Porcupine inhibitor LGK-974 inhibits Wnt/β-catenin signaling and modifies tumor-associated macrophages resulting in inhibition of the malignant behaviors of non-small cell lung cancer cells

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Abstract. Tumor-associated macrophages (TAMs) are critical components of the tumor microenvironment that are tightly associated with malignancies in human cancers, including lung cancer. LGK-974, a small molecular inhibitor of Wnt secretion, was reported to block Wnt/β-catenin signaling and exert anti-inflammatory effects by suppressing pro-inflammatory gene expression in cancer cells. Although it was reported that Wnt/β-catenin was critical in regulating TAMs, it is still largely unknown whether LGK-974 regulates tumor malignancies by regulating TAMs. The present study firstly verified that the polarization of TAMs was regulated by LGK-974. LGK-974 increased M1 macrophage functional markers and decreased M2 macrophage functional markers. The addition of Wnt3a and Wnt5a, two canonical Wnt signaling inducers, reversed the decrease in M1 macrophage functional markers, including mannose receptor, IL-10 and Arg1, by activating Wnt/β-catenin signaling. Conditioned medium from LGK-974-modified TAMs inhibited the malignant behaviors in A549 and H1299 cells, including proliferation, colony formation and invasion, by blocking Wnt/β-catenin signaling. LGK-974-modified TAMs blocked the cell cycle at the G_1/G_0 phase, which was reversed by the addition of Wnt3a/5a, indicating that LGK-974 regulates the microenvironment by blocking Wnt/β-catenin signaling. Taken together, the results indicate that LGK-974 indirectly inhibited the malignant behaviors of A549 and H1299 cells by regulating the inflammatory microenvironment by inhibiting Wnt/β-catenin signaling in TAMs.

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Introduction

Non-small-cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases and is a leading cause of cancer-associated death worldwide (1). Despite the development of early detection techniques, novel therapeutic strategies and standard therapeutic procedures, the 5-year relative survival rate for lung cancer is only 17%, and the survival rate of patients with tumors is still poor due to a high proportion of early metastases, recurrence after surgery, or the development of chemoresistance or radioresistance (2). In the past years, the main treatment options for lung cancer include surgery, radiation therapy, chemotherapy and targeted therapy, despite of poor prognosis (3). For decades, the tumor microenvironment, which is considered an emerging hallmark of cancer, including in lung cancer, has attracted attention for its critical role in regulating cancer malignancies (4).

The tumor microenvironment is characterized by the complex interactions of various cell types, including tumor-associated macrophages (TAMs), dendritic cell (DC) subsets, cytotoxic and regulatory T cells (CTLs and Tregs, respectively), natural killer (NK) cells, and myeloid-derived suppressor cells (MDSCs) (3). Among patients, the tumor microenvironment can vary dramatically and lead to different therapeutic outcomes and prognoses due to the different compositions of immune cell subsets (5,6). Among these subsets of immune cells, TAMs play critical roles in regulating the microenvironment through tumor-associated immune cells, which accumulate in the tumor stroma and are associated with poor therapeutic outcome and prognosis (7). Recruited macrophages adapt to their environment by developing one of two major polarization phenotypes: M1 (classical) and M2 (alternative). M1 macrophages produce inflammatory cytokines such as IL-1, 6, 12 and 23, TNF-α, reactive oxygen species and nitric oxide. However, M2 macrophages produce IL-10, transforming growth factor-β, vascular endothelial growth factor, and matrix metalloproteinase 9 and

express argininase-1 (ARG-1), scavenger receptors (CD163 and CD204), and C-type lectin (CD301) (8). Among TAMs, there is an increase in the frequency of the pro-tumor M2 phenotype and impaired antigen presentation and subsequent T cell stimulation (9). Among TAMs inhibiting the M1 phenotype, which is characterized by antitumor functions exerted through the expression of HLA-DR, inducible nitric oxide synthase (iNOS) and TNF-α were found to promote malignancies in NSCLC (10).

Accumulating evidence has shown that crosstalk between tumor cells and macrophage polarization is involved in tumor progression (11). Tumor cells tightly promote TAMs by secreting several types of soluble factors, such as Wnts, thus activating Wnt/β-catenin signaling (12). Wnts that are secreted by tumor cells bind to the Frizzled (Fzd) family and mediate noncanonical Wnt signaling pathway activation in macrophages, resulting in an M2-like phenotype (13), demonstrating that Wnt/β-catenin is critical for TAM polarization. LGK-974, which is also known as Wnt974, is a novel inhibitor of Porcupine, which is an O-acyltransferase responsible for the palmitoylation of Wnt ligands (14). In lung adenocarcinoma (LUAD), LGK-974 was found to exert antitumor effects on tumor growth and proliferation and extended the survival of mice with advanced LUAD tumors by inhibiting ligand-driven Wnt signaling (15). Although the antitumor effects of LGK-974 via the direct inhibition of Wnt/β-catenin signaling in lung cancer have been well researched, little is known about whether LGK-974 affects tumors by regulating the microenvironment through TAM polarization, which depends on inhibiting Wnt/β-catenin signaling.

The present study used human monocytic THP-1 cells and the NSCLC cell lines A549 and H1299 for TAM stimulation, in order to explore the effects of LGK-974 on TAM polarization, regulation of the microenvironment and subsequent NSCLC malignancies. It was shown that LGK-974 indirectly affected NSCLC by regulating TAM polarization. These findings provide novel insight into the antitumor role of LGK-974.

