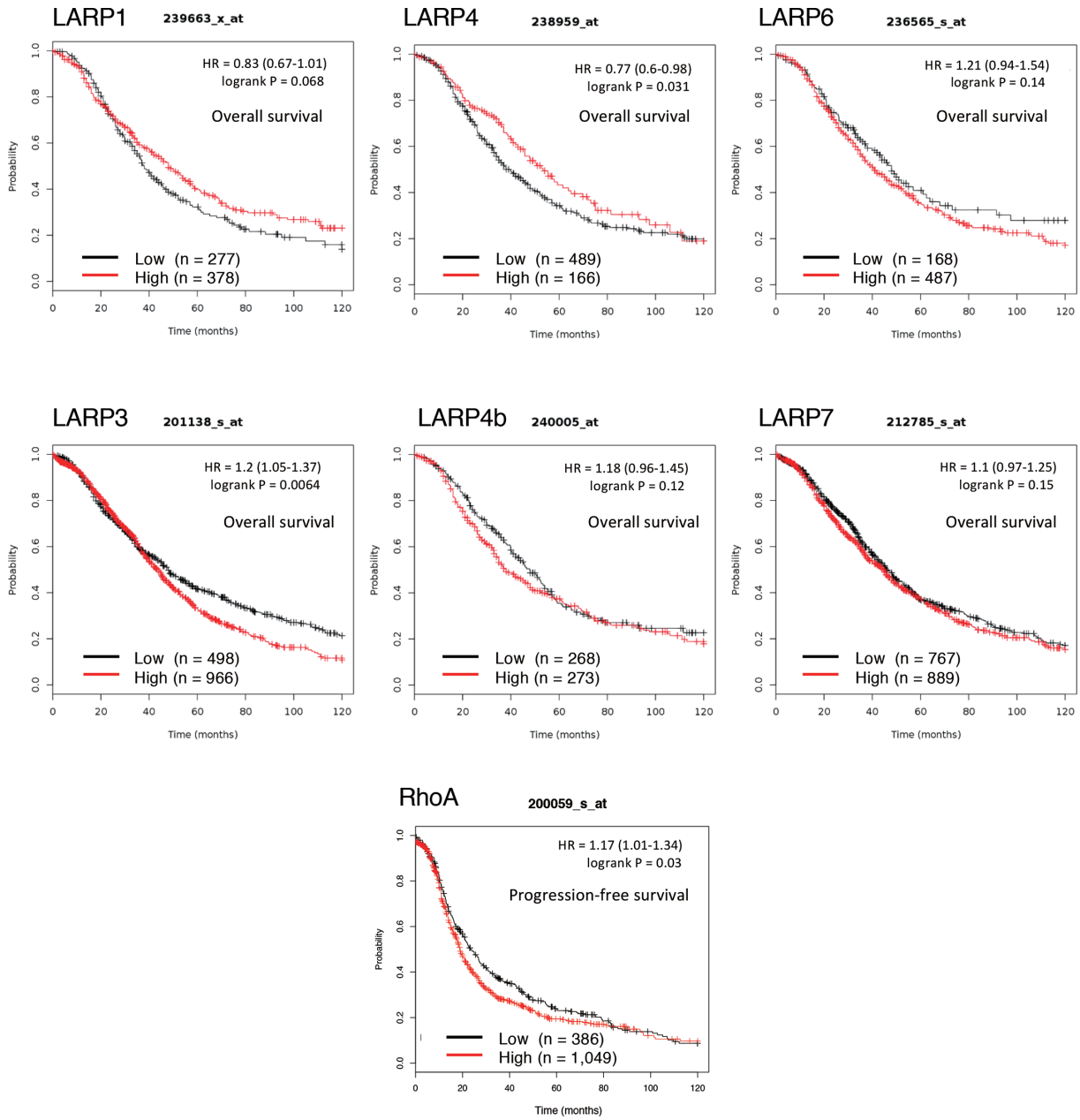

Fig. 6. Correlation of LARP mRNA expression with the overall survival probability in ovarian cancer patients. The graphs show the correlation between overall survival and mRNA expression of LARPs in ovarian cancer patients. The red line represents patients with higher expression and the black line indicates those with lower expression. The numbers of patients used for the analyses are shown in the individual panels. One graph shows the correlation between progression-free survival and expression of RhoA mRNA in ovarian cancer patients (bottom center).

ing a positive correlation of LARP4 mRNA in ovarian cancer tissues with patient prognosis. Conversely, LARP3 mRNA expression was inversely correlated with overall survival ($P < 0.007$). No significant correlation of overall survival with the mRNA expression of other LARPs was observed (Fig. 6). mRNA expression of RhoA was significantly correlated with progression-free survival (Fig. 6), but not with overall survival (data not shown).

