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Original Paper

MORC2 Enhances Tumor Growth by Promoting Angiogenesis and Tumor-Associated Macrophage Recruitment via Wnt/β-Catenin in Lung Cancer

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Key Words
MORC2 • Growth • TAM • Lung cancer • Stem-like cells

Abstract
Background/Aims: In this study, we aimed to investigate how MORC family CW-type zinc finger 2 (MORC2) affects tumor progression of lung cancer. ***Methods:*** The MORC2 level was analyzed by real-time RT-PCR and immunohistochemistry (IHC) in normal control tissues and lung cancers. LL/2 cells overexpressing MORC2 were used to study how MORC2 expression influences lung cancer progression. The effects of MORC2 on cell viability, migration and invasion were assessed by MTT assay, Western blotting, and transwell assays, respectively. Afterwards, the effects of MORC2 on the activation of the Wnt/β-catenin pathway were explored by Western blotting. The effects of MORC2 on tumor-associated macrophages (TAM) were determined by immunofluorescence (IF) staining, real-time RT-PCR and Western blotting. ***Results:*** Our results showed that MORC2 was upregulated in lung cancers relative to adjacent tissues. The results also demonstrated that MORC2 promoted lung cancer tumor growth *in vivo*. Additionally, MORC2 overexpression stimulated the upregulation of vascular endothelial growth factor (VEGF), driving angiogenesis. MORC2 overexpression in LL/2 also increased the amount of aldehyde dehydrogenase-1 (ALDH1) protein, indicating that MORC2 increased cancer stem cell features. We further determined that MORC2 activated Wnt/β-catenin signaling in lung cancer cells. Upregulation of macrophage-recruiting genes including VEGF and Macrophage-specific colony stimulating factor (CSF-1) recruits TAMs to the tumor site, which has the net effect of promoting additional tumor growth and metastasis. ***Conclusion:*** Our data suggest that MORC2 overexpression can drive lung cancer growth by stimulating the recruitment of TAMs in addition to angiogenesis and that activation of Wnt/β-signaling may be a key pathway underlying this phenotype that is amenable to pharmacological intervention.

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Introduction

Lung cancer is the most prominent cause of cancer-related death both in the US, with an estimated 224, 390 new diagnoses in 2016 and a 5-year survival rate < 20%, and worldwide [1, 2]. Non-small cell lung cancer (NSCLC) accounts for 80-85% of these cancers, with 10-15% being SCLCs [3-5]. Smoking and the inhalation of related noxious particulates have been identified as major risk factors for lung cancer, in part due to chronic inflammation that occurs as a consequence of exposure to these compounds [6, 7]. At the molecular level, upregulation of a number of oncogenes, including BRAF (B-Raf proto-oncogene, serine/threonine kinase), human epidermal growth factor receptor 2 (HER2), and ROS1 (ROS proto-oncogene 1, receptor tyrosine kinase), as well as loss of the expression of tumor suppressor genes, such as GPRC5A (G protein-coupled receptor class C group 5 member A) can all promote oncogenesis in the lung [8-11]. While many of the drivers of lung cancer are understood, additional research is needed to produce a comprehensive understanding of the development of this lethal disease.

The microrchidia (MORC) protein family comprises a group of conserved proteins that are key for multiple biological processes [12, 13]. MORC family CW-type zinc finger protein 2 (MORC2), also known as ZCWCC1, ZCW3, KIAA0852 and AC004542.C22.1, is a member of this family [14]. MORC2 contains a zinc finger type CW domain, coiled-coil domains, an ATPase domain, and a nuclear localization signal [15]. A limited number of studies have shown that MORC2 functions in chromatin remodeling, facilitating DNA damage repair and promoting lipogenesis; however, its function in cancer remains to be fully elucidated [16]. As abnormal chromatin dynamics, enhanced DNA damage repair and de novo lipogenesis are crucial events in cancer cells, MORC2 exhibits oncogenic actions by promoting the malignant phenotype of cancer cells [17, 18]. MORC2 is linked to promoting the invasion and metastasis of breast cancer cells [17, 19]. However, to date, no studies have reported the clinical and pathological significance and functions of MORC2 in lung cancer.

The Wnt/β-catenin signaling pathway is conserved throughout evolution and is crucial for maintaining homeostasis in virtually every tissue during normal embryonic development and throughout life [20]. The Wnt/β-catenin pathway is initiated by Wnt family ligands, which are secreted lipoglycoproteins that act on the frizzled (FZD) family receptors [21]. In the resting state without Wnt stimulation, cytoplasmic β-catenin is destroyed by a structure that contains casein kinase 1α (CK1α), APC, Axin, and glycogen synthase kinase 3β (GSK3β), and its level is maintained low by a series of events including priming phosphorylation at Ser45 by CK1α and subsequent phosphorylation at Thr41, Ser37, and Ser33 by GSK3β and by the recruitment of the β-TrCP E3 linker, followed by degradation through the polyubiquitination-mediated proteasomal degradation pathway [22, 23]. When the secreted Wnt ligands accumulate, they interact with the FZD receptor together with the coreceptor lipoprotein receptor-related protein (LRP)-5/6, resulting in activation of dishevelled (DVL) protein [24]. The accumulated nuclear β-catenin can interact directly with T-cell transcription factor (TCF) and other factors to drive specific gene expression, potentially activating genes involved in proliferation and transformation [25, 26].

In this current work, we examined how MORC2 contributes to tumor growth and metastasis in lung cancer cells from both humans and mice. We ultimately found that MORC2 promoted tumor growth *in vivo*, drove vascular endothelial growth factor (VEGF)-mediated angiogenesis, and increased the frequency of cancer stem cell (CSC) features. MORC2 further drove VEGF, chemokine (CC-motif) ligand 2/5 (CCL2/5) and macrophage-specific colony stimulating factor (CSF-1)-mediated recruitment of tumor-associated macrophages (TAMs), which in turn promoted additional growth of lung tumors.

Materials and Methods

Cell culture

Human bronchial epithelial cells HBEC3-KT and BEAS-2B and lung cancer cell lines A549, PC-9, H1299, NCI-H2170, NCI-H226 and LL/2 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose and 10% FBS (Gibco). All cells were grown in a humidified incubator with 5% CO_2 at 37°C.

MORC2 expression modulation

Murine MORC2 cDNA (Origene, Rockville, MD, USA) was cloned into a pcDNA3.1 plasmid (pcDNA3.1-mMORC2). Lipofecamine 2000 (Invitrogen, Carlsbad, CA, USA) was then used to stably transfect LL/2 cells with pcDNA3.1-mMORC2 or vector. The isolated stable transfectants, LL/2-V and LL/2-MORC2, were isolated by antibiotic selection in media supplemented with 500 μg/ml Zeocin (Invitrogen, Carlsbad, CA, USA). PC-9 cells were transfected with lentiviral constructs tagged with green fluorescent protein (GFP) containing MORC2 shRNA (shMORC2) or scrambled control (shCon) and packaging plasmids (addgene, Cambridge, MA, USA) using Lipofectamine 2000. At 48 hours posttransfection, supernatants were gathered, and lung cancer cells were immediately infected in media containing 10 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Puromycin (5 μg/ml) was used as an antibiotic selection agent for cells containing MORC2 shRNA, and these cells were subsequently sorted for the top 10-20% of GFP-positive cells.