Materials and methods

Cell culture and reagents. The human monocyte cell line THP-1, normal human bronchial epithelial cells 16HBE, human NSCLC cell lines H1299 and A549 were purchased from the Chinese Academy of Sciences in Shanghai. THP-1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. Aiming to stimulate macrophage polarization, $3x10^5$ THP-1 cells were seeded in 6-well plate with the addition of 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; Merck KGaA). To obtain TAMs, THP-1 macrophages were cultured using 50% original media and 50% conditioned media (CM) from H1299 or A549 cell lines for another 48 h. TAMs and NSCLC cell lines co-culture was conducted using the non-contact co-culture Transwell system (Corning, Inc.). TAMs or THP-1 were cultured in upper inserts and co-cultured with NSCLC cells ($1x10^5$ cells per well) (16). After 48 h of co-culture, TAMs or NSCLC cells were harvested for further analyses.

Migration assay. In total, $5x10^5$ cultured cells were seeded into 12-well plate and incubated at 37˚C and 5% CO_2 overnight. When cells reached 100% confluence in wells, a 200-μl pipette tip was used to obtain the cell-free lane. Cells were washed with serum-free RPMI-1640 medium and imaged to record the wound width at 0 h. Then, cells were cultured in serum-free RPMI-1640 medium for 24 h. A ruler was used as a guide to obtain a straight line. Images were taken at 0 and 24 h using a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at magnification of x1,000.

Invasion assays. H1299 or A549 cells ($2x10^4$) suspended in RPMI-1640 medium without FBS were seeded into the 24-well Transwell plate, which was precoated with Matrigel (Thermo Fisher Scientific, Inc.) at 37˚C for 4 h, in lower chambers for 24-h culture. Then, the Transwell was plated into 24-well plate filled with CM from TAMs supplemented with 10% FBS. After incubating at 37˚C for 24 h, the non-invading cells were removed using a cotton swab. The cells that had penetrated through the filter were fixed using 4% paraformaldehyde for 10 min at room temperature and stained with 0.05% crystal violet at room temperature for 30 min and counted under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at magnification of x40. In total 10 randomly selected fields were used to count the number of invading cells number in each insert.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For RT-qPCR analysis, reverse transcription was performed from 1 μg total RNA using RT kit (Thermo Fisher Scientific, Inc.) at 37˚C for 1 h. qPCR was performed using the Fast start Universal SYBR Green master mix (Roche Diagnostics). Then, 0.5 μl cDNA was used as the template for each reaction under the following conditions: 40 cycles of 95˚C for 30 sec; 55˚C for 30 sec; and 72˚C for 1 min. ABI7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for qPCR. The primers used for qPCR were as follows: Actin forward, 5'-CATGTACGTTGCTATCCAGGC-3'; actin reverse, 5'-CTCCTTAATGTCACGCACGAT-3'; CD68 forward, 5'-GGAAATGCCACGGTTCATCCA-3'; CD68 reverse, 5'-TGGGGTTCAGTACAGAGATGC-3'; CD163 forward, 5'-TTTGTCAACTTGAGTCCCTTCAC-3'; CD163 reverse, 5'-TCCCGCTACACTTGTTTTCAC-3'; CD206 forward, 5'-TCCGGGTGCTGTTCTCCTA-3'; CD206 reverse, 5'-CCAGTCTGTTTTTGATGGCACT-3'; Arg1 forward, 5'-GTGGAAACTTGCATGGACAAC-3'; Arg1 reverse, 5'-AATCCTGGCACATCGGGAATC-3'; CD86 forward, 5'-CTGCTCATCTATACACGGTTACC-3'; CD86 reverse, 5'-GGAAACGTCGTACAGTTCTGTG-3'; HLA-DR forward, 5'-AGTCCCTGTGCTAGGATTTTTCA-3'; HLA-DR reverse, 5'-ACATAAACTCGCCTGATTGGTC-3'; TNF-α forward, 5'-CCTCTCTCTAATCAGCCCTCTG-3'; TNF-α reverse, 5'-GAGGACCTGGGAGTAGATGAG-3'; IL-12 forward, 5'-ACCCTGACCATCCAAGTCAAA-3'; IL-12 reverse, 5'-TTGGCCTCGCATCTTAGAAAG-3'; iNOS forward, 5'-TTCAGTATCACAACCTCAGCAAG-3'; iNOS reverse, 5'-TGGACCTGCAAGTTAAAATCCC-3'; mannose receptor (MR) forward, 5'-GATGGCGTTCCTGTTACCTCT-3'; MR reverse, 5'-GCCCAGGCGAAAATATCTCAG-3'; IL-10 forward, 5'-GACTTTAAGGGTTACCTGGGTTG-3'; reverse, 5'-TCACATGCGCCTTGATGTCTG-3'; Fzd4 forward, 5'-CCTCGGCTACAACGTGACC-3'; Fzd4 reverse, 5'-TGCACATTGGCACATAAACAGA-3'; Fzd7 forward, 5'-GTGCCAACGGCCTGATGTA-3'; Fzd7 reverse, 5'-AGGTGAGAACGGTAAAGA

GCG-3'; Fzd9 forward, 5'-TGCGAGAACCCCGAGAAGT-3'; Fzd9 reverse, 5'-GGGACCAGAACACCTCGAC-3'; β-catenin forward, 5'-AAAGCGGCTGTTAGTCACTGG-3'; β-catenin reverse, 5'-CGAGTCATTGCATACTGTCCAT-3'; c-Myc forward, 5'-GGCTCCTGGCAAAAGGTCA-3'; c-Myc reverse, 5'-CTGCGTAGTTGTGCTGATGT-3'; Axin2 forward, 5'-CAA CACCAGGCGGAACGAA-3'; and Axin2 reverse, 5'-GCCCAA TAAGGAGTGTAAGGACT-3'. The qPCR results were analyzed and expressed relative to the CT (threshold cycle) values and then converted to fold changes, using the $2^{-\Delta\Delta Cq}$ method; 2.0-fold change was considered as significant (17).