Discussion

Seetharaman et al. (2016) demonstrated that LARP4 depletion increased cell motility and invasiveness in PC3 prostate cancer cells and MDA-MB-231 breast cancer cells, and RhoA was reported to promote cell motility and invasiveness in those cell lines (Pille et al. 2005; Hwang et al. 2006; Zhu et al. 2012). In our study, LARP4 suppressed RhoA-dependent cell motility but not invasiveness of ovarian cancer cells. Although the reason for this difference remains unknown, the regulatory mechanism of invasiveness in SKOV3 cells might differ from those in PC3 and MDA-MB-231 cells. Evaluating the formation of invadopodia and podosomes might be a suitable approach

for understanding the roles of LARP4 in the invasiveness of ovarian cancer cells.

In the present study, we suggest that LARP4 may regulate RhoA expression. However, the molecular bases by which LARP4 downregulates RhoA protein expression remain unknown. Rho GTPase signaling is controlled by multiple regulators, including Rho GTPase-specific guanine nucleotide exchange factors, GTPase activating enzymes, and Rho guanine nucleotide dissociation inhibitors (Schmitz et al. 2000; Ridley 2015). We did not determine RhoA activities in the present study. Further investigations need to be performed to uncover the molecular mechanisms involved.

RhoA is widely implicated in the progression of several types of human cancer, including breast, prostatic, testicular, and esophageal cancers (Somlyo et al. 2000; Fritz et al. 2002; Kamai et al. 2004; Pille et al. 2005; Faried et al. 2007; Matsuoka and Yashiro 2014). Similarly, RhoA has emerged as a key player in the progression of ovarian cancer associated with metastasis (Horiuchi et al. 2003, 2008; Chen et al. 2013; Wang et al. 2015). Considering these studies, the regulation of RhoA by LARP4 is suggested to play an important role in ovarian cancer progression.

LARP3 was shown to promote cell proliferation in cervical, prostatic, and hypopharyngeal cancers (Sommer et al. 2011a, b). In addition, LARP3 has been implicated in the upregulation of cell migration in hypopharyngeal squamous adenocarcinoma cells. However, the pathobiological roles of LARP3 in ovarian cancer remain poorly understood. We provide evidence that LARP3 promotes cell motility in ovarian cancer cells (Fig. 1A and B). In addition, we explored the clinical importance of LARP3 mRNA expression in ovarian cancer (Fig. 6). Taken the obtained findings together, LARP3 might serve as an oncogenic RBP in ovarian cancer.

In this study, we provide evidence that LARP4 suppresses RhoA-dependent cell motility and metastatic potential in ovarian cancer. This anti-metastatic effect might be associated with the clinical significance of LARP4 expression in ovarian cancer.

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Conflict of Interest

The authors declare no conflict of interest.

References

Bai, H., Li, H., Li, W., Gui, T., Yang, J., Cao, D. & Shen, K. (2015) The PI3K/AKT/mTOR pathway is a potential predictor of distinct invasive and migratory capacities in human ovarian cancer cell lines. *Oncotarget*, **6**, 25520-25532.

Bayfield, M.A., Yang, R. & Maraia, R.J. (2010) Conserved and divergent features of the structure and function of La and La-related proteins (LARPs). *Biochim. Biophys. Acta*, **1799**, 365-378.

Bisi, S., Disanza, A., Malinverno, C., Frittoli, E., Palamidessi, A. & Scita, G. (2013) Membrane and actin dynamics interplay at lamellipodia leading edge. *Curr. Opin. Cell Biol.*, **25**, 565-573.

Burrows, C., Abd Latip, N., Lam, S.J., Carpenter, L., Sawicka, K., Tzolovsky, G., Gabra, H., Bushell, M., Glover, D.M., Willis, A.E. & Blagden, S.P. (2010) The RNA binding protein Larp1 regulates cell division, apoptosis and cell migration. *Nucleic Acids Res.*, **38**, 5542-5553.

Chen, S., Wang, J., Gou, W.F., Xiu, Y.L., Zheng, H.C., Zong, Z.H., Takano, Y. & Zhao, Y. (2013) The involvement of RhoA and Wnt-5a in the tumorigenesis and progression of ovarian epithelial carcinoma. *Int. J. Mol. Sci.*, **14**, 24187-24199.

Cheng, Y., Jin, Z., Agarwal, R., Ma, K., Yang, J., Ibrahim, S., Olaru, A.V., David, S., Ashktorab, H., Smoot, D.T., Duncan, M.D., Hutcheon, D.F., Abraham, J.M., Meltzer, S.J. & Mori, Y. (2012) LARP7 is a potential tumor suppressor gene in gastric cancer. *Lab. Invest.*, **92**, 1013-1019.

