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R2-8018 reduces the proliferation and migration of non-small cell lung cancer cells by disturbing transactivation between M3R and EGFR

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ABSTRACT

Aims: The M3 muscarinic acetylcholine receptor (M3R) is a G protein-coupled receptor that is expressed in cases of non-small cell lung cancer (NSCLC). Previous studies demonstrated that M3R antagonists reduce the proliferation of NSCLC. However, how antagonists inhibit the NSCLC proliferation and migration is still little known. This study aims to investigate the mechanism of M3R involved in the growth of NSCLC.

Main methods: The CRISPR/Cas9 was used to knock out (KO) the M3R gene. A real-time cell analyzer (RTCA) was used to record the proliferation of NSCLC cells. The migration and cell cycle of NSCLC cells were evaluated with scratch test and flow cytometry (FCM), respectively. Antibody microarray analysis was performed to detect the expression of proteins after antagonizing M3R and knocking out of M3R, subsequently some of these important proteins were verified by western blot.

Key findings: The proliferation and migration of NSCLC cells were inhibited by M3R antagonist R2-8018 and knocking out of M3R. Antagonism or knocking out of M3R reduced the phosphorylation of EGFR. Moreover, c-Src and β-arrestin-1 are involved in the mechanism of how the inhibition of M3R affects EGFR in NSCLC. Further study demonstrated that PI3K/AKT and MEK/ERK signal pathways are involved in M3R-induced EGFR transactivation in NSCLC, and the molecules involved in the cell cycle progression and migration of NSCLC cells were identified.

Significance: This further understanding of the relationship between M3R and NSCLC facilitates the design of therapeutic strategy with M3R antagonist as an adjuvant drug for NSCLC treatment.

1. Introduction

Lung cancer is one of the most common types of cancer in both men and women and a leading cause of cancer-related death worldwide [1]. Lung cancer can be categorized as small cell lung cancer or non-small cell lung cancer (NSCLC), the latter of which accounts for approximately 80% of all cases and includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [2,3]. Although current treatments such as surgery, radiotherapy, and chemotherapy are well developed, the overall 5-year survival rate of patients with NSCLC remains extremely low [4]. Tumor proliferation, invasion, and metastasis play pivotal roles in the progression of NSCLC and contribute to the primary mechanisms underlying cancer-related death. Thus, investigating the molecular basis of NSCLC cell proliferation and metastasis is crucial for its detection and treatment.

Acetylcholine (ACh) is an important neurotransmitter in the central and peripheral nervous systems where it modulates cell function via production and synaptic release by neurons as well as non-neuronal cells. Furthermore, recent findings support the importance of muscarinic ACh receptor (M1–M5R) expression and activation in various cancers. For example, muscarinic stimulation of cancer growth is observed in colon [5], lung [4], breast [6], and prostate [7] cancers. As one subtype of muscarinic receptors, M3 muscarinic acetylcholine receptor (M3R) plays key roles in the proliferation and metastasis of NSCLC. Previous studies from our research group and others suggested that M3R expression is closely related to tumor progression in NSCLC

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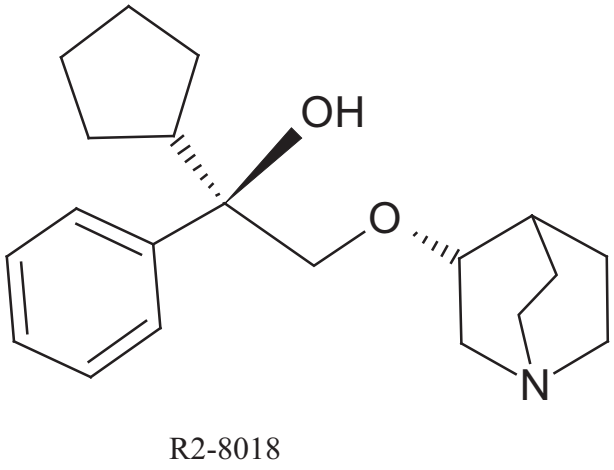

Fig. 1. Structure of R2-8018.

