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LMO2 Enhances Lamellipodia/Filopodia Formation in Basal-Type Breast Cancer Cells by Mediating ARP3-Profilin1 Interaction

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The human LMO2 gene was first cloned from an acute T lymphocytic leukemia patient; it is primarily expressed in hematopoietic and vascular endothelial systems, and functions as a pivotal transcriptional regulator during embryonic hematopoiesis and angiogenesis. However, some recent reports indicated that LMO2 is widely expressed in many tissues and tumors, predominantly in cytoplasm, and revealed complicated functions on tumor behaviors in a variety of cancer types. As an adaptor molecule, binding partners and function details of LMO2 in these solid tumors need to be further investigated.




Material/Methods: In this study, we used yeast two-hybrid method to screen potential LMO2 interacting partners, MBP-pulldown, and co-immunoprecipitation assay to confirm protein-protein interactions, and confocal microscopy to reveal the subcellular localization of relevant proteins and actin cytoskeleton changes in relevant cells.

Results: We found that ARP3 and profilin1 were 2 binding partners of LMO2, primarily in cytoplasm. LMO2. Functionally, LMO2 mediated the assembly of a complex including ARP3, profilin1, and actin monomer, increased actin monomer binding to profilin1, and promoted lamellipodia/filopodia formation in basal-type breast cancer cells.

Conclusions: Our data indicate a novel functional mechanism of LMO2 in facilitating the delivery of actin monomers to the branched microfilament and increasing lamellipodia/filopodia formation in basal-type breast cancer cells, suggesting a cancer-promoting role of LMO2 in a subtype-dependent manner and its potential as a subtype-specific biomarker for clinical treatment of breast cancers.

MeSH Keywords: **ARP3 • LMO2 • Profilin1**

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Background

The human LMO2 gene was first cloned from an acute T lymphocytic leukemia (T-ALL) patient with an (11;14)(p13;q11) translocation [1]. It was a pivotal factor for both embryonic and adult hematopoiesis, as well as angiogenesis [2,3]. Moreover, LMO2 is expressed in all hematopoietic cells except for mature T cells [4], and its forced ectopic expression caused T-ALL-like syndrome in mouse models [5] and several X-SCID patients who were treated with lentivirus-mediated gene therapy [6]. In classic studies, LMO2 was recognized as a transcription factor and performed bi-directional regulation functions in its different target genes [7–9]. However, the LMO2 protein, which consists of only 2 tandem LIM domains, lacks the direct DNA-binding ability and functions as a bridge molecular in a transcriptional complex usually including LDB1, GATA1, TAL1, and E47 [10,11]. Notably, recent studies revealed that LMO2 was expressed in a variety of solid tissues and tumors, with either nuclear or cytoplasmic localization [12], and played dual functions in tumor behaviors in different cancer types [13]. As an adaptor molecule, many details about LMO2 binding partners, as well as molecular and cytological functions, need to be further investigated.

Invasion and metastasis are hallmarks of highly aggressive tumor cells [14,15], and result primarily from enhanced cell motility controlled by actin cytoskeleton remodeling [16]. Notably, in actin cytoskeleton regulation, profilin1 is an actin monomer-binding protein that participates in free actin monomer capture and delivery [16], while ARP3, together with other members of the ARP2/3 complex, functions as a core nucleation component for branched microfilament growth during cell migration [17]. In a recent study, we found that LMO2 had a function of promoting tumor cell invasion and metastasis specifically in basal-type breast cancers [18]. In the present study, we confirmed that ARP3 and profilin1 were 2 binding partners of LMO2 in cytoplasm, and LMO2 facilitated the assembly of the complex including ARP3, profilin1, and actin monomer, promoting lamellipodia/filopodia formation in basal-type breast cancer cells.