Western blotting. Cells ($1x10^6$) were lysed with 500 μl RIPA buffer (cat. no. R0278; Sigma-Aldrich; Merck KGaA) containing 1X protease cocktail (Thermo Fisher Scientific, Inc.), and then total protein sample was denatured at 100˚C for 15 min. The protein was measured using the BCA method, and 20 μg total protein was fractionated in each lane by performing electrophoresis using 6-12% SDS-polyacrylamide gradient gel, and then the proteins from the gel were transferred to PVDF membranes. After being blocked with 5% non-fat dry milk for 1 h at room temperature, the membrane was incubated with the primary antibodies at 4˚C overnight, followed by secondary antibody incubation at room temperature for 1 h. After three washes with 0.1% TBS-Tween-20, membrane was imaged using an ECL luminescence kit (MilliporeSigma) on X-ray file. The dilution ratios of the primary antibodies were as follows: Anti-Arg-1 antibody (1:1,000 dilution; cat. no. A4923, ABclonal Biotech Co., Ltd.) and anti-β-actin antibody (1:1,000 dilution; cat. no. ab8226; Abcam). The HRP-conjugated goat anti-mouse secondary antibody and HRP-conjugated goat anti-rabbit secondary antibody (cat. nos. ab205719 and ab205718; Abcam) were diluted at a ratio of 1:5,000. Blots were semi-quantitatively analyzed using ImageJ software (version-2.0; National Institutes of Health).

ELISA. The cytokines contained in the supernatant of cultured medium, including TNF-α, IL-12 and IL-10 were examined by ELISA kits: Human-TNF-α- ELISA-Kit (cat. no. ml077385), Human-IL-10-ELISA-Kit, (cat. no. ml064299), Human-IL-12-ELISA-Kit, (cat. no. ml058044), according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

Cell viability assays. Cells ($5x10^3$) were seeded in 96-well plates for each well and allowed to attach overnight. Then, cells were cultured using 50% RPMI-1640 medium and 50% CM supplemented with 10% FBS for 1-5 days. Medium was refreshed every 2 days. For each day, 10 μl tetrazolium salt Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd.) was added to each well and allowed for an additional 1-h incubation at 37˚C. Optical density was measured at a wavelength of 450 nm (OD_{450}).

Cell cycle distribution. Cells ($1x10^6$) were pelleted and washed by pre-cold PBS three times and fixed using 75% ethyl alcohol (1 ml) for 16 h at 4˚C. Then, cells were pelleted and washed using ice-cold PBS two times. RNase A (100 μl) and 400 μl PI (Sigma-Aldrich; Merck KGaA) were added in the dark and incubated for 30 min at room temperature. After 30 min incubation at 4˚C, cell cycle phases were measured using the FACS LSRII flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (FlowJo Treestar; version 9.7.4).

Colony formation. Cells ($5x10^3$) were suspended in 50% original medium and 50% CM in a 12-well plate, with the addition of antibiotic-antimycotic mixture (final concentration, 1%). Medium was refreshed every 3 days. Cells were cultured for 14 days and colonies were fixed using 4% paraformaldehyde at room temperature for 10 min and stained with 0.05% crystal violet at room temperature for 10 min and counted under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at magnification of x40. Then, colonies were identified as those containing >50 cells.

Statistical analysis. Statistical analysis was performed with SPSS version 15.1 (SPSS, Inc.). Data are expressed as mean ± SD (x±s). The statistical significance of the difference between groups was assessed by unpaired Student's t-tests and one-way ANOVA followed by Bonferroni post hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LGK-974 regulates the polarization of tumor-associated macrophages. In order to determine the effects of LGK-974 on the polarization of tumor-associated macrophages co-cultured with lung cancer cells, an *in vitro* model of tumor-associated macrophages TAMs was utilized. The human monocyte cell line THP-1 pretreated with PMA for 24 h was cultured in CM from NSCLC cell lines (A549 or H1299) or normal human bronchial epithelial cells 16HBE, which was considered as a negative control, to generate TAMs. The polarization of TAMs was validated on the basis of marker expression and cytokine profile. As shown in Fig. 1A-C, by detecting CD68, CD163, CD206, Arginase 1, HLA-DR mRNA levels and Arginase 1 protein level, co-culture with both A549 and H1299 demonstrated upregulated expression levels of M1 markers (arginase 1, CD86, HLA-DR) and M2 markers (CD163, CD206) compared with that with 16HBE, without disturbing pan-macrophage marker CD68. This indicates that both A549 and H1299 induced TAMs of a mixed M1/M2 phenotype. The addition of 10 nM of LGK-974 significantly decreased the expression levels of all these markers, demonstrating that LGK-974 potentially blocks the polarization stimulated by tumor cell co-culture.

In order to explore the roles of LGK-974 in macrophage polarization, the expression of macrophage surface and functional markers for M1 such as TNF-α, IL-12 and iNOS, for M2 such as MR, IL-10 and Arg1 were assessed. As shown in Fig. 1D and E, the addition of LGK-974 affected TNF-α, IL-12 and iNOS slightly, but significantly decreased MR, IL-10 and Arg1 (Fig. 1F and G), indicating that LGK-974 may inhibit M2-type phenotype and M2-like function.

LGK-974 blocks Wnt signaling and regulates the function of TAMs. LGK-974 specifically inhibits Wnt secretion, and thus regulates Wnt/β-catenin pathway (18). The two main canonical Wnt signaling inducers (19,20), Wnt3a and Wnt5a, were chosen to investigate whether the effect of LGK-974 on macrophage polarization is dependent on the inhibition of Wnt secretion. As shown in Fig. 2A, the