Animal models

An orthotopic murine model of lung cancer in mice was used based on a previous description [27]. Briefly, 2% isoflurane was used to anesthetize C57BL/6 mice, and LL/2-V or LL/2-MORC2 cells were injected into the left pleural cavities of C57BL/6 mice (8 weeks old) to construct the orthotopic model. Mice were euthanized by CO_2 overexposure six weeks after implantation, and lungs were removed and fixed in 10% formalin. Animal work was approved by the ethics committee of China-Japan Union Hospital of Jilin University.

Histology and immunofluorescence

Processing of tumor sections was consistent with previously described protocols [28]. Normal horse serum was used to block samples for 1 hour, and then samples were incubated overnight at 4°C with appropriate antibodies. For immunofluorescence, a fluorochrome-conjugated secondary antibody (Invitrogen) was instead used for 1 hour, followed by staining with DAPI (Invitrogen) prior to mounting. A fluorescence microscope of model Olympus×81 was used to image samples. For immunohistochemistry, samples had a HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) incubated with them for 1 hour, and antigen detection was performed with a 2-Solution Diaminobenzidine Kit (Invitrogen) using hematoxylin counterstaining and Acrymount (StatLab, Mckinney, TX, USA).

MTT assay

For MTT assays, 5×10^3 cells per well were added to 96-well plates, and cell viability was measured via addition of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution. Absorbance of each well at 560 nm was then measured using a microplate reader.

Western blot analysis

Western blotting was conducted in accordance with previously described approaches [29, 30] with appropriate antibodies. Equivalent quantities of total protein were separated by SDS-PAGE on 10% gels prior to transferring to PVDF membranes (Thermo Fisher Scientific). After blocking with 5% milk, primary antibodies were added and incubated overnight (4°C). Subsequent to washing thrice, one-hour incubation was performed with secondary antibody conjugated to HRP. ECL kits (Amersham, Piscataway, NJ, USA) were used to visualize protein bands. Antibodies were specific for MORC2, ALDH1, c-Myc, Sox2, E-cadherin, ZO-1, β-Actin (Santa Cruz Biotechnology), Nanog, β-catenin, active β-catenin, cyclin D1, KLF4, ABCG2, vimentin (Cell Signaling Technology), MMP9, MMP2, VEGF (Abcam), CSF-1, CCL2, and CCL5 (R&D Systems).

In vitro tumor sphere formation assay

Indicated cells (1×10^4/well) were plated in 2 mL serum-free DMEM/F12 media supplemented with 2% B27, 20 ng/mL EGF, and 20 ng/mL bFGF into 6-well ultralow attachment plates (Corning, Lowell, MA). Cells were grown for 10 days, after which the numbers of tumorspheres in each well were counted.

Cell migration and cell invasion assays

Transwell inserts for 24-well plates with porous filters without coating (the pore size was 8 μm) were used for the evaluation of cell migration, and Matrigel (BD Biosciences) porous filters with coating were used for the examination of cell invasion. Briefly, 2 × 10^4 cells in serum-free DMEM (0.2 ml) were seeded into the inserts and 0.6 ml DMEM with 10% FBS was added in the lower portion of the well.

Real-time PCR

The Total RNA Isolation Mini Kit (Agilent, Wilmington, DE, USA) was used to collect cell RNA, and then the iScript cDNA Synthesis Kit (Bio-Rad) was used to reverse transcribe the RNA. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), with β-actin as a control gene and triplicate assessments of samples. The results were expressed as the threshold cycle (Ct). The relative quantification of the target transcripts was determined by the comparative Ct method (ΔΔCt) according to the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression. Control experiments were conducted without reverse transcription to confirm that the total RNA was not contaminated with genomic DNA.

Statistical analysis

All data are presented as the means ± SEM. All experiments were performed a minimum of three times prior to statistical analyses. A two-tailed student's *t*-test and ANOVA were utilized to compare two groups using the GraphPad Prism software with significance thresholds of * $P<0.05$.

Results

MORC2 expression is upregulated in lung cancer

MORC2 has been shown to play critical roles in the progression of several cancers [31, 32], but how it contributes to lung cancer remains unclear. To better clarify the role of MORC2 in lung cancer, real-time PCR analysis was used to analyze MORC2 expression in lung cancer tissues and adjacent normal tissues obtained from patients at our hospital. Higher mRNA levels were found in the tumor group (n=25) than in the normal group (n=25) (Fig. 1A). We then performed immunohistochemistry (IHC) to assess the MORC2 protein abundance in the available lung cancer samples. Normal tissues expressed little MORC2, whereas there was a clear increase in MORC2 levels in clinical lung cancer specimens (Fig. 1B). This elevation in MORC2 expression was significantly higher in these clinical samples than in the healthy control samples (Table 1). We further used real-time PCR and Western blotting to assess MORC2 expression in lung cancer cell lines, and we observed elevated expression in these cells relative to human bronchial epithelial cell lines (Fig. 1C and 1D).

We next examined the correlation between MORC2 levels and clinical pathological findings in lung cancer tissue samples. We identified a positive correlation between MORC2 expression and tumor size, positive nodal status, and TNM stage, suggesting that MORC2 is associated with tumor progression and metastasis (Table 2). A Kaplan-Meier survival analysis showed that elevated MORC2 levels were linked to a significantly decreased chance of progression-free survival of patients with lung cancer, as well as of patients with lung adenocarcinoma (LUAD) or lung squamous cell carcinoma (LUSC) (Fig. 1E). These results show that MORC2 plays a critical function in the progression and/or development of lung cancer.