[8–10], high levels of M3R expression are correlated with poor survival in NSCLC patients, and M3R may be a novel antineoplastic target in the future [9–11]. M3R is a kind of G protein-coupled receptor (GPCR) that can transactivate the epidermal growth factor receptor [12,13] and is shown to induce epidermal growth factor receptor (EGFR) transactivation in salivary gland cells and colon cancer cells [5,14]. However, the relationship between M3R and EGFR and the exact signaling pathway post-EGFR in NSCLC remain little known. Only one available research reported that ACh-induced activation of M3R promotes NSCLC proliferation and invasion probably via the EGFR/PI3K/AKT pathway [11]. Thereby, more extensive and profound investigations are needed to elucidate the partnership between M3R and NSCLC.

3-(2′-phenyl-2′-cyclopentyl-2′-hydroxyl-ethoxy) quinuclidine (Penehyclidine hydrochloride; 8018) is a new cholinolytic with two chiral carbonic atoms. It has 4 optical isomers among which the RR′ configuration, namely R2-8018 (Fig. 1), showed most potent pharmacological activity, mainly blocking the M3R and M1 muscarinic acetylcholine receptor (M1R) [15–18].

The present study mainly investigated the mechanisms underlying M3R-induced transactivation of EGFR. Additionally, NSCLC proliferation and invasion and the molecular mechanisms supporting these processes were assessed after inhibiting M3R by R2-8018 or knocking out M3R by CRISPR/Cas9.

2. Materials and methods

2.1. Cell lines, antibodies and reagents

Human NSCLC cell lines H1299 and H460 were purchased from the National Infrastructure of Cell Line Resource (Beijing, China). Cells were cultured in 1640 medium supplemented with 10% FBS at 37 °C and 5% CO_2 incubator. Antibodies of EGFR, AKT, Phospho-AKT Pathway Antibody Sampler Kit, mitogen-activated protein kinase (MAPK) Family Antibody Sampler Kit, β-arrestin-1, phospho-PI3K and other antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Phospho-EGFR (Tyr-1110), Phospho-MEK1 (Thr291), Phospho-MEK1/2 (Ser217), Phospho-Src (Tyr529), β-arrestin-2 and c-Src antibody were purchased from Absin Bioscience (Shanghai, China). Acetylcholine was obtained from Abcam (Cambridge, UK). R2-8018 is synthesized in our institute, EGFR specific inhibitor AG-1478 was purchased from Sigma (St Louis, MO, USA), VU 0255035 (hereinafter referred to as VU) was purchased from Tocris Bioscience (Bristol, UK).

2.2. M3R gene knocked out by CRISPR/Cas9

M3R gene knockout was performed as previously described [19,20]. Briefly, all procedure, gRAN design, plasmids construction, lentivirus package and transfected, as well as positive clones screen and identification were performed by Beijing Syngentech Co., Ltd.

2.3. Real-time cell analyzer (RTCA) monitoring proliferation assay

The study used an electrical impedance-based technique, the real-time cell analyzer (RTCA, Roche Applied Science, Penzberg, Germany). The system consists of an RTCA analyzer, an RTCA SP station and RTCA software. The experiment was performed as previously described [21]. Briefly, cell lines were cultured in *E*-Plate 48 of RTCA for at least 96 h. After the first 24 h, cells were treated with R2-8018 or AG-1478 for 72 h. Cell index was recorded every 15 min.

2.4. Migration assay

Cells were seeded in P6-well culture dishes at a density of 3×10^5 cells per well. Confluent cell monolayers were then gently scratched with a pipette tip across the entire diameter of the dish. Next, cells were rinsed with medium to remove all cellular debris, and treatment with R2-8018 or AG-1478, whereas the rest of the wells served as control. Cultures were observed immediately after wounding and 24 h later the phase-contrast pictures of the wounded area were taken using a high-resolution camera (Olympus, Tokyo, Japan) connected to the microscope. Quantification of migrated cells was done by measuring the healed distance between the borders of the scratches [22].

2.5. Cell cycle analysis

Alterations in cell cycle were determined by using flow cytometric assay. For time-dependent response, cells were incubated in 2 ml fresh RPMI-1640 containing 10% FBS with 20 μM R2-8018 for 24, 48, 72 h. For concentration dependent response, cells were incubated for 48 h with R2-8018 at final concentrations of 5, 10 and 20 μM in 2 ml fresh RPMI-1640 containing 10% FBS. Fresh media was added in the control well simultaneously. After treatment, the cells were harvested with trypsin, washed once with PBS, and then fixed in 70% ethanol overnight at 4 °C. Before flow cytometry analysis, cells were stained with 200 mg/ml Ribonuclease A and 50 mg/ml propidium iodide (Sigma) for 30 min at room temperature away from light. 16×10^4 cells per sample were analyzed using a FACSC alibur flow cytometer (BD Biosciences). Data were evaluated using Modfit software.