Material and Methods

Yeast two-hybrid assay

The Matchmaker™ Gold Yeast Two-Hybrid system (Clontech, Palo Alto, CA, USA) was used for yeast two-hybrid assays. The LMO2 coding sequence was inserted into the pGBKT7 vector to express the GAD4_BD-LMO2 fusion protein as bait. A pre-transformed library of human universal cDNA cloned into pGADT7 vector was purchased from Clontech. The yeast strain mating and screening procedures were performed following the manufacturer's instructions. Positive clones, which presented as blue

colonies, were re-seeded onto new selection medium plates and each inserted cDNA fragment was PCR-amplified and sequenced. The potential LMO2 binding partners were validated by BLAST searches on the NCBI website.

Plasmid construct

The human profilin1 and ARP3 expression vectors were constructed by amplification of their coding sequences from human peripheral blood cDNA and inserting into pcDNA6B vector with a myc-tag. The coding region of LMO2 was inserted into the pMAL-c2x vector for the expression of recombinant MBP-LMO2 fusion proteins. The LMO2 expression and LMO2-shRNA lentivirus vector, as well as the control lentivirus vector, and the Lenti-Pac™ HIV Expression Packaging Kit, were purchased from GeneCopoeia (Rockville, MD, USA). HEK293T packaging cell line was used for lentiviral amplification following GeneCopoeia's instructions.

Cell culture and transfection

The breast cancer cell line MDA-MB-231 and human embryonic kidney cell line HEK-293T were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM medium supplemented with 10% fetal bovine serum (Life Technologies, Austin, TX, USA). Transfections were performed using Lipofectamine 2000 following the manufacturer's instructions (Life Technologies). Stable cell strains of MDA-MB-231-LMO2, MDA-MB-231-control and MDA-MB-231-sh-LMO2 were selected and maintained by cultured in the medium with puromycin (2 µg/ml) 3 days after lentivirus infection. Cells transfected with Rac1(Q61L) or Cdc42(Q61L) expression vector were cultured in the presence of 500 µg/ml G418 for 1 week for selection to remove the non-transfected cells before assay.

Protein extraction and western blotting

Total proteins were extracted using a protein extraction kit (CWBI, Beijing, China). Protein concentrations were assayed with a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). SDS-PAGE was performed using 50 µg of each sample. The antibodies used for each detection are listed in the Supplementary Table 1. Immunostaining was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The gray-scale quantification of immunoblotting bands was performed using ImageJ software.

MBP-pulldown assay

DE3 *E. coli* cells were transformed with pMAL-c2x/pMAL-LMO2, and then activated and cultured in a 1-L flask. IPTG (at a final concentration of 0.1 mmol/L) was added to cultures at a density of OD 0.6–0.8 for 4 h to induce the expression of the