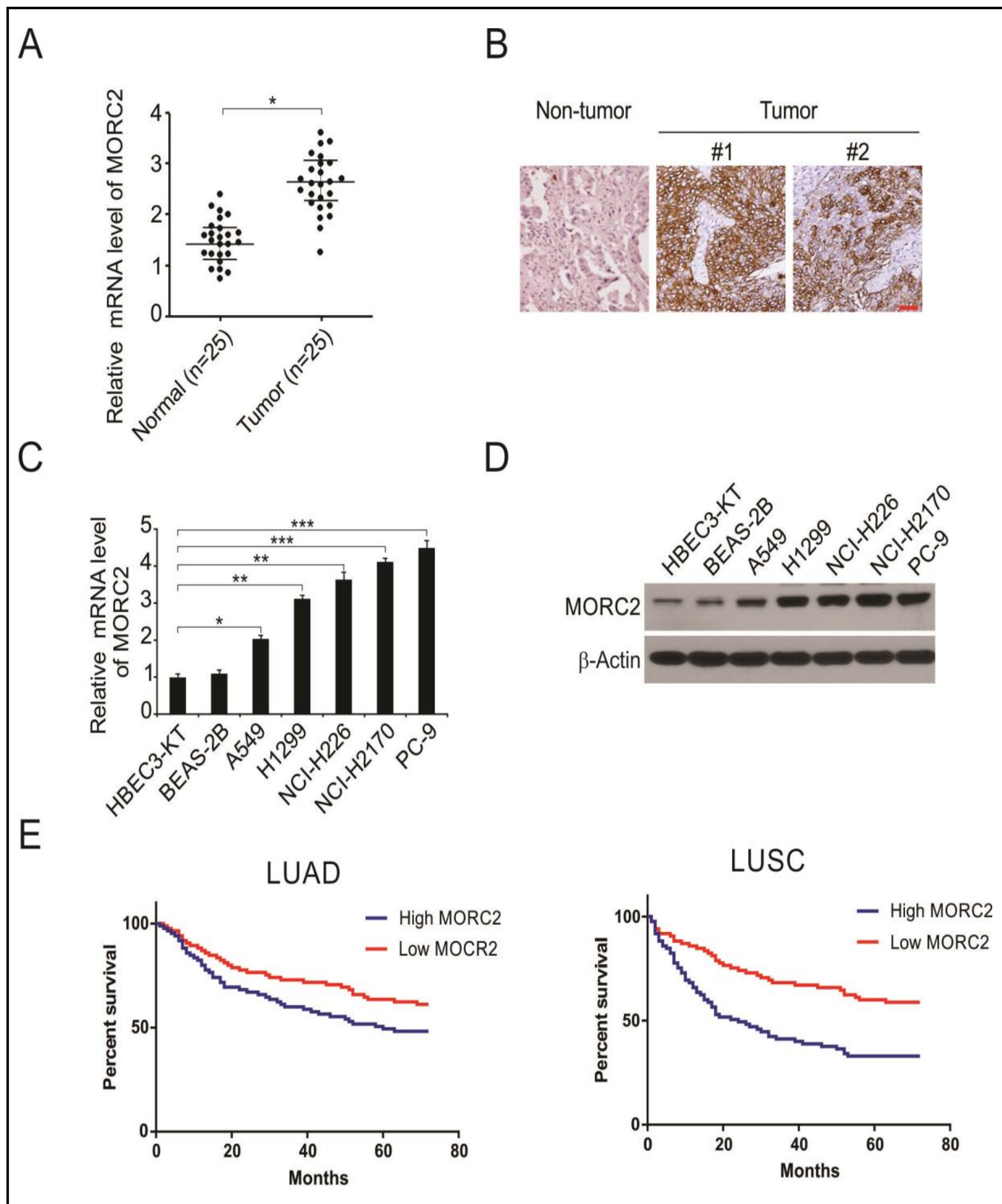

Fig. 1. MORC2 expression in lung cancer tissues and relative to poor survival. (A) Relative MORC2 mRNA expression level in 25 paired lung cancer tissues measured by RT-PCR. *, P<0.05. (B) Representative images of IHC staining of MORC2 in lung cancer and adjacent normal mucosal tissues. Data represent the mean ± SEM of three independent experiments. *, P<0.05. Scale bar: 50 µm. (C) Relative mRNA level of MORC2 in lung cancer cell lines and Human bronchial epithelial cells. (D) Relative protein level of MORC2 in lung cancer cell lines and Human bronchial epithelial cells (p<0.05). (E) Kaplan-Meier analysis of overall survival for patients with lung cancer (p<0.05).

Table 1. IHC staining of MORC2 in lung cancer specimens and normal lung tissues

Tissue	Low expression	High expression	P
Tumor tissues (n=26)	3 (11.5%)	23 (88.5%)	p<0.001
Normal tissues (n=12)	10 (83.3%)	2 (16.7%)	

Table 2. Clinical parameters and their correlation with MOCR2 level

Variables	All cases (N=112; %)	MORC2 low expression (56)	MORC2 high expression (56)	p values
Male	60 (53.6)	27 (48.2)	33 (58.9)	0.487
Female	52 (46.4)	29 (51.8)	23 (41.1)	
Age (Year)				
<65	46 (41.1)	22 (39.3)	24 (42.9)	0.356
≥65	66 (58.9)	34 (60.7)	32 (57.1)	
Smoking status				
Smoker	45 (40.2)	22 (39.3)	23 (40.1)	0.512
Non-smoker	67 (59.8)	34 (60.7)	33 (58.9)	
Tumor size (cm)				
<5	52 (55.1)	33 (58.9)	19 (33.9)	0.015
≥5	60 (44.9)	23 (41.1)	37 (66.1)	
Tumor status				
T1	45 (40.2)	38 (67.8)	7 (12.5)	0.002
T2-T4	67 (59.8)	18 (32.1)	49 (87.5)	
Lymph node status				
N0	66 (58.9)	43 (76.8)	23 (41.1)	0.019
N1 N2 N3	46 (41.1)	13 (23.2)	33 (58.9)	
TNM stage				
I-II	60 (53.6)	36 (64.3)	24 (42.9)	0.022
III-IV	52 (46.4)	20 (35.7)	32 (57.1)	

Overexpression of MORC2 enhances lung cancer tumor growth and stimulates angiogenesis in lung cancer tumors

To probe the link between MORC2 overexpression and lung cancer development, we next chose LL/2 cells to investigate lung cancer progression in response to MORC2 overexpression. MORC2 overexpression slightly increased cell growth in LL/2 in vitro assays (Fig. 2A). To explore how MORC2 influences tumor progression in vivo, we used an orthotopic mouse model in which LL/2 vector control cells (LL/2-V) or MORC2-overexpressing LL/2 cells (LL/2-MORC2) were injected into the lungs of C57BL/6 mice, leading to primary tumor formation, and tumor weights were analyzed. As shown in Fig. 2B, the tumor weight of the LL/2-V group was less than that of the LL/2-MORC2 group at six weeks postinjection. Thus, MORC2 overexpression in LL/2 significantly increased tumor growth.

LL/2-MORC2 tumors exhibited more α-SMA- and CD31-positive vascular endothelial cells and pericytes than the the LL/2-V control tumors (Fig. 2C), suggesting that overexpression of MORC2 is associated with angiogenesis in tumors. We next isolated serum from LL/2-MORC2- and LL/2-V tumor-bearing mice to measure protein content in an effort to assess the levels of proteins linked to metastasis or angiogenesis. The levels of LOX, MMP9, MMP2 and VEGF were all in LL/2-MORC2 tumor-bearing mouse serum relative to the LL/2-V control (Fig. 2D). Immunohistochemical analysis demonstrated these same results in tumor tissues (Fig. 2E), suggesting that the elevated serum content of these proteins is due to their increased expression within LL/2-MORC2 tumors and not due to the relative increase in LL/2-MORC2 tumor size compared with the LL/2-V control size. These results suggest that MORC2 overexpression in lung cancer can promote tumor angiogenesis through elevated expression of LOX, MMP9, and VEGF.