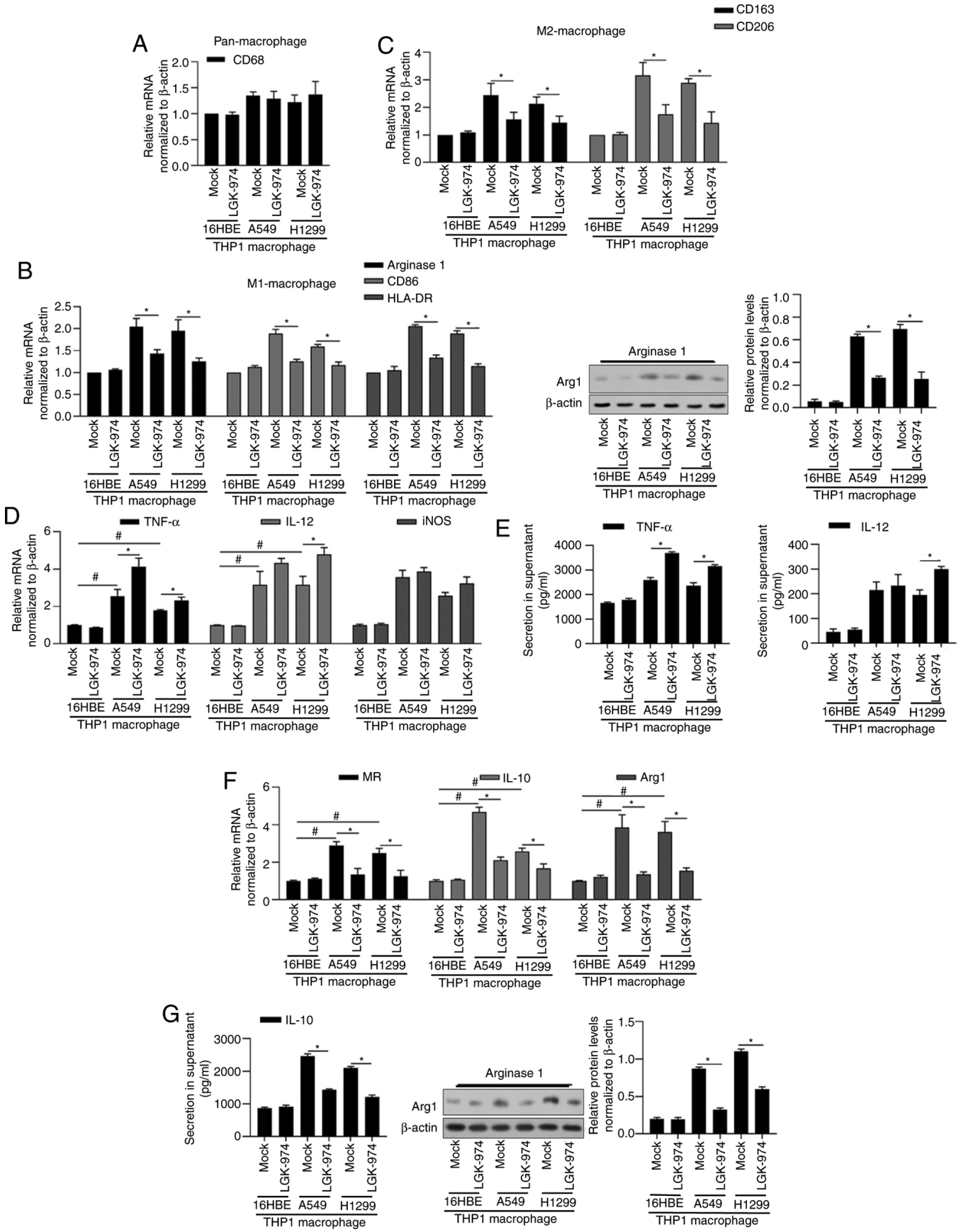

Figure 1. Addition of LGK-974 regulates TAMs and its inflammation-associated cytokines. After 24-h co-incubation with 16HBE, A549 or H1299, total RNA or protein was isolated and the expression levels of cell markers for pan-macrophage CD68 (A), for M1-macrophage Arginase 1, CD86 and HLA-DR (B), and for M2-macrophage CD163 and CD206 (C) were determined. (D-G) Expression levels of functional markers for M1-macrophage (TNF-α, IL-12 and iNOS) and for M2-macrophage (MR, IL-10 and Arg1) were measured by reverse transcription-quantitative PCR. *P<0.05 vs. mock group; #P<0.05 vs. mock group/16HBE. TAMS, tumor-associated macrophages; iNOS, inducible nitric oxide synthase; MR, mannose receptor; Arg1, argininase-1.