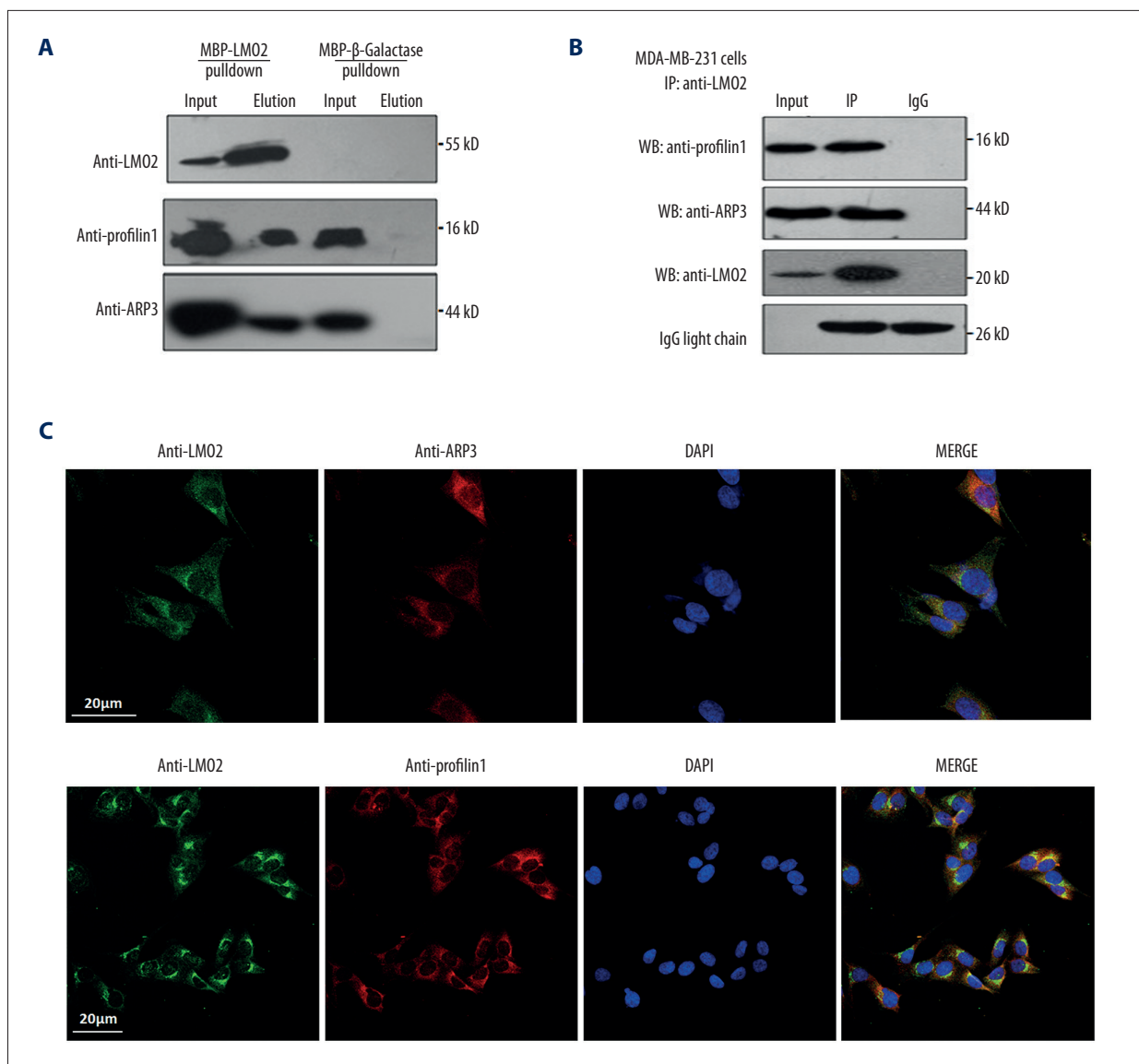


Figure 2. Confirmation of LMO2-ARP3 and LMO2-profilin1 interactions in breast cancer cells. **(A)** MBP-pull-down assay measuring binding between LMO2 and profilin1 and ARP3. MDA-MB-231 cell lysate was incubated with purified recombinant MBP-LMO2 fusion protein or MBP-β-galactase fusion protein as a control. **(B)** Co-immunoprecipitation assay confirming the interaction between endogenous profilin1/ARP3 and LMO2 in MDA-MB-231 cells. **(C)** Immunofluorescence images showing the cytoplasmic co-localization of LMO2 and profilin1/ARP3 in MDA-MB-231 cells. LMO2 protein was labeled with Fluor-488 fluorescence and profilin1/ARP3 was labeled with Fluor-546 fluorescence. The nuclei were stained by DAPI.

equal volume of 2×SDS loading buffer, and heat-denatured for 10 min. Finally, the prepared samples were subjected to SDS-PAGE and analyzed by Western blotting with the relevant antibodies. A total of 1/20 of the total protein mixture of each sample was loaded as the input.

Co-immunoprecipitation (Co-IP)

Each co-IP was performed with 1 mg of total protein. After pre-clearing with 5 μL of 50% protein G beads (Life Technologies),

the supernatant of each sample was incubated with 5 μg anti-LMO2 or -V5 antibodies (1: 100 dilution) or rabbit IgG as the control at 4°C overnight with rotation. Then, 10 μL of 50% protein G beads (Life Technologies) was added to each sample and rotated for an additional 2 h. After washing 3 times with PBST, the beads were mixed with an equal volume of 2×SDS loading buffer and heat-denatured for 10 min. The prepared samples were then subjected to SDS-PAGE and analyzed using Western blotting with relevant antibodies. A total of 1/20 of the proteins from each sample was used as the input.