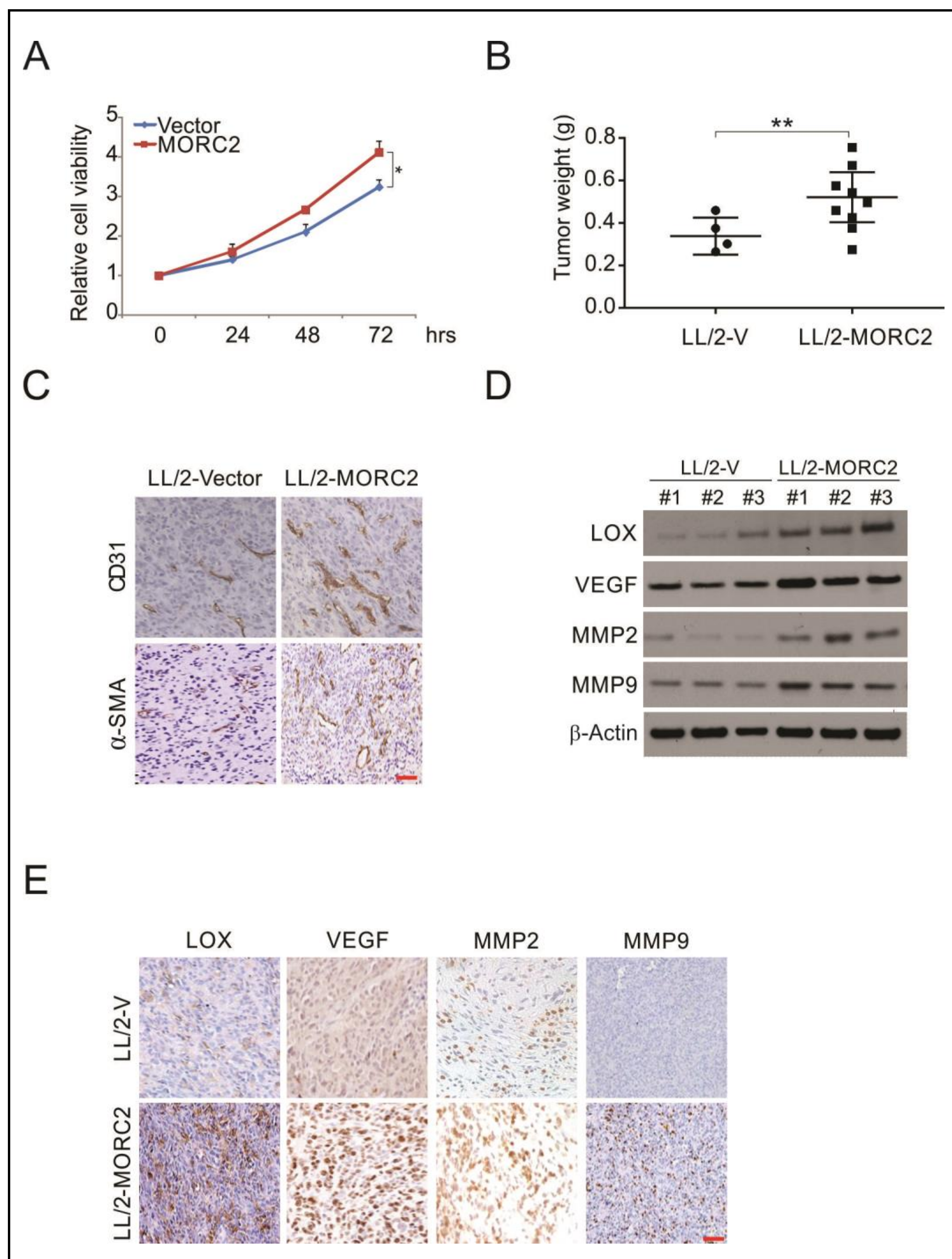

Fig. 2. MORC2 overexpression increases lung cancer tumor growth in vivo. (A) Relative cell viability was analyzed by MTT in the cells transfected with Vector or MORC2. Data represent the mean ± SEM of three independent experiments. *, P<0.05. (B) LL/2-MORC2 or LL/2-V were orthotopically injected into the pleural cavities of mice and mice were killed after 6 weeks. The weight of tumors in mice injected in the cecum with LL/2-V or isolates of LL/2-MORC2 cells. *, P<0.05. (C) Representative images of CD31 and α-SMA staining in LL/2-V and LL/2-MORC2 tumors. Scale bar: 50 μm. (D) Western blotting of VEGF, MMP9, MMP2 and LOX in LL/2-MORC2 tumor-bearing mice and LL/2-V mice. (E) The expression of MMP9, VEGF and LOX in LL/2-MORC2 and LL/2-V tumors as assessed by immunohistochemistry. Scale bar: 50 μm.