Dhillon, A.S., Hagan, S., Rath, O. & Kolch, W. (2007) MAP kinase signalling pathways in cancer. *Oncogene*, **26**, 3279-3290.

Faried, A., Faried, L.S., Usman, N., Kato, H. & Kuwano, H. (2007) Clinical and prognostic significance of RhoA and RhoC gene expression in esophageal squamous cell carcinoma. *Ann. Surg. Oncol.*, **14**, 3593-3601.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M. & Kaina, B. (2002) Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br. J. Cancer*, **87**, 635-644.

Glisovic, T., Bachorik, J.L., Yong, J. & Dreyfuss, G. (2008) RNA-binding proteins and post-transcriptional gene regulation. *FEBS Lett.*, **582**, 1977-1986.

Guan, X. (2015) Cancer metastases: challenges and opportunities. *Acta Pharm. Sin. B*, **5**, 402-418.

Gyorffy, B., Lanczky, A. & Szallasi, Z. (2012) Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr. Relat. Cancer*, **19**, 197-208.

Heerboth, S., Housman, G., Leary, M., Longacre, M., Byler, S., Lapinska, K., Willbanks, A. & Sarkar, S. (2015) EMT and tumor metastasis. *Clin. Transl. Med.*, **4**, 6.

Hopkins, T.G., Mura, M., Al-Ashtal, H.A., Lahr, R.M., Abd-Latip, N., Sweeney, K., Lu, H., Weir, J., El-Bahrawy, M., Steel, J.H., Ghaem-Maghami, S., Aboagye, E.O., Berman, A.J. & Blagden, S.P. (2016) The RNA-binding protein LARP1 is a post-transcriptional regulator of survival and tumorigenesis in ovarian cancer. *Nucleic Acids Res.*, **44**, 1227-1246.

Horiuchi, A., Imai, T., Wang, C., Ohira, S., Feng, Y., Nikaido, T. & Konishi, I. (2003) Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma. *Lab. Invest.*, **83**, 861-870.

Horiuchi, A., Kikuchi, N., Osada, R., Wang, C., Hayashi, A., Nikaido, T. & Konishi, I. (2008) Overexpression of RhoA enhances peritoneal dissemination: RhoA suppression with Lovastatin may be useful for ovarian cancer. *Cancer Sci.*, **99**, 2532-2539.

Hwang, Y.S., Hodge, J.C., Sivapurapu, N. & Lindholm, P.F. (2006) Lysophosphatidic acid stimulates PC-3 prostate cancer cell

Matrigel invasion through activation of RhoA and NF-kappaB activity. *Mol. Carcinog.*, **45**, 518-529.

Jelovac, D. & Armstrong, D.K. (2011) Recent progress in the diagnosis and treatment of ovarian cancer. *CA Cancer J. Clin.*, **61**, 183-203.

Kamai, T., Yamanishi, T., Shirataki, H., Takagi, K., Asami, H., Ito, Y. & Yoshida, K. (2004) Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clin. Cancer Res.*, **10**, 4799-4805.

Kitatani, K., Usui, T., Sriraman, S.K., Toyoshima, M., Ishibashi, M., Shigeta, S., Nagase, S., Sakamoto, M., Ogiso, H., Okazaki, T., Hannun, Y.A., Torchilin, V.P. & Yaegashi, N. (2016) Ceramide limits phosphatidylinositol-3-kinase C2beta-controlled cell motility in ovarian cancer: potential of ceramide as a metastasis-suppressor lipid. *Oncogene*, **35**, 2801-2812.

Krause, M. & Gautreau, A. (2014) Steering cell migration: lamellipodium dynamics and the regulation of directional persistence. *Nat. Rev. Mol. Cell Biol.*, **15**, 577-590.

Lee, Y.K. & Park, N.H. (2009) Prognostic value and clinicopathological significance of p53 and PTEN in epithelial ovarian cancers. *Gynecol. Oncol.*, **112**, 475-480.

Liao, B., Hu, Y. & Brewer, G. (2007) Competitive binding of AUF1 and TIAR to MYC mRNA controls its translation. *Nat. Struct. Mol. Biol.*, **14**, 511-518.

Machesky, L.M. (2008) Lamellipodia and filopodia in metastasis and invasion. *FEBS Lett.*, **582**, 2102-2111.

Matsuoka, T. & Yashiro, M. (2014) Rho/ROCK signaling in motility and metastasis of gastric cancer. *World J. Gastroenterol.*, **20**, 13756-13766.