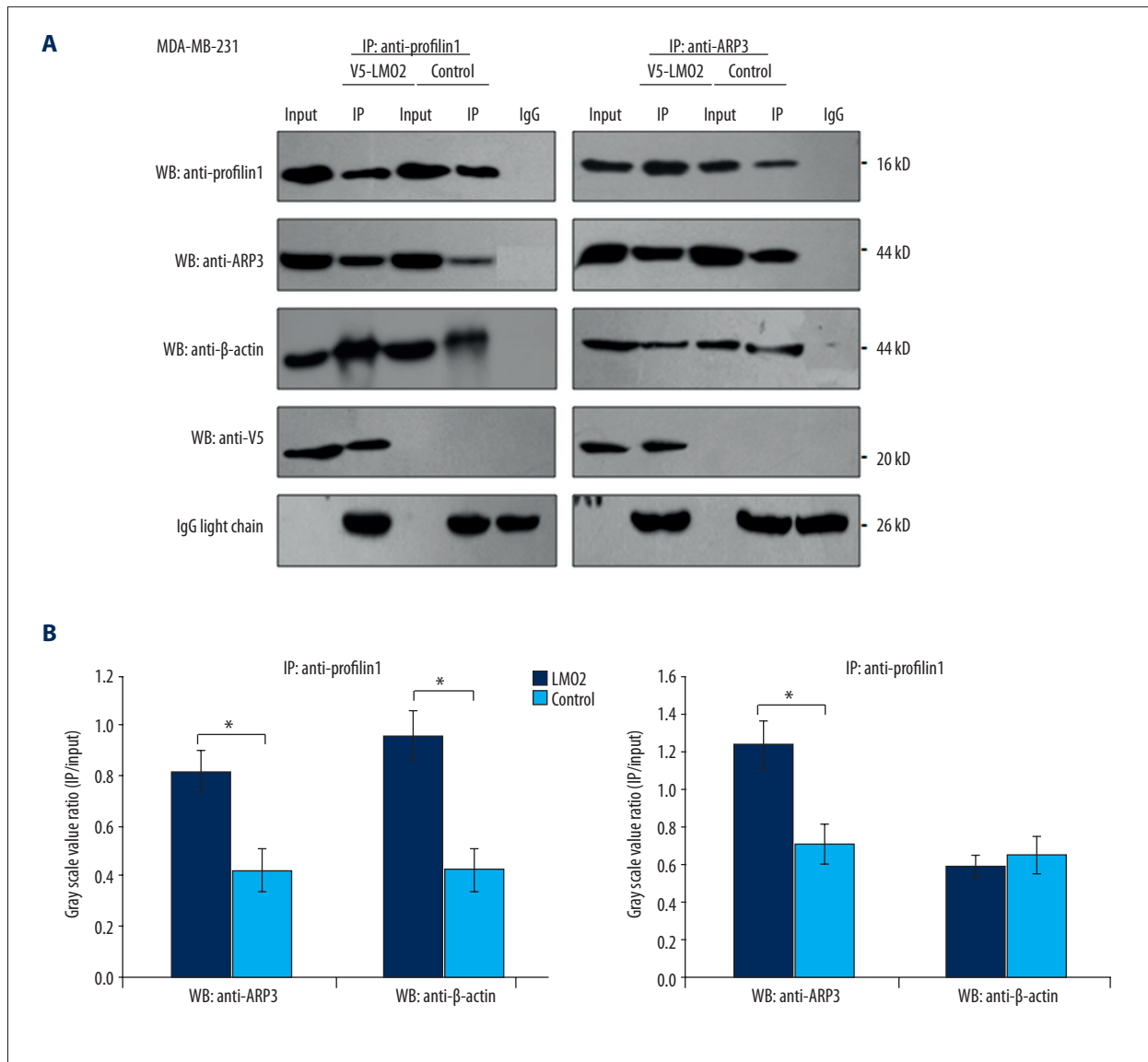


Figure 3. LMO2 facilitated ARP3-profilin1 complex formation and actin monomer delivery in breast cancer cells. **(A)** Co-immunoprecipitation assay confirming interactions between profilin1 and ARP3 in LMO2-overexpressing and control MDA-MB-231 cells. Cell lysate was immunoprecipitated as indicated, and co-immunoprecipitated β-actin was immunoblotted with anti-β-actin antibody. **(B)** Quantification of immunoblot band gray-scale values for co-immunoprecipitated profilin1, ARP3, and β-actin in LMO2-overexpressing and control MDA-MB-231 cells. Bars represent the means of 3 independent experiments for each sample, and error bars indicate standard errors. * Student's *t*-test, $p < 0.05$.

Immunocytofluorescence and confocal microscopy

MDA-MB-231 cells (2×10^4) were seeded onto cell chamber slides (Corning, Tewksbury, MA, USA) in 24-well plates 24 h before assaying. The cells on the slides were fixed with 4% formaldehyde and then stained by FITC-labeled Phalloidin (Sigma-Aldrich, St. Louis, MO, USA, 1: 500 dilution), or stained with anti-LMO2 and anti-ARP3/anti-profilin1 antibodies (1: 200 dilution) at 4°C overnight, followed by incubating with the appropriate fluorescent secondary antibodies at room temperature

for 1 h. Images were captured using an FV1000 confocal microscope (Olympus, Center Valley, PA, USA).