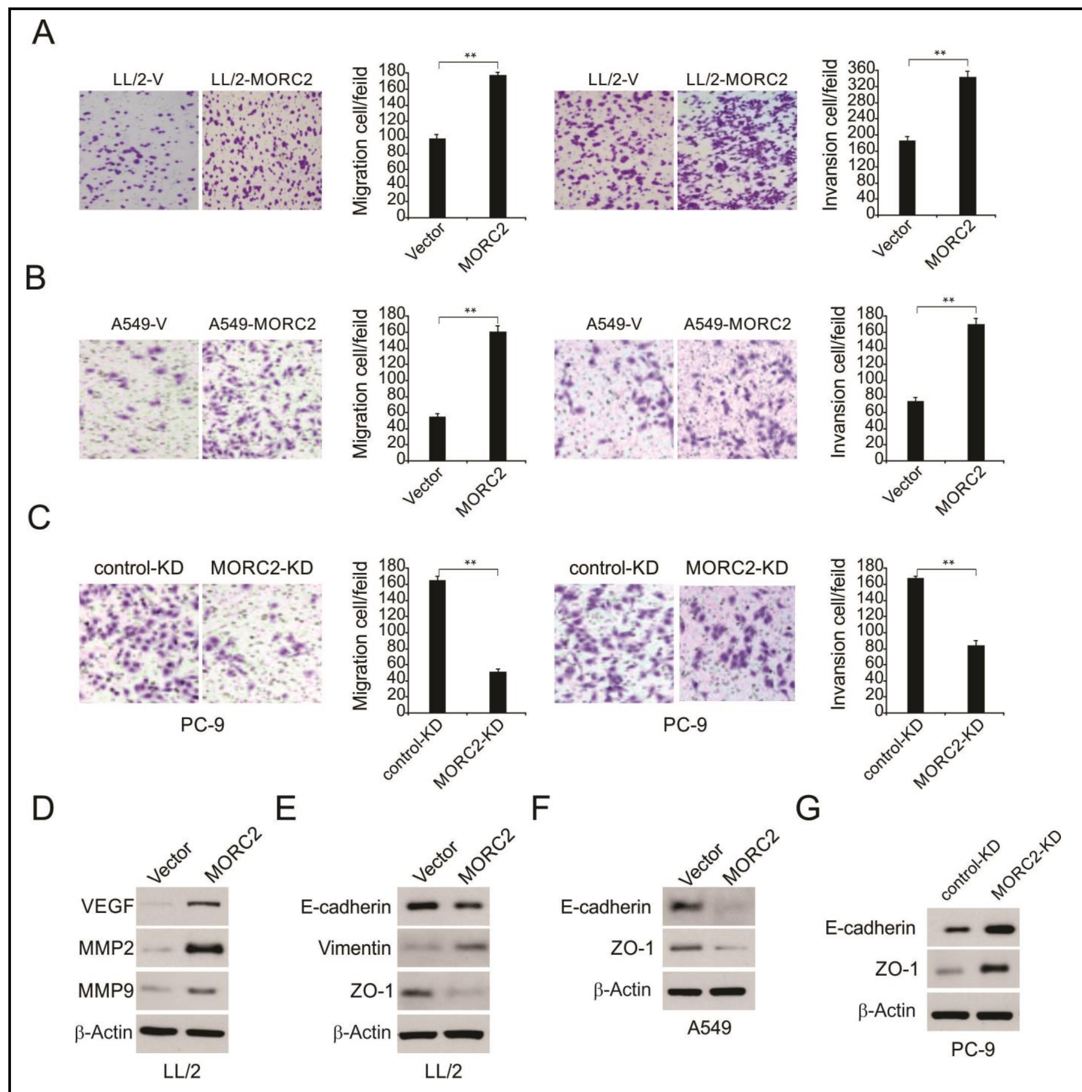

Fig. 3. MORC2 promotes migration and invasion in lung cancer cells in vitro. (A) Cell migration and invasion were determined in LL/2-V and LL/2-MORC2 using a Matrigel transwell invasion assay. Data represent the mean ± SEM of three independent experiments. **, P<0.01. (B) Cell invasion and migration in A549-V and A549-MORC2 were determined using a transwell assay. Data represent the mean ± SEM of three independent experiments. **, P<0.01. (C) Cell invasion and migration in control-KD and MORC2-KD PC-9 cells were determined using a transwell assay. Data represent the mean ± SEM of three independent experiments. **, P<0.01. (D) The indicated protein level was detected by Western blotting in LL/2-V and LL/2-MORC2 cells. (E) The indicated protein level was detected by Western blotting in LL/2-V and LL/2-MORC2 cells. (F) The indicated protein level was detected by Western blotting in A549-V and A549-MORC2 cells. (G) The indicated protein level was detected by Western blotting in control-KD and MORC2-KD PC-9 cells.

MORC2 overexpression promotes in vitro lung cancer cell invasion and migration

As MORC2 overexpression was linked to an increase in LL/2 tumor progression in vivo, we next aimed to understand the cellular changes arising due to MORC2 overexpression. When placed in Matrigel, LL/2-MORC2 cells exhibited superior invasion and migration capabilities to those of the LL/2-V controls (Fig. 3A). This finding was confirmed in MORC2-overexpressing A549 cells (Fig. 3B). In contrast, the knockdown of MORC2 in PC-9 led to impaired migration and invasion capabilities (Fig. 3C). Consistent with the in vivo results, LL/2-MORC2 also presented with elevated MMP9, MMP2 and VEGF in comparison to LL/2-V

cells (Fig. 3D). These data indicate that in vitro MORC overexpression is linked to an increase in lung cancer migration and invasion.

The epithelial-mesenchymal transition (EMT) is an important tumor progression step wherein epithelial cells are able to acquire characteristics that better allow them to migrate and become invasive [33, 34]. We assessed the relative levels of EMT-associated genes in LL/2-MORC2 with LL/2-V cells by Western blotting, and in so doing, we were unexpectedly only able to detect decreased E-cadherin and ZO-1 and increased expression of vimentin in LL/2-MORC2 cells (Fig. 3E). In human lung cancer (A549) cells, overexpression of MORC2 led to significantly lower protein levels of E-cadherin and ZO-1 (Fig. 3F). Inhibiting MORC2 expression using shRNA, in contrast, led to increased E-cadherin and ZO-1 levels in PC-9 (Fig. 3G). Given the limited nature of these findings, these results suggest that changes in EMT are not key components of the role played by MORC2 overexpression in lung cancer cells.

MORC2 increases CSC features of lung cancer

To better understand how overexpression of MORC2 leads to enhanced lung tumor growth, we isolated tumors from mice bearing tumors comprising LL/2-V or LL/2-MORC2 cells. The larger tumor size observed in the case of LL/2-MORC2 could stem either from enhanced cell proliferation or decreased cell death/apoptosis, so we compared the levels of Ki67 (a marker of proliferation) and cleaved caspase 3 (a marker of apoptosis) in LL/2-V and LL/2-MORC2 tumors by immunohistochemistry. There was no apparent change in the frequency of cells exhibiting cleaved caspase 3, whereas LL/2-MORC2 tumors exhibited more Ki67-positive cells (Fig. 4A), suggesting that MORC2 affects tumor growth by enhancing cell proliferation rather than by reducing apoptosis. We next assessed the expression of stem cell markers, including Nanog, Sox2, ALDH1, c-Myc, KLF4, and ABCG2, in LL/2-V and LL/2-MORC2 tumors. We observed significantly increased protein and mRNA levels of ALDH1, KLF4 and ABCG2 in LL/2-MORC2 tumors relative to LL/2-V controls (Fig. 4B and 4C). We next assessed the potential for LL/2-V and LL/2-MORC2 cells to form tumorspheres. Significantly more tumorspheres were present in the case of LL/2-MORC2 cells than in the case of the LL/2-V controls (Fig. 4D). MORC2 overexpression similarly led to a slight increase in tumorsphere formation by A549 cells (Fig. 4E). In addition, inhibition of MORC2 by shRNA in PC-9 decreased tumorsphere formation (Fig. 4F). Overexpression of MORC2 therefore leads to an increase in CSC-like populations among lung cancer cells.

MORC2 overexpression activates the Wnt/β-catenin signaling pathway

We next investigated the signaling pathway in MORC2-overexpressing cells and found that β-catenin expression increased considerably in LL/2-MORC2 cells compared with the LL/2-V controls (Fig. 5A).

We further assessed the molecular changes linked to MORC2 expression levels using MORC2-overexpressing cell lines of human lung cancers. We observed that the knockdown of MORC2 in PC-9 cells clearly decreased the protein and mRNA levels of β-catenin, cyclin D1, c-myc, and MMP7. Overexpression of MORC2 increased the levels of MMP7, c-myc, cyclin D1 and β-catenin in A549 cells (Fig. 5B and 5C). This supports the model in which MORC2 regulates the Wnt/β-catenin signaling pathway. To reinforce the evidence of this regulation, the level of active β-catenin (non-phospho β-catenin (Ser33/37/Thr41)) was assessed and found to be significantly elevated in MORC2-overexpressing cells and reduced in PC-9 cells lacking MORC2 (Fig. 5D). The effect of MORC2 on β-catenin signaling was also analyzed using the TOP/FOP Flash dual-luciferase reporter assay for TCF/β-catenin. The reporter contains both active TCF sites and mutant TCF-binding sites, which serve as negative controls. Based on these results, we found that TOP Flash luciferase activity was significantly reduced upon MORC2 knockdown, whereas MORC2 overexpression promoted the activation of the TCF reporter (Fig. 5E and 5F). Therefore, the above findings indicate that MORC2 plays a key role as a positive mediator of Wnt/β-catenin activity. Thus, MORC2 activates the Wnt/β-catenin pathway in LL/2 and human lung cancer cells.