2.6. Antibody microarray analysis

As reported previously [23], cell lysates obtained from NSCLC cells (treated with R2-8018 and M3R knockout, with PBS as a control) were applied to the Signal Pathway Phosphorylation Antibody Array (Full Moon BioSystems, USA). Each of the antibodies has 6 replicates that are printed on standard-size coated glass slides. In brief, the Antibody Array was first blocked with blocking solution (Full Moon BioSystems, USA) for 30 min at room temperature, followed by incubation with the biotin-labeled cell lysates at 4 °C overnight. After three times of washing, the conjugated labeled proteins were detected using Cy3-conjugated streptavidin. For each antibody, phosphorylation ratio was computed as the equation below:

$$Phospho\ \ Ratio\ \ = \ \ \frac{Phospho\ \ Ratio_{Exp.}}{Phospho\ \ Ratio_{Con.}}$$

(phospho: phosphorylation; con.: control group; Exp.: experimental group)

Some results of the Phospho-antibody array were further confirmed

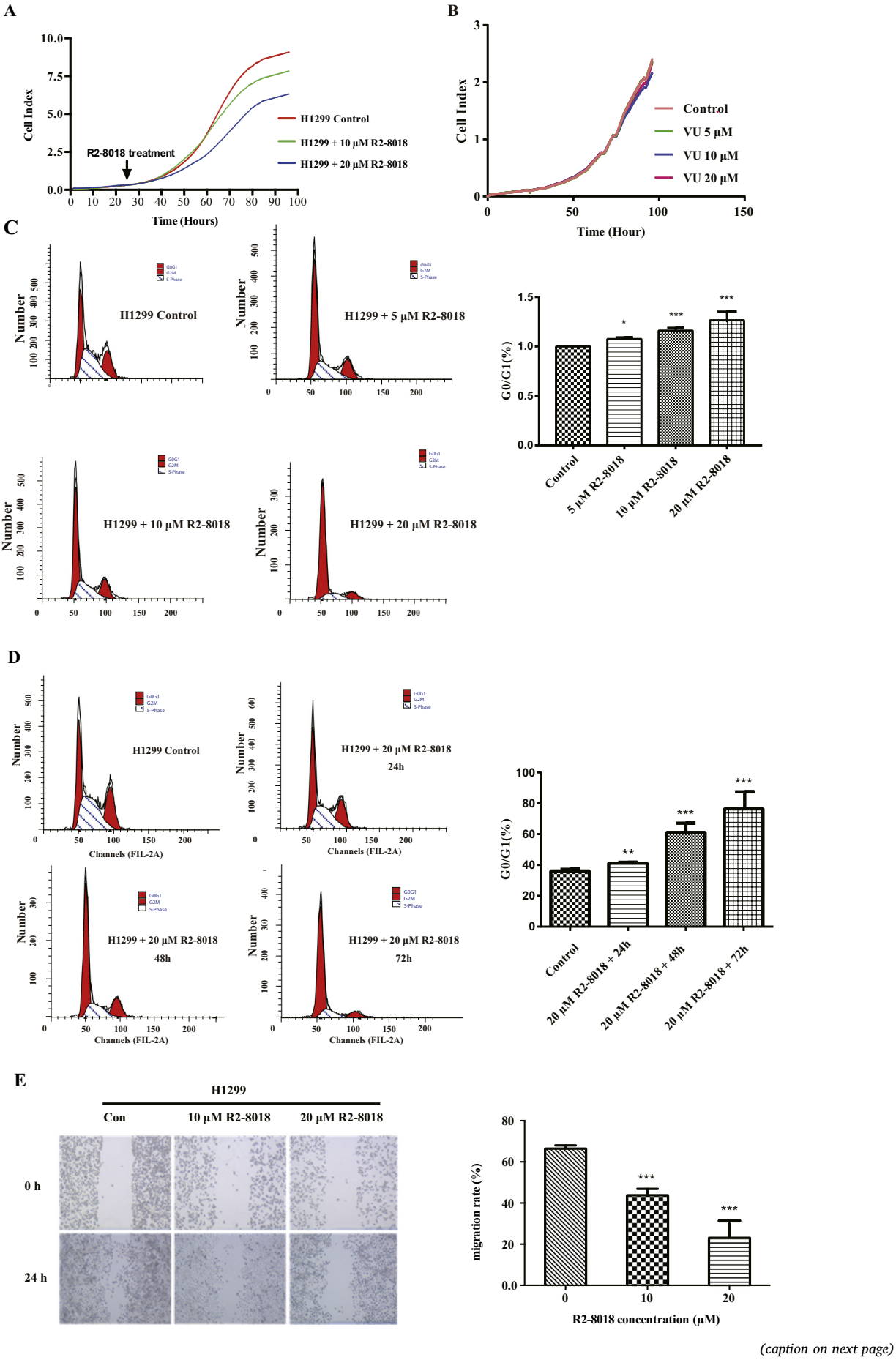

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Fig. 2. R2-8018 inhibited NSCLC cells proliferation and migration, and induced G0/G1 arrest. **A** R2-8018 reduced H1299 cells proliferation at different concentrations ($n = 3$). B M1R selective antagonist VU had no inhibitory effects on the proliferation of H1299 cells ($n = 3$). **C** R2-8018 arrested H1299 cells in G_0/G_1 phase in a concentration dependent manner ($^{*}P < 0.05$, $^{***}P < 0.001$, n = 3, one-way analysis of variance (ANOVA)). D R2-8018 arrested H1299 cells in G_0/G_1 phase in a time dependent manner ($^{**}P < 0.01$, $^{***}P < 0.001$, n = 3, one-way analysis of variance (ANOVA)) **E** R2-8018 could concentration dependently inhibit H1299 cell migration. The cells were treated with the indicated concentrations of R2-8018 for 24 h ($^{***}P < 0.001$, $n = 3$, one-way ANOVA).