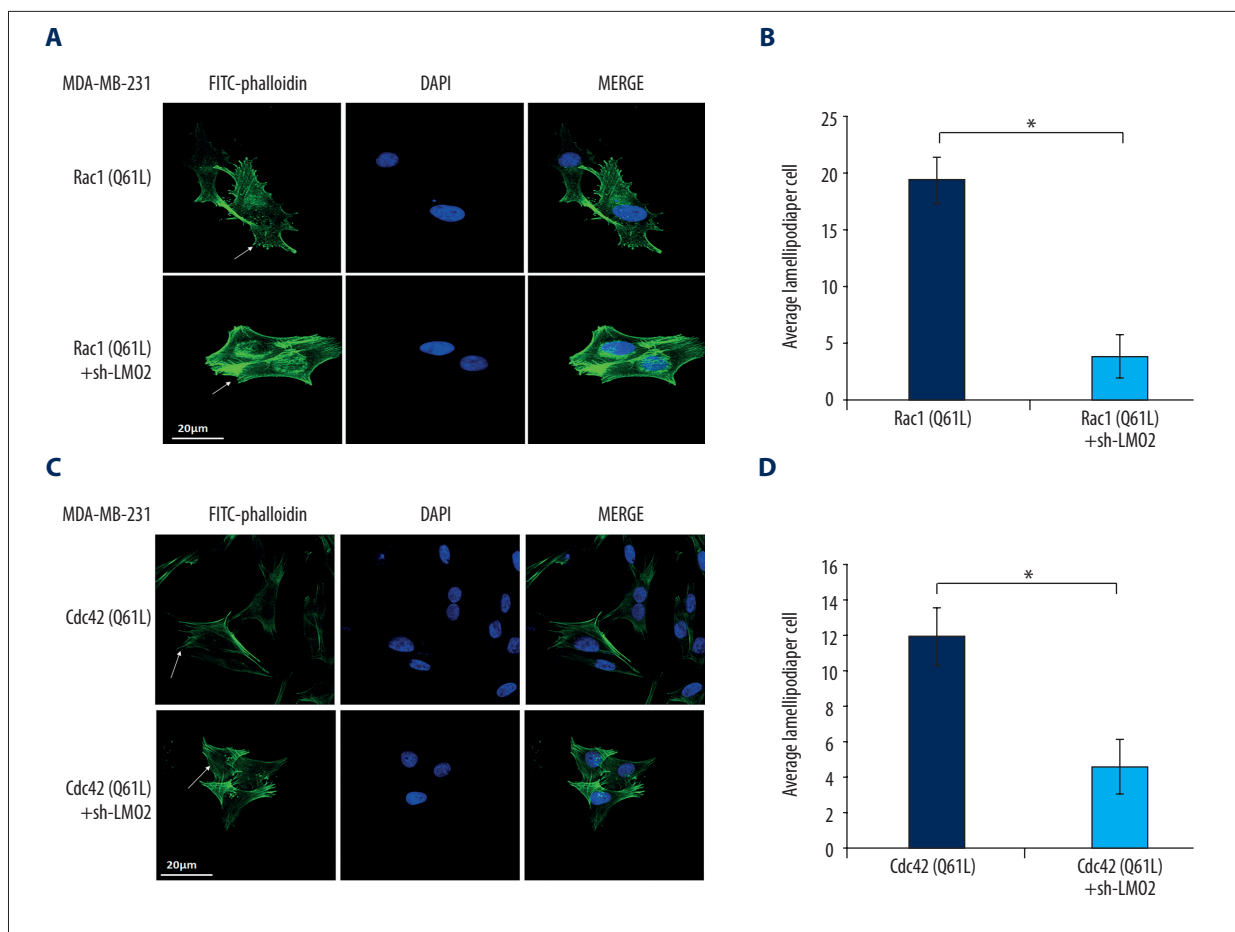


Figure 4. Knocking-down of LMO2 blocked the lamellipodia/filopodia-promoting effect of Rac1/Cdc42 in breast cancer cells. **(A)** Images of immunofluorescent staining of actin cytoskeleton with FITC-phalloidin after forced expression of Rac1 (Q61L) in sh-LMO2 or control MDA-MB-231 cells. Nuclei were stained with DAPI. Arrows indicate changes in lamellipodia in control and sh-LMO2 MDA-MB-231 cells. **(B)** Bar plot showing mean numbers of lamellipodia in different cells. Lamellipodia were counted for each cell in all images; bars represent mean numbers/cell from 10 different images, and error bars indicate standard error. * Student's *t*-test, $p < 0.05$. **(C)** Images of immunofluorescent staining of the actin cytoskeleton with FITC-phalloidin after forced expression of Cdc42 (Q61L) in sh-LMO2 and control MDA-MB-231 cells; arrows indicate changes of filopodia. **(D)** Bar plot quantification of mean numbers of filopodia in different cells. Mean filopodia were quantified with the same protocol as described in **(B)**.

Results

ARP3 and profilin1 are identified as binding partners of LMO2 and co-localize with LMO2 in cytoplasm