Mura, M., Hopkins, T.G., Michael, T., Abd-Latip, N., Weir, J., Aboagye, E., Mauri, F., Jameson, C., Sturge, J., Gabra, H., Bushell, M., Willis, A.E., Curry, E. & Blagden, S.P. (2015) LARP1 post-transcriptionally regulates mTOR and contributes to cancer progression. *Oncogene*, **34**, 5025-5036.

Naora, H. & Montell, D.J. (2005) Ovarian cancer metastasis: integrating insights from disparate model organisms. *Nat. Rev. Cancer*, **5**, 355-366.

Parsons, J.T., Horwitz, A.R. & Schwartz, M.A. (2010) Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.*, **11**, 633-643.

Pille, J.Y., Denoyelle, C., Varet, J., Bertrand, J.R., Soria, J., Opolon, P., Lu, H., Pritchard, L.L., Vannier, J.P., Malvy, C., Soria, C. & Li, H. (2005) Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Mol. Ther.*, **11**, 267-274.

Raineri, I., Wegmueller, D., Gross, B., Certa, U. & Moroni, C. (2004) Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference. *Nucleic Acids Res.*, **32**, 1279-1288.

Rajkowitsch, L., Chen, D., Stampfl, S., Semrad, K., Waldsich, C., Mayer, O., Jantsch, M.F., Konrat, R., Blasi, U. & Schroeder, R. (2007) RNA chaperones, RNA annealers and RNA helicases. *RNA Biol.*, **4**, 118-130.

Ridley, A.J. (2015) Rho GTPase signalling in cell migration. *Curr. Opin. Cell Biol.*, **36**, 103-112.

Schmitz, A.A., Govek, E.E., Bottner, B. & Van Aelst, L. (2000) Rho GTPases: signaling, migration, and invasion. *Exp. Cell Res.*, **261**, 1-12.

Seetharaman, S., Flemyng, E., Shen, J., Conte, M.R. & Ridley, A.J. (2016) The RNA-binding protein LARP4 regulates cancer cell migration and invasion. *Cytoskeleton* (*Hoboken*), **73**, 680-690.

Somlyo, A.V., Bradshaw, D., Ramos, S., Murphy, C., Myers, C.E. & Somlyo, A.P. (2000) Rho-kinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells. *Biochem. Biophys. Res. Commun.*, **269**, 652-659.

Sommer, G., Dittmann, J., Kuehnert, J., Reumann, K., Schwartz, P.E., Will, H., Coulter, B.L., Smith, M.T. & Heise, T. (2011a) The RNA-binding protein La contributes to cell proliferation and CCND1 expression. *Oncogene*, **30**, 434-444.

Sommer, G., Rossa, C., Chi, A.C., Neville, B.W. & Heise, T. (2011b) Implication of RNA-binding protein La in proliferation, migration and invasion of lymph node-metastasized hypopharyngeal SCC cells. *PLoS One*, **6**, e25402.

Vignjevic, D. & Montagnac, G. (2008) Reorganisation of the dendritic actin network during cancer cell migration and invasion. *Semin. Cancer Biol.*, **18**, 12-22.

Wang, X., Jiang, W., Kang, J., Liu, Q. & Nie, M. (2015) Knockdown of RhoA expression alters ovarian cancer biological behavior in vitro and in nude mice. *Oncol. Rep.*, **34**, 891-899.

Ye, L., Lin, S.T., Mi, Y.S., Liu, Y., Ma, Y., Sun, H.M., Peng, Z.H. & Fan, J.W. (2016) Overexpression of LARP1 predicts poor prognosis of colorectal cancer and is expected to be a potential therapeutic target. *Tumour Biol.*, **37**, 14585-14594.

Yeung, T.L., Leung, C.S., Yip, K.P., Au Yeung, C.L., Wong, S.T. & Mok, S.C. (2015) Cellular and molecular processes in ovarian cancer metastasis. A review in the theme: cell and molecular processes in cancer metastasis. *Am. J. Physiol. Cell Physiol.*, **309**, C444-456.

Zhang, X., Kitatani, K., Toyoshima, M., Ishibashi, M., Usui, T., Minato, J., Egiz, M., Shigeta, S., Fox, T., Deering, T., Kester, M. & Yaegashi, N. (2018) Ceramide nanoliposomes as a MLKL-dependent, necroptosis-inducing, chemotherapeutic reagent in ovarian cancer. *Mol. Cancer Ther.*, **17**, 50-59.

Zhu, Y., Tian, Y., Du, J., Hu, Z., Yang, L., Liu, J. & Gu, L. (2012) Dvl2-dependent activation of Daam1 and RhoA regulates Wnt5a-induced breast cancer cell migration. *PLoS One*, **7**, e37823.