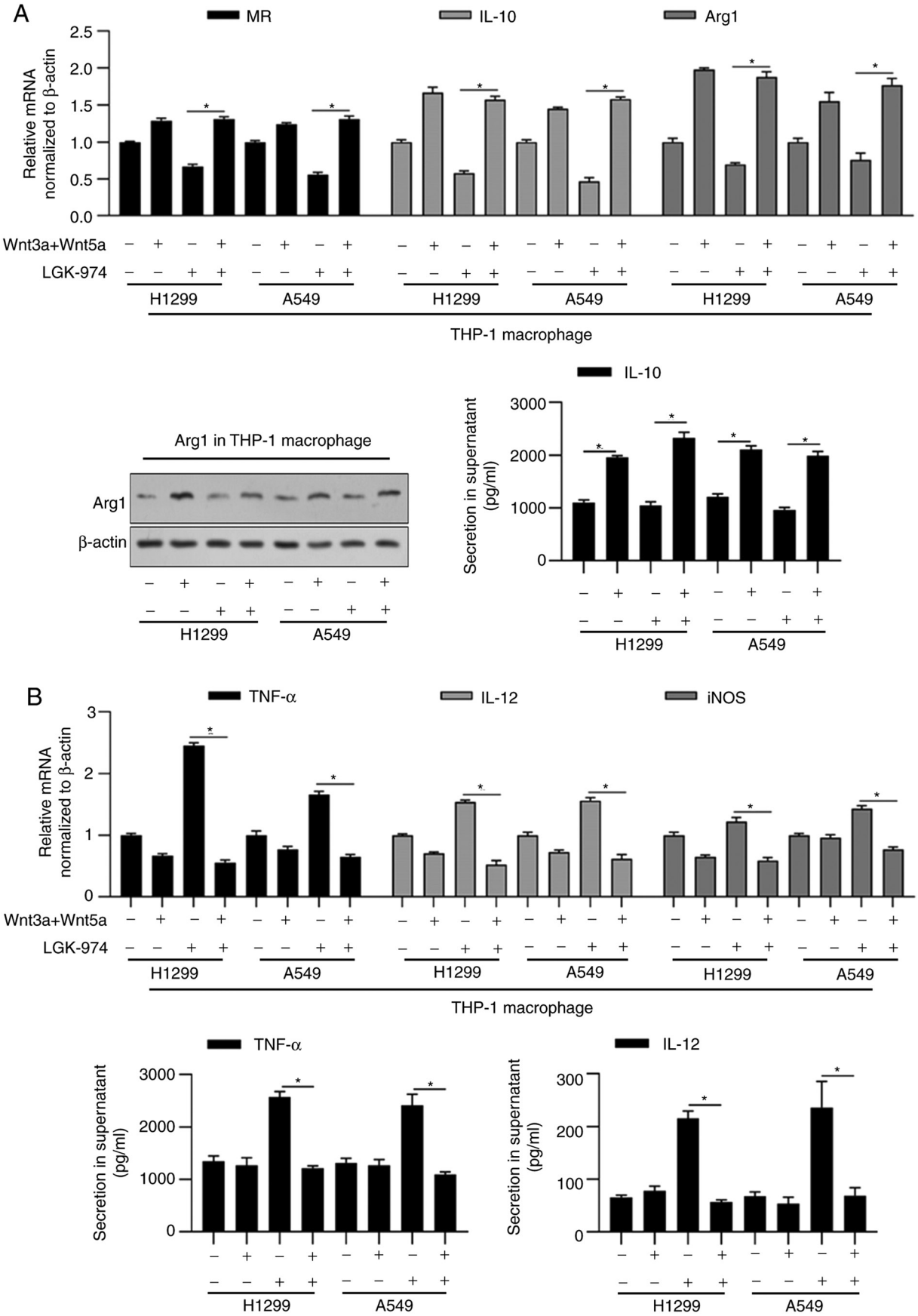

Figure 2. LGK-974 regulates functional markers for macrophage via blocking Wnt/β-catenin signaling. 16HBE, A549 or H1299 cells were co-cultured with LGK-974 and/or Wnt3a/5aA for 24 h. Then, total RNA was isolated and the expression levels of functional markers for MR, IL-10 and Arg1 (A), and functional markers for M1-macrophage (TNF-α, IL-12 and iNOS) (B) were measured by performing reverse transcription-quantitative PCR. Arg1 protein level was detected by western blotting and IL-10 section in supernatant was measured by performing ELISA (A, right panel). TNF-α and IL-12 section in supernatant was measured by performing ELISA (B, right panel). *P<0.05 vs. mock group. MR, mannose receptor; iNOS, inducible nitric oxide synthase; Arg1, argininase-1.

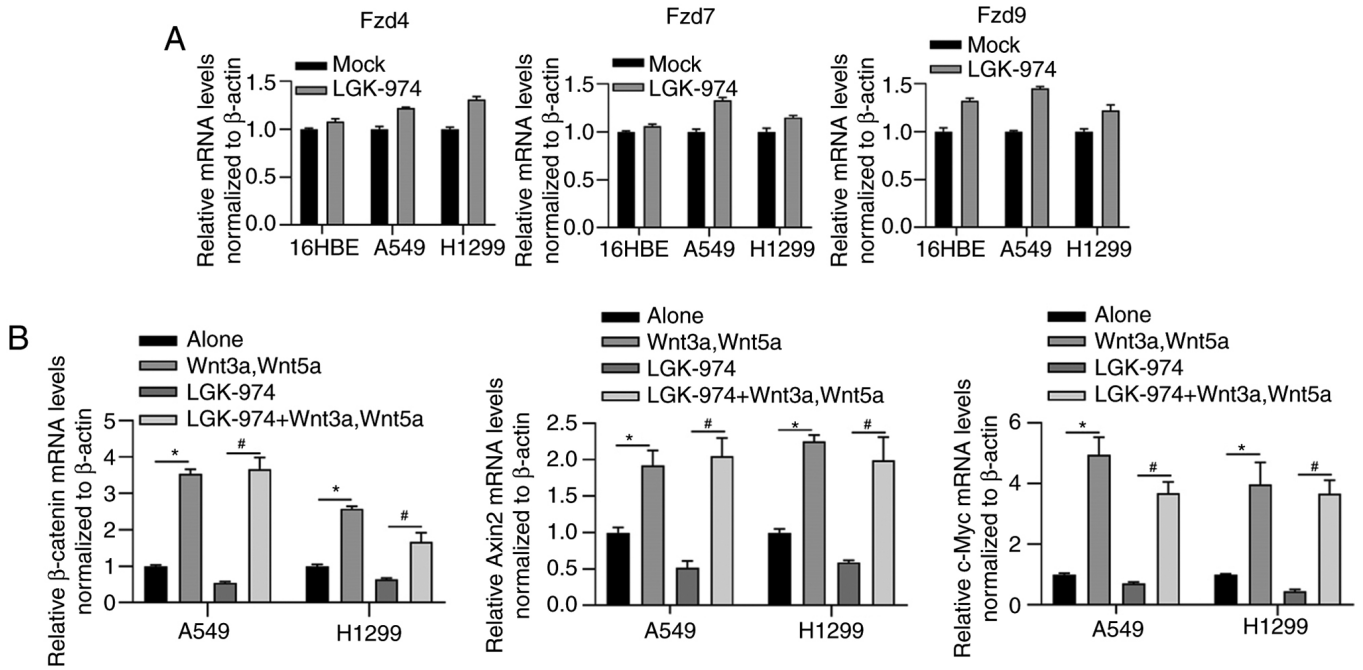

Figure 3. LGK-974 regulates Wnt/β-catenin signaling in tumor-associated macrophages. (A) In 16HBE, A549 and H1299 cells treated with LGK-974, mRNA levels of Wnt receptors, including Fzd4, Fzd7 and Fzd9, were determined. Then, the downstream genes of Wnt signaling including β-catenin, Axin2 and c-Myc (B) were determined by reverse transcription-quantitative PCR. *P<0.05 vs. alone; #P<0.05 vs. LGK-974 group. Fzd, Frizzled.