In a yeast two-hybrid assay using LMO2 as the bait, dozens of candidates that interacted with LMO2 were identified (presented as blue colonies; Figure 1A). After PCR amplification and sequencing of the inserted cDNA fragments for each clone, 2 fragments aligned to the coding sequence of profilin1 and ARP3, respectively, were screened out (Figure 1B), suggesting the potential binding between LMO2 and ARP3/profilin1.

Further, using MBP-pulldown assay, binding between ARP3/profilin1 and recombinant MBP-LMO2 fusion proteins

were confirmed (Figure 2A). Moreover, co-immunoprecipitation assay also confirmed the endogenous LMO2-ARP3 and LMO2-profilin1 interaction (Figure 2B). Immunofluorescence staining indicated strongly cytoplasmic co-localization of LMO2 and profilin1/ARP3 (Figure 2C), suggesting that these interactions occurred primarily in cytoplasm.

LMO2 facilitates actin monomer delivery and lamellipodia/filopodia formation in basal-type breast cancer cells

Considering that both profilin1 and ARP3 can bind with the actin monomer, LMO2, profilin1, ARP3, and actin monomer may form a complex. Correspondingly, as shown in Figure 3A, co-immunoprecipitation assay revealed that profilin1 and ARP3 could indeed be immunoprecipitated by each other, while the