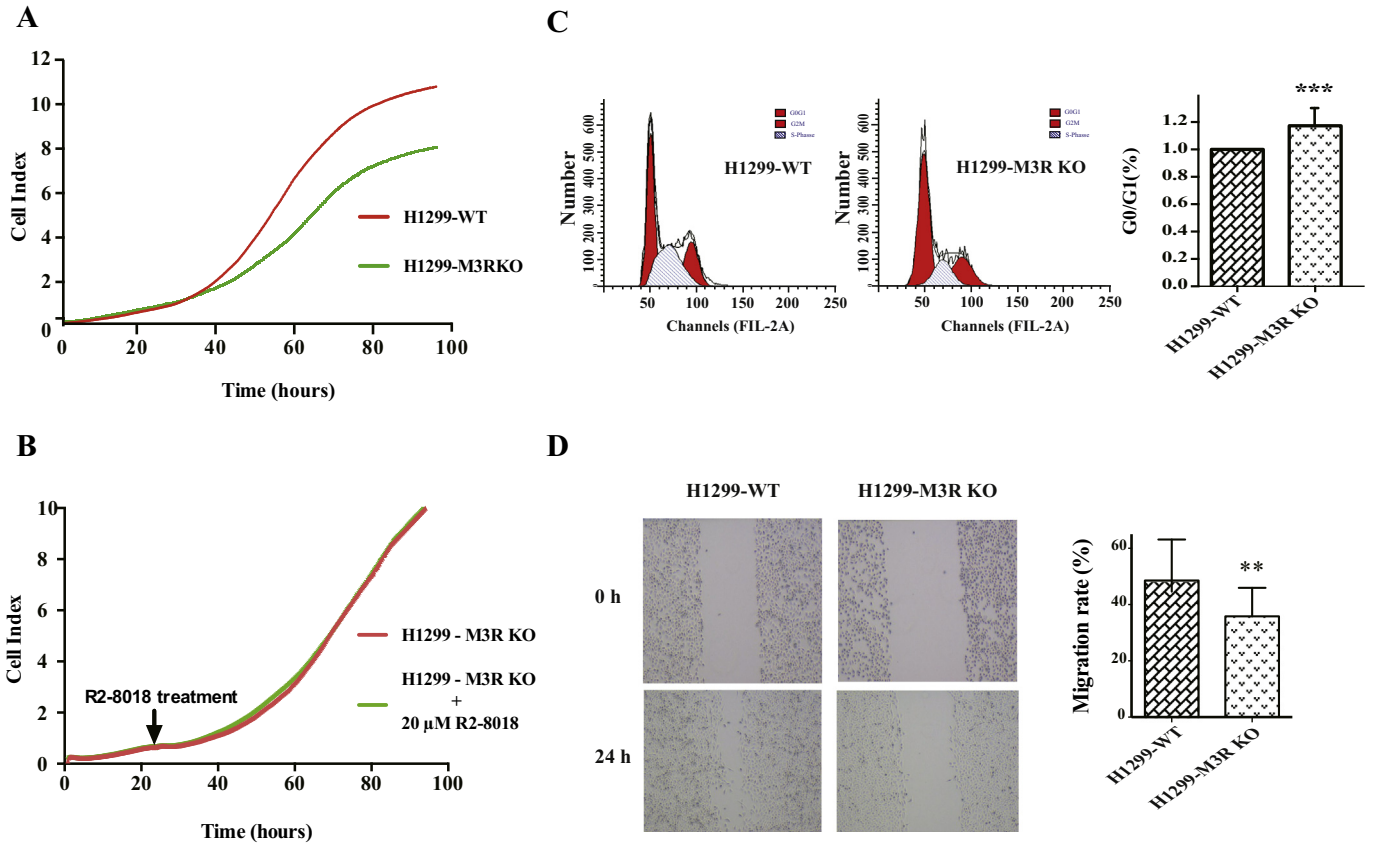

Fig. 3. Knocking out of M3R inhibited the proliferation and migration of H1299 cells. **A**. M3R knockout reduced H1299 cells proliferation (n = 3). **B**. There was no difference between wild-type and M3R knockout cell line under R2-8018 treatment (n = 3). **C**. M3R knockout induced G_0/G_1 arrest in H1299 cells ($P < 0.001$, $n = 4$, Student's *t*-test). **D**. M3R knockout suppressed H1299 cell migration. The cells were treated with the indicated concentrations of R2-8018 for 72 h or 24 h ($^{**}P < 0.01$, n = 3, student's t-test).

by Western Blot assay.

2.7. Western blot

Cells were harvested and subjected to lysis in RIPA Lysis Buffer. Equal amounts of lysates (30 μg) were separated on 10% SDS-PAGE and then transferred to nitrocellulose membrane (Roche). Membranes were then blocked with PBS buffer containing 5% low fat milk and 0.1% Tween 20 (PBST) for 1 h at room temperature and then incubated with primary antibodies (1:1000–2000) overnight at 4 °C. After been washed three times with PBST, membranes were incubated with IRDye secondary antibodies (1:15000) for 1 h at room temperature. The membranes were washed with PBST again and imaging (odyssey, Li-COR).

2.8. Data analysis

Data analysis was performed with software GraphPad Prism Version 6.0, SPSS 21 and Minitab 17 Statistical Software. All results were presented as Mean ± standard error of mean (SEM.). Statistical analysis was performed using student's *t*-test, the one-way analysis