addition of commercially available Wnt3a and Wnt5a recombinant proteins reversed the decrease in IL-10 and Arg1 by LGK-974. Expectedly, the addition of Wnt3a and Wnt5a increased MR, IL-10 and Arg1 (Fig. 2A). It was also observed that the addition of Wnt3a and Wnt5a increased M1-macrophage markers, including TNF-α, IL-12 and iNOS, which may be due to the promoting effect of Wnt on M2-type phenotype (Fig. 2B).

In order to assess whether LGK-974 modulates the expression level of Wnt receptors, the mRNA levels of Fzd4, Fzd7 and Fzd9 in THP-1 macrophage co-cultured with 16HBE, A549 or H1299, respectively, were examined. As shown in Fig. 3A, Fzd4, Fzd7 and Fzd9 were not obviously affected by the addition of LGK-974, suggesting that Wnt signaling could not be modified in LGK-974 regulation via regulating Wnt receptors. The addition of LGK-974 significantly decreased the mRNA levels of three downstream genes of the Wnt/β-catenin signaling pathway including β-catenin, Axin2, and c-Myc, which were reversed by co-culturing with Wnt3a and Wnt5a (Fig. 3B), further indicated that the regulatory role of LGK-974 was dependent on regulating Wnt secretion.

LGK-974 modulates TAMs' effects on proliferation, colony formation and invasion. Since TAMs play critical roles in tumor malignant behaviors, including proliferation, invasion, metastasis and immunosuppression, the ability of TAMs modified by co-culture with LGK-974 to regulate tumor cell proliferation was detected by a CCK-8 assay and colony-formation assay. The results showed that the cell viability of both H1299 and A549 cultured with CM, which was collected from LGK-974-modified TAMs culture medium and contained no LGK-974, were significantly decreased (Fig. 4A). Secondly, colony-formation assay of H1299 and A549 cultured with CM from LGK-974-modified TAMs was performed, which indicated that TAM-modified CM inhibited cell proliferation without LGK-974. Consistently, CM from TAMs with the addition of LGK-974 significantly inhibited the colony-formation ability in these two cell lines (Fig. 4B). Thirdly, the invasion of tumor cells was also measured using Transwell inserts with a Matrigel layer for 24 h. The results showed that CM from LGK-974-modified TAMs significantly limited the numbers of invaded cells (Fig. 4C). Expectedly, by performing scratch assay, it was also observed that CM from LGK-974-modified TAMs markedly limited cell migration (Fig. 4D). Notably, CM from TAMs without LGK-974 pre-modification slightly affected cell proliferation, colony formation, invasion or migration, which is potentially due to the high malignancies of H1299 and A549.

Wnt/β-catenin signaling is critical for LGK-974's effects on TAMs. In order to further confirm whether LGK-974 regulates TAMs via inhibiting Wnt/β-catenin, Wnt3a and Wnt 5a in were employed for TAM culturing. Consistent with previous results, CM from TAMs with LGK-974 resulted in decreased cell viability. CM from TAMs with LGK-974 and Wnt3a/5a significantly promoted cell viability compared with that without the addition of Wnt3a/5a (Fig. 5A). By detecting cell cycle phase distribution, it was also observed that CM from TAMs with LGK-974 significantly increased the proportion of G_1/G_0 phase, which was reversed by the addition of LGK-974 (Fig. 5B). Taken together, all these data demonstrated that the modulation of LGK-974 on TAMs is potentially via blocking Wnt/β-catenin by inhibiting Wnt secretion.