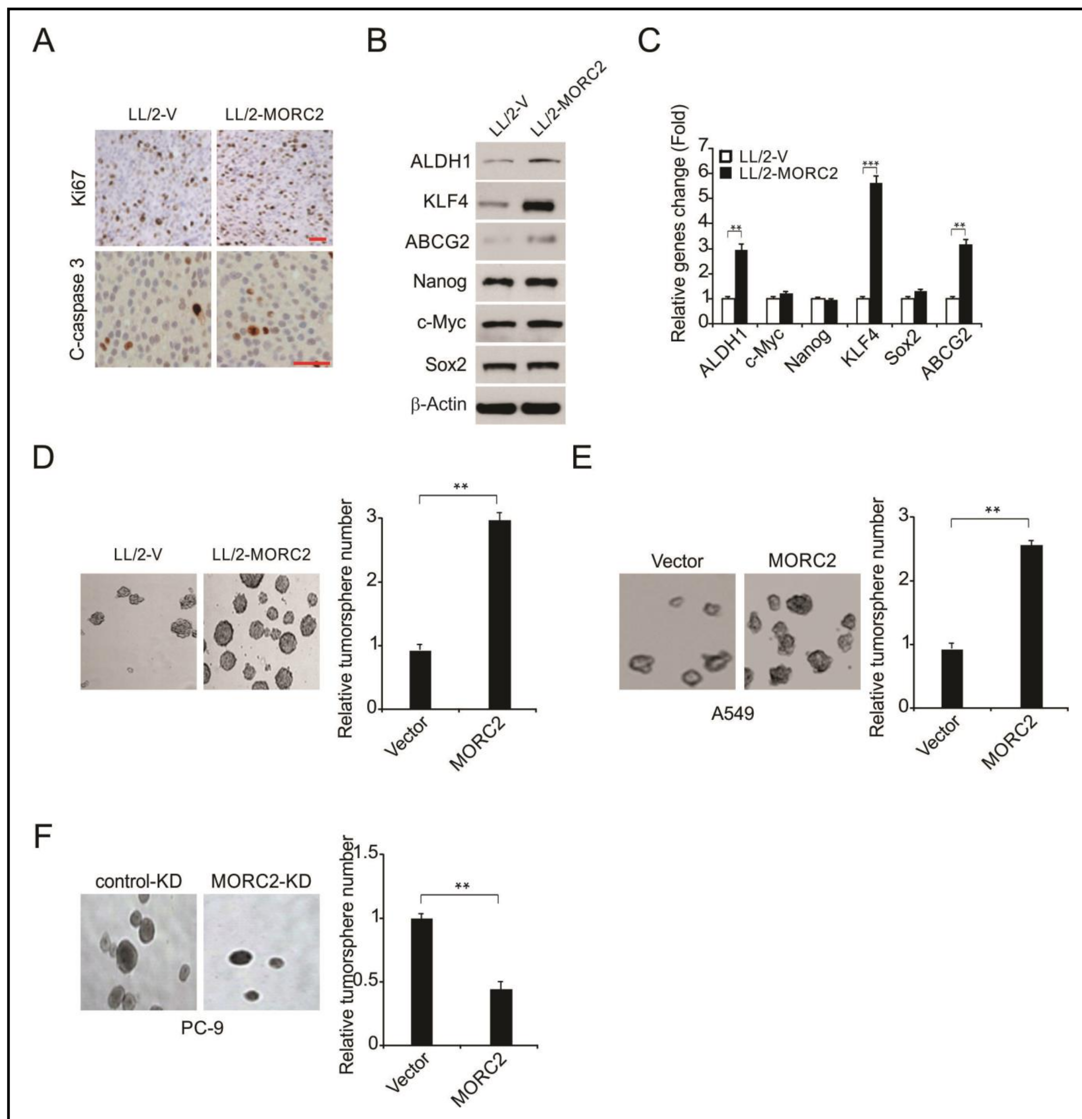

Fig. 4. MORC2 overexpression in LL/2 cells increases CSC characteristics. (A) The expression of Ki67 and cleaved caspase-3 in tumors of LL/2-V and LL/2-MORC2 as detected by immunohistochemistry. Scale bar: 50 μm. (B) The indicated protein level was detected by Western blotting in LL/2-V and LL/2-MORC2 tumors. (C) The indicated mRNA level was detected by real-time PCR in LL/2-V and LL/2-MORC2 tumors. Data represent the mean ± SEM of three independent experiments. ***, $P<0.001$; **, $P<0.01$. (D) Tumorsphere formation ability in LL/2-V and LL/2-MORC2 cells. Data represent the mean ± SEM of three independent experiments. **, $P<0.01$. (E) Tumorsphere formation ability in A549-V and A549-MORC2Cells. Data represent the mean ± SEM of three independent experiments. **, $P<0.01$. (F) Tumorsphere formation ability in control-KD and MORC2-KD PC-9 cells. Data represent the mean ± SEM of three independent experiments. **, $P<0.01$.

We then treated cells that overexpressed MORC2 with XAV-939, a Wnt/β-catenin signaling inhibitor, and found that cell growth was significantly suppressed following XAV-939 treatment in cells overexpressing MORC2 (Fig. 5G). Furthermore, the results from transwell assays showed that blockade of the Wnt/β-catenin pathway significantly abolished the MORC2-enhanced migration and invasion in A549 cells (Fig. 5H and 5I).