of variance (ANOVA), and the three-way factorial design ANOVA. Outliers are analyzed and rejected, kept or adjusted based on actual conditions. Difference of $P < 0.05$ was considered statistically significant, all reported *P* values are two-sided and were adjusted for multiple testing.

3. Results

3.1. Treatment with R2-8018, a novel M3R antagonist, suppressed the proliferation and migration of H1299 cells

To investigate the biological functions of M3R associated with the proliferation of NSCLC H1299 cells. First, an SRB (Sulforhodamine B) assay was performed to observe the inhibitory proliferation effects of R2-8018 in H1299 cells. The results showed that R2-8018 can inhibit H1299 cells in a concentration- and time-dependent manner (Supplementary materials, Fig. S1). Then a real-time cell analysis device was used to monitor the cell index in real time (Fig. 2A). Quantitative analyses revealed that the M3R inhibitor R2-8018 significantly decreased the proliferative activity of H1299 cells at different concentrations (Fig. 2A), and the same results were observed in H460 cells [24]. Because R2-8018 is also antagonistic to M1 receptor, present work further investigated whether M1R involved in the proliferation of H1299 cells, the RTCA method was used to real time monitor the effects of M1R selective antagonist VU [25] at concentrations of 5, 10 and 20 μM. Results showed that VU did not inhibit the proliferation of H1299 cells (Fig. 2B), which revealed that effects of R2-8018 on H1299