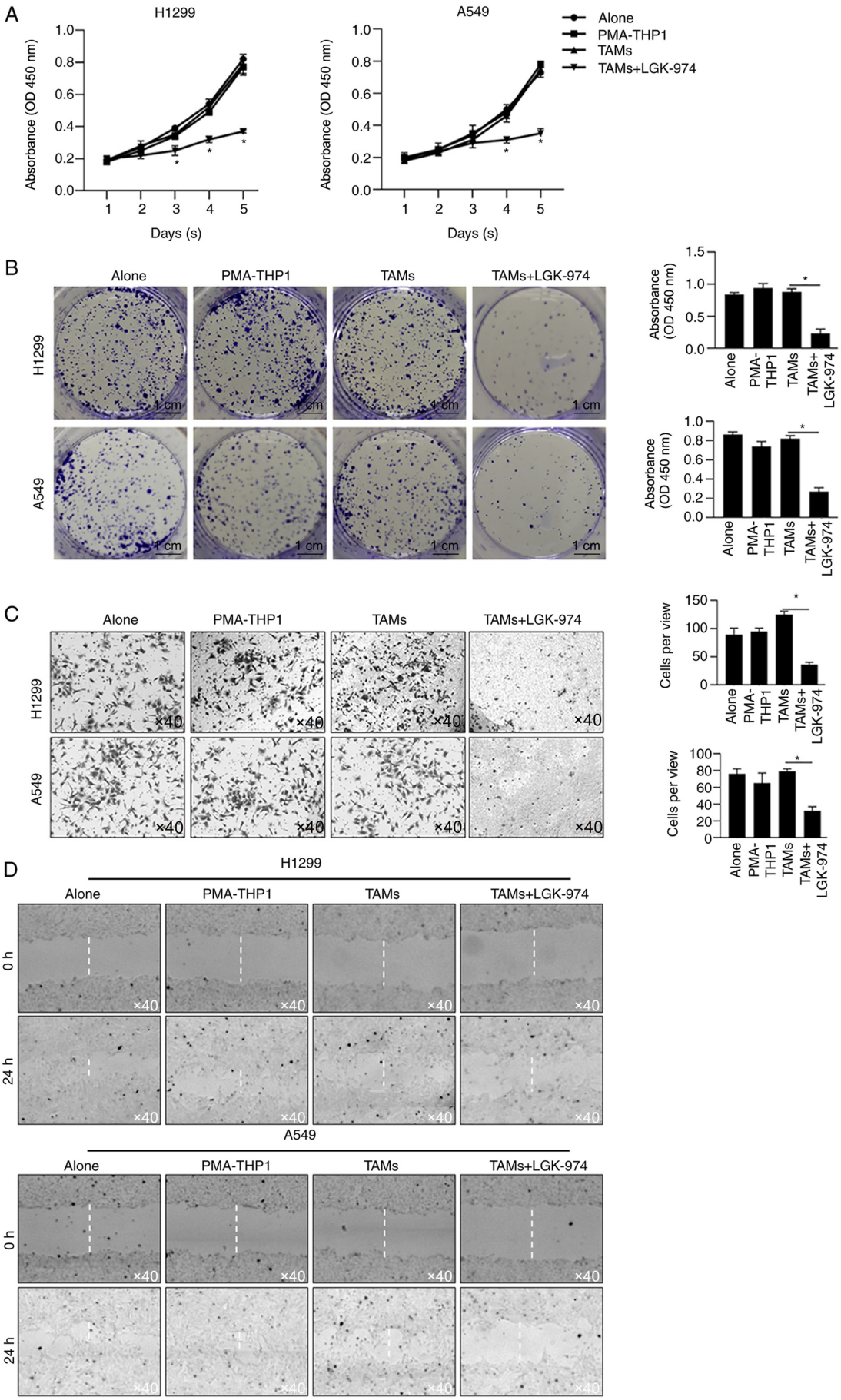

Figure 4. LGK-974 modifies TAMs-inhibited malignant behaviors of A549 and H1299. (A) The cell viability of A549 or H1299 cultured in conditioned media from PMA-THP1, TAMs or TAMs + LGK-974 was measured from day 1 to 5. Then, colony formation, (B) migration (C) and invasion (D) were measured. Magnification, x40. *P<0.05 vs. TAMs group. TAMs, tumor-associated macrophages; PMA, phorbol 12-myristate 13-acetate.