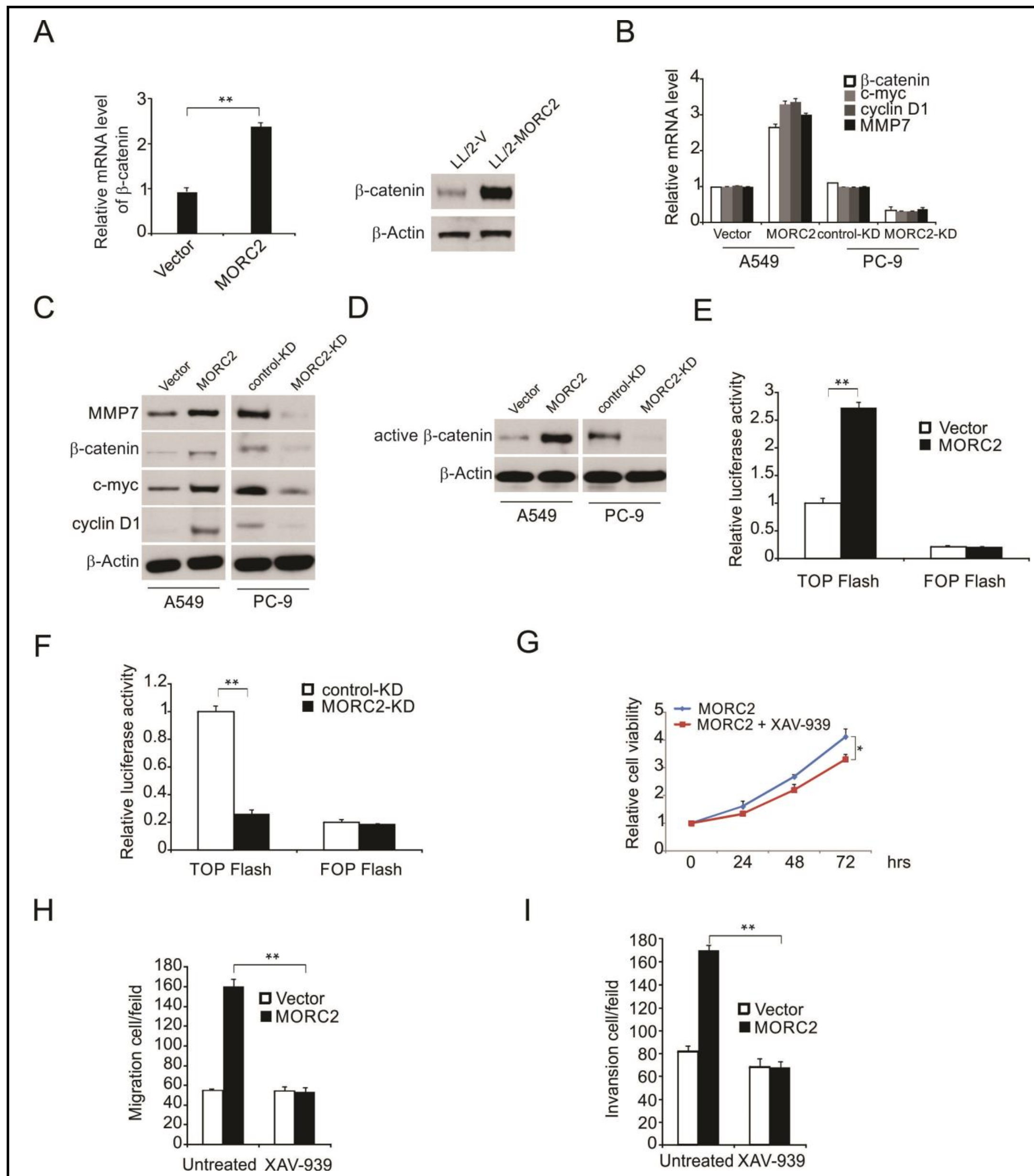

Fig. 5. MORC2 actives Wnt/β-catenin signaling pathway in lung cancer cell lines. (A) The indicated mRNA and protein level were detected in LL/2-V and LL/2-MORC2 cells. Data represent the mean ± SEM of three independent experiments. **, P<0.01. (B) The indicated mRNA level was detected in A549-V, A549-MORC2, control-KD and MORC2-KD PC-9 cells. (C) The indicated protein level was detected by Western blotting in A549-V, A549-MORC2, control-KD and MORC2-KD PC-9 cells. (D) The level of active β-catenin was detected by Western blotting in A549-V, A549-MORC2, control-KD and MORC2-KD PC-9 cells. (E and F) The activity of TCF/β-catenin reporter (TOP/FOP Flash) in (E) MORC2-overexpressing and MORC2-knockdown cells (F). Data represent the mean ± SEM of three independent experiments. **, P<0.01. (G-I) Effects of XAV-939, on MORC2-enhanced cell proliferation (G), migration (H) and invasion (I). Cells were treated with XAV-939 or DMSO during the migration and invasion assays. Data represent the mean ± SEM of three independent experiments. **, P<0.01, *P <0.05.

The overexpression of MORC2 in LL/2 enhances the recruitment of TAMs

Next, we investigated the effects of the stromal cells that make up the tumor microenvironment on MORC2-induced tumor progression. Cells present in the tumor microenvironment, particularly infiltrating immune cells and associated cytokines, are directly linked to many aspects of tumorigenesis. We therefore assessed whether MORC2 was associated with any inflammatory changes in the lung tumor microenvironment that may contribute to tumor progression. There was a significant increase in CD206⁺ cell infiltration in LL/2-MORC2 tumors (Fig. 6A and 6B). These cells were also positive for CD11c, suggesting that they are of the macrophage lineage (Fig. 6C), suggesting that MORC2 overexpression drives TAM recruitment in the LL/2 model.