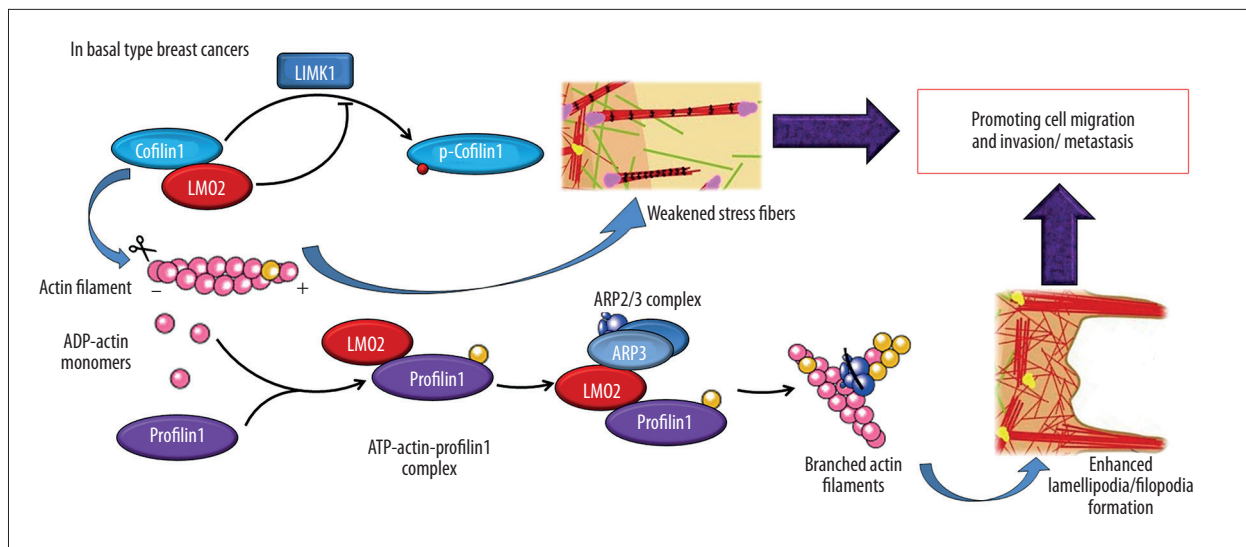


Figure 5. Sketch diagram of the molecular mechanism of LMO2 on regulating lamellipodia/filopodia formation in basal-type breast cancer cells. LMO2 increased actin monomer release by targeting the LIMK1-cofilin pathway and enhanced profilin1-ARP3 interactions, which facilitated actin monomer delivery and increased lamellipodia/filopodia formation.

amount of ARP3 or profilin1 co-immunoprecipitated by each other was increased in LMO2-overexpressing MDA-MB-231 cells compared to control cells (Figure 3A, 3B). β -actin, an actin monomer, could also be co-immunoprecipitated by ARP3 or profilin1. The amount of β -actin co-precipitated by profilin1 was increased in LMO2-overexpressing MDA-MB-231 cells compared to the control, but the change in β -actin amount co-precipitated by ARP3 was not obvious (Figure 3A, 3B). Taken together, these data indicated that LMO2 could enhance the interaction between ARP3 and profilin1 and facilitated the profilin1-mediated actin monomer delivery to the growing branched microfilaments marked by ARP3, which primarily contributed to cell protrusion formation and cell movement.

Notably, in a previous study, we found that basal-type breast cancer cell line MDA-MB-231 with LMO2 knocking-down showed more tightly-attached cell morphologies and fewer cell protrusions, while overexpression of LMO2 in MDA-MB-231 cells caused the opposite effect [18]. Moreover, the small RhoGTPase family member Rac1 and Cdc42 are key positive regulators of lamellipodia and filopodia formation, respectively [16]. In general, forced expression of the constitutively active form of Rac1 (Rac1(Q61L)) or Cdc42 (Cdc42(Q61L)) in MDA-MB-231 cells increased lamellipodia (Figure 4A, 4B) or filopodia (Figure 4C, 4D), respectively. However, knocking-down of LMO2 largely eliminated these effects and maintained the relatively tightly-attached, less-protruding cell morphology (Figure 4A-4D). These results indicated the cytological function of LMO2 as a positive regulator of lamellipodia/filopodia formation and cell motility in basal-type breast cancer cells.