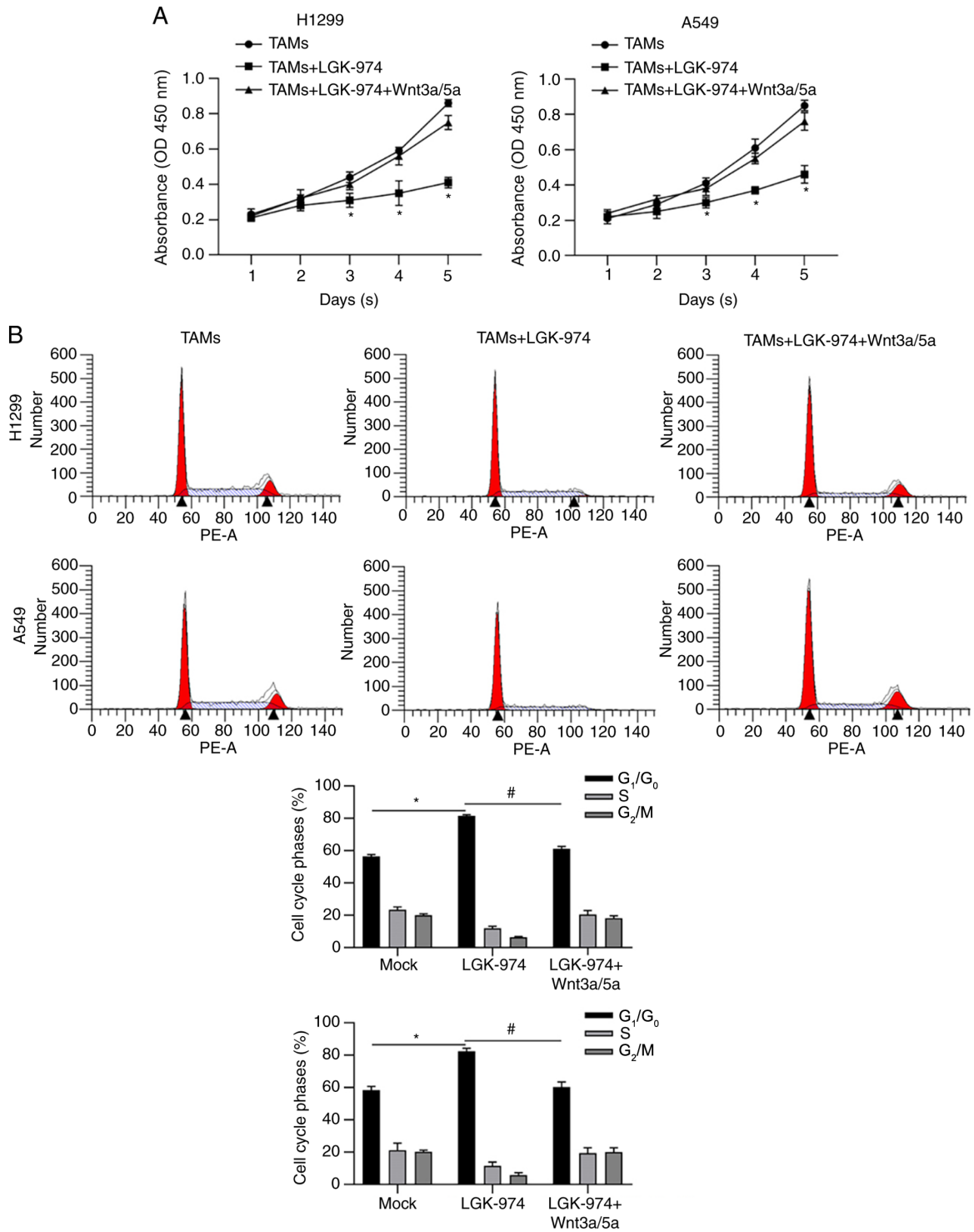

Figure 5. LGK-974 modifies TAMs via blocking Wnt/β-catenin signaling. (A) The effect of LGK-974 on cell viability was measured by Cell Counting Kit-8 assay. Then, the addition of Wnt3a/5a on reversing this effect was measured. (B) Cell cycle phase distribution was measured by performing PI staining followed by flow cytometry. *P<0.05 vs. mock group; #P<0.05 vs. TAMs + LGK-974 group. TAMs, tumor-associated macrophages.

Discussion

The present study results showed that LGK-974, a novel inhibitor of Porcupine, which is an O-acyltransferase responsible for the palmitoylation of Wnt ligands, regulated the polarization of macrophages and thus regulated the microenvironment and subsequently inhibited tumor malignancies, including proliferation, colony-formation and invasion. Numerous reports have described the antitumor effects of LGK-974 in several types of cancer, including NSCLC. However, little is known as to whether LGK-974 affects tumor malignancies indirectly by regulating macrophage polarization. As expected, LGK-974 regulated macrophage polarization by inhibiting Wnt/β-catenin signaling, and the modified microenvironment significantly inhibited tumor malignancies. Taken together, these results suggest that LGK-974 not only directly

regulates tumor malignancies but also indirectly regulates tumor malignancies by modifying macrophage polarization and the microenvironment.

The porcupine-selective inhibitor LGK-974 blocks Wnt/β-catenin signaling by inhibiting the secretion of Wnt ligands. LGK-974 has been proven to block tumor growth and malignancies *in vivo* (14). Giefing *et al* (21) reported that in head and neck squamous cell carcinoma cell lines with NOTCH1 mutations, LGK-974 exerts antitumor effects by blocking Wnt signaling. Wnt/β-catenin signaling is critical in promoting the proliferation and maintenance of cancer stem-like cells (CSCs) in various cancer types, including NSCLC (22). This indicated that LGK-974 may affect stemness in CSCs derived from NSCLC via blocking Wnt signaling and the absence of the investigation of the effect of LGK-974 on stemness maintenance is a limitation in the present study. In NSCLC, hyperactivation of Wnt signaling is necessary for cancer progression and the maintenance of self-renewal capacity, resulting in the poor prognosis of lung cancer patients (23,24). Guimaraes *et al* (25) found that in NSCLC, LGK-974 treatment significantly inhibited cell migration and invasion by blocking Wnt signaling. In LUAD, despite intestinal toxicity, LGK-974 markedly decreased cell viability, indicating that it is a promising antitumor treatment (25).

TAMs not only affect the intrinsic characteristics of tumor cells, but also affect the tumor microenvironment. TAMs can stimulate the proliferation, migration and genetic instability of tumor cells. TAMs act on the primary tumor site or secondary localization site, and promote the invasion and the metastasis. TAMs promote angiogenesis and lymph angiogenesis, as well as tissue remodeling of fibrous tissue deposition (26). TAMs contribute to immunoregulation observed in the tumor microenvironment. Therefore, TAMs targeting may complement the action of checkpoint blockade inhibitors. LGK-974 was accepted as a tumor inhibitor by blocking Wnt signaling (19), without knowing its exact role in regulating microenvironment via TAMs. The present study focused on the effects of LGK-974 on TAMs polarization by inhibiting Wnt signaling. However, molecular evaluation by identifying TAM polarization in clinical sample set it is failed was not performed, which is a limitation of the present study.

β-catenin is one of the critical components of canonical Wnt signaling. In the inactivated state, β-catenin is at a low level, localized in the cytoplasm and binds to a destruction complex that is composed of APC, axins, CK1α and GSK3β (27). Canonical Wnt ligands interact with specific receptors, such Fzd4, Fzd7 and Fzd9, and inactivate this destruction complex, which leads to the nuclear translocation of cytoplasmic β-catenin. In the nucleus, β-catenin then activates the transcription factors LEF and TCF, resulting in the upregulation of downstream target genes, including c-Myc and Axin2 (27). The present results showed that the addition of LGK-974 notably decreased c-Myc and Axin2 without disturbing the expression of Fzd4, Fzd7 or Fzd9, and this effect was reversed by the addition of the Wnt ligands Wnt3a and Wnt5a, which are known to promote the activation of Wnt/β-catenin signaling. Interestingly, the addition of the Wnt ligands Wnt3a and Wnt5a induced TAMs to adopt the M1 phenotype. However, the effects of Wnt3a and Wnt5a on the malignant behaviors in A549 and H1299 were not demonstrated, which is a limitation in the present study.

Overall, the present study shows that LGK-974 exerts antitumor effects not only by blocking Wnt/β-catenin signaling in cancer cells but also by regulating macrophage polarization and thus modifying the tumor microenvironment. The present study results are the first to demonstrate that LGK-974 indirectly regulates lung cancer malignancies via TAM-derived Wnt ligands and thus activates canonical Wnt/β-catenin signaling in TAMs, resulting in the inhibition of cell proliferation, colony formation and invasion. Blocking Wnt secretion from TAMs or inactivating Wnt signaling in TAMs should be a novel strategy for NSCLC therapy in the future.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT, YS, CC and XK designed the experiments. YT, MJ, AC, WQ and XH performed cell culture-associated experiments. JZ and GX are responsible for data collection and performed the statistical analysis. All authors read and approved the final manuscript. YT, CC and XK confirmed the authenticity of all the raw data

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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