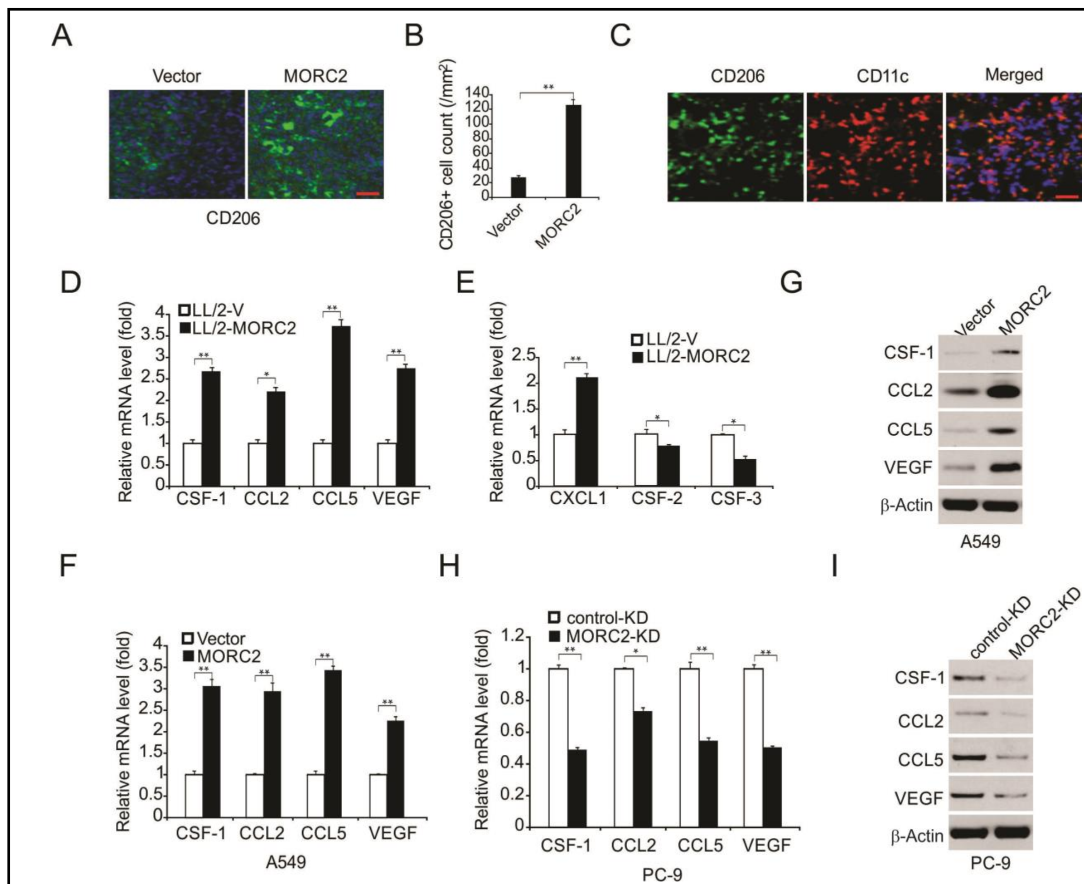

Fig. 6. Tim4 overexpression recruited macrophages to tumors. (A) CD206 staining in tumors. Scale bar: 50 μm. (B) CD206 ⁺ cell count averages per mm². Data represent the mean ± SEM of three independent experiments. **, P<0.01. (C) CD206⁺/CD11c⁺ cell staining in tumors. Scale bar: 50 μm. (D) mRNA expression of CSF-1, CCL2, CCL5 and VEGF was determined by real-time PCR in LL/2-V and LL/2-MORC2. Data represent the mean ± SEM of three independent experiments. **, P<0.01, *P <0.05. (E) mRNA expression of CSF-2, CSF-3 and CXCL1 was determined by real-time PCR in LL/2-V and LL/2-MORC2. Data represent the mean ± SEM of three independent experiments. **, P<0.01, *P <0.05. (F) mRNA expression of CCL2, CCL5 and CSF-1 in A549-V and A549-MORC2 cells. Data represent the mean ± SEM of three independent experiments. **, P<0.01. (G) Protein level of VEGF, CCL2, CCL5 and CSF-1 was analyzed Western blotting in A549-V and A549-MORC2 cells. (H) mRNA expression of VEGF, CSF-1, CCL2 and CCL5 was determined by real-time PCR in control-KD and MORC2-KD PC-9 cells. Data represent the mean ± SEM of three independent experiments. **, P<0.01, *P <0.05. (I) Protein level of CCL2, CCL5 and CSF-1 was analyzed Western blotting in control-KD and MORC2-KD PC-9 cells.

MORC2 recruits TAMS via increased expression of CSF-1 and CCL2/5

Although TAMs function as an immune lineage associated with clearing threats from the body, TAMs can ultimately drive tumor progression via tissue remodeling and angiogenesis once recruited to the tumor stroma. Chemokines known to recruit TAMs include VEGF, CSF-1, CCL2, and CCL5. The mRNA levels of all four of these chemokines were increased in LL/2-MORC2 tumors compared with the LL/2-V controls (Fig. 6D). We further assessed the expression levels of neutrophil-recruiting chemokines, including CXCL1, CSF-2, and CSF-3. CXCL1 levels were increased in LL/2 MORC2 tumors, whereas CSF-2 and CSF-3 levels were decreased (Fig. 6E). In A549 cells, we also observed elevated mRNA (Fig. 6F) and protein levels of VEGF, CSF-1, CCL2 and CCL5 (Fig. 6G) in A549-MORC2 cells compared with the corresponding controls. The knockdown of MORC2 in PC-9 cells significantly decreased the protein and mRNA levels of CSF-1, CCL5 and CCL2 (Fig. 6H and 6I). Together, these data demonstrate that MORC2 promotes the production of cytokines that drive TAM infiltration, in turn driving lung cancer growth and progression.

Discussion

MORC2 is a poorly characterized, highly conserved protein with known nuclear localization [35]. Based on studies of MORC homologs in prokaryotes, MORC2 is predicted to play a role in chromatin remodeling in eukaryotic cells, although its exact function remains to be assessed [17, 36]. It has been reported that p21 protein (Cdc42/Rac)-activated kinase 1 phosphorylates serine 739 of MORC2 and thereby modulates ATPase-dependent chromatin remodeling following damage stemming from double-stranded breaks, promoting DNA damage repair [37]. Based on evidence that MORC2 was upregulated in liver cancer tissue [16], it was hypothesized that liver cancer cells with elevated MORC2 expression levels may facilitate DNA repair and homologous recombination and may additionally be less sensitive to apoptotic signals, leading to aberrant cell cycle progression, a higher survival ability and chemoresistance [32]. Accordingly, in patients with liver cancer, the present study showed that a higher fraction of copy number alterations in the genome was detected with higher expression of MORC2 [16]. It was also found that MORC2 knockdown induced cell cycle arrest and endogenous apoptotic pathways [16]. The knockdown of MORC2 also sensitized cancer cells to doxorubicin, 5-fluorouracil and cisplatin and markedly increased IC50 values, which suggested that MORC2 may be involved in the chemoresistance of cancer [16].

Thus, in the current study, the oncogenic role of MORC2 was elucidated in association with angiogenesis and proliferation. We found that MORC2 overexpression is linked to decreased survival of lung cancer patients. Moreover, MORC2 overexpression in the murine lung cancer cell line LL/2 led to increased tumorigenic potential and angiogenesis *in vivo*, in addition to elevating the frequency of CSCs. Furthermore, MORC2 overexpression led to increased macrophage infiltration of the tumor, likely due to elevated expression of chemokines. We also found evidence that MORC2 may further be associated with EMT, suggesting that in some instances, MORC2 may facilitate EMT to further potentiate tumor cell migration and metastasis. Elevated VEGF and MMP9 levels in MORC2-overexpressing tumors may further promote angiogenesis and metastasis, providing a direct molecular link between MORC2 expression and tumor progression. We also investigated how MORC2 overexpression affects lung cancer progression with a mouse model of lung cancer by implanting tumor cells into immunocompetent mice. Consistent with *in vitro* observations, MORC2 enhanced the malignant capacity of tumor cells by enhancing angiogenesis and TAM recruitment to the local tumor stromal microenvironment.

Enhanced Wnt/β-catenin signaling is common to many cancer types, directly linking aberrant cellular signaling to enhanced angiogenesis and survival [38]. Wnt/β-catenin signaling regulates MMP9 expression, which in turn can promote the invasive potential of many types of cancers, including lung cancer [39]. We demonstrated that overexpression of MORC2 leads to β-catenin upregulation in both murine and human lung cancer cells,

whereas MORC2 knockdown results in decreased β-catenin activation. Aberrant Wnt/β-catenin signaling may thus be a common link among MORC2-expressing tumors, potentially serving as an ideal therapeutic target.

Numerous clinical and experimental studies have highlighted the ability of TAMs to drive tumor progression, owing to the fact that although TAMs initially play a proinflammatory role in the tumor microenvironment, they often later transition into a distinct phenotype that promotes angiogenesis, immune evasion, and tumor metastasis [40, 41]. Underscoring this fact, studies have shown that high levels of the macrophage regulatory protein CSF-1 are linked to poorer prognoses for a range of cancer types, including lung cancer [42, 43]. Tumor cells can further exacerbate this process by promoting MMP9 secretion, leading to extracellular matrix cleavage and facilitating tumor cell invasion [44]. In the current study, we found that MORC2 overexpression in orthotopic lung tumors was linked to enhanced TAM infiltration and that these TAMs reciprocally secreted MMP9 and VEGF, promoting further angiogenesis and tumor invasion, thereby driving tumor progression.

Conclusion

Overall, our findings demonstrate that MORC2 is upregulated in lung cancer cells, and the upregulation is correlated with the aggressiveness of lung cancer. Our results also show that MORC2 plays an important part in regulating the proliferation, migration, invasion and stem-like features of tumor cells. Overexpression of MORC2 leads to Wnt/β-catenin activation in both mouse and human cancer cells. This pathway may serve as a common signaling mediator of the effects of MORC2 overexpression, potentially making Wnt/β-catenin signaling an ideal therapeutic target.

Disclosure Statement

The authors declare no conflicts of interest.

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