Discussion

Breast cancer is a highly heterogeneous disease with diverse biological and clinical characteristics. Based on gene expression features, breast cancers can be subdivided into luminal A, luminal B, Her2, and basal subtypes (the PAM50 subtyping system) [19]. Among all these subtypes, basal-type breast cancer is a prognostically unfavorable subtype characterized by high aggressiveness and metastasis [20]. In a previous study, we demonstrated that, specifically in basal-type breast cancer, LMO2 promoted tumor cell migration, invasion, and metastasis, and this function was partially achieved by blocking LIM kinase 1 (LIMK1)-mediated phosphorylation of cofilin1 [18]. Herein, we found that high LMO2 expression also contributed to the regulation of cell protrusions by interaction with ARP3 and profilin1. Because LMO2 increased the release of actin monomers by targeting cofilin1 [18], it can be speculated that interactions between LMO2, ARP3/profilin1, and actin monomers may accelerate free actin monomer delivery by profilin1 as the materials for the growth of branched microfilaments marked by the ARP2/3 complex, and these molecular interactions finally promote the branched microfilament growth and lamellipodia/filopodia formation (Figure 5).

Traditionally, LMO2 was primarily considered as a transcription factor in cell nuclei in hematopoietic cells and vascular endothelia [10]; however, some recent studies revealed that LMO2 is expressed in a variety of other tissues and tumors, with either nuclear or cytoplasmic location [13]. Moreover, LMO2 showed predominantly cytoplasmic location in most epithelial-derived tumors [12,13,18] and dual effects on tumor behaviors: some reports indicated that LMO2 played an oncogenic

role in glioblastoma [21] and prostate carcinoma [22], but was a good prognostic marker for diffuse large B cell lymphoma (DLBCL) [23], acute B lymphocytic leukemia (B-ALL) [24], and pancreatic carcinoma [25]. In breast cancers, LMO2 primarily is located in cytoplasm and plays additionally divergent functions in different breast cancer subtypes [18]. Our data in the current study further support this viewpoint and provided 2 novel cytoplasmic LMO2 binding partners, ARP3 and profilin1, for the annotation of the function of LMO2 in basal-type breast cancers.

Conclusions

The current study identified 2 novel cytoplasmic LMO2-binding partners, ARP3 and profilin1, and revealed a novel functional

mechanism of LMO2 in promoting lamellipodia/filopodia formation via LMO2-ARP3 and LMO2-profilin1 interactions in basal-type breast cancer cells. In general, these findings contribute to optimizing the knowledge of LMO2 in biochemistry and cell-level biology. Specifically, LMO2 exhibited complicated effects on tumor behaviors in different cancer types, and our study confirmed a basal-type breast cancer-specific tumor-promoting role of LMO2. These findings also suggest the potential of LMO2 for use as a cancer-type-dependent biomarker for precision medicine in clinical practice.

Conflict of interest

There is no conflict of interest in relation to this article.

Supplementary Table

Supplementary Table 1. Supplementary antibody information.

Product Name	Source	Product number	Company	Application
V5-Probe (G-14)	Rabbit pAb	sc-83849	Santa Cruz	WB, IP
Profilin-1 antibody	Rabbit pAb	3237	CST	WB, IP, IF
Arp3 antibody [FMS338]	Mouse mAb	ab49671	Abcam	WB, IP, IF
β -actin antibody	Rabbit pAb	21338	SAB	WB
Anti-LMO2 antibody [1A9-3B11]	Mouse mAb	ab81988	Abcam	IF
Anti-LMO2 antibody [EP3257]	Rabbit mAb	ab91652	Abcam	WB, IP
Anti-rabbit IgG	HRP-linked antibody	7074	CST	WB
Anti-mouse IgG	HRP-linked antibody	7076	CST	WB
Alexa fluor 488 donkey anti-mouse IgG		CA21202s	Invitrogen	IF
Alexa fluor 546 donkey anti-rabbit IgG		A10040	Invitrogen	IF

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