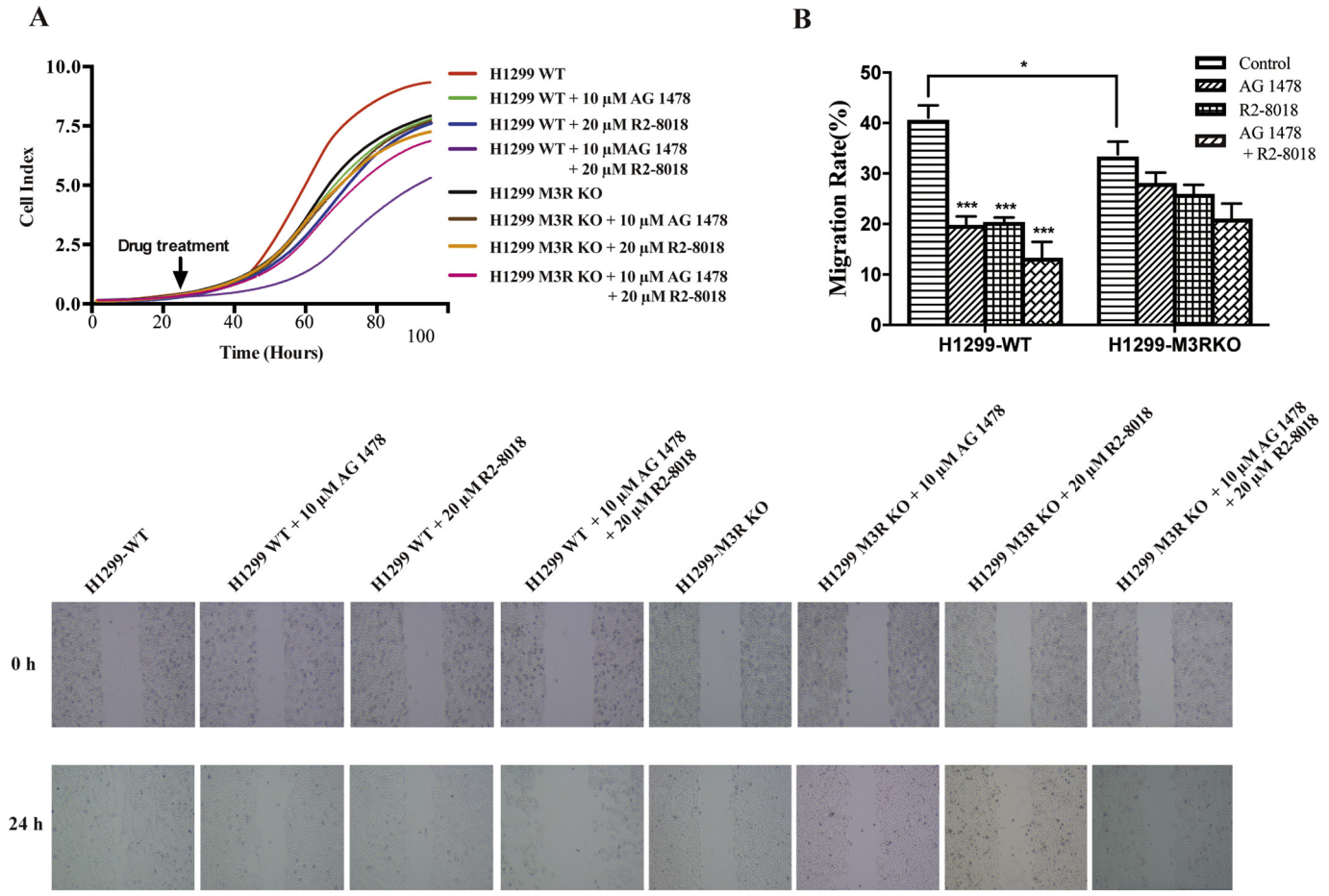

Fig. 4. R2-8018 and AG-1478 combined application increased the inhibition of H1299 cells proliferation and migration. **A**, **B** Coincubation of R2-8018 and AG-1478 dramatically reduced the proliferation and migration compared with R2-8018 and AG-1478 used alone in wild type H1299 cells, but neither R2-8018 nor AG-1478, nor combined of both had any effects on M3R knockout H1299 cell line. The cells of administration group were treated with 20 μM R2-8018 or 10 μM AG-1478 or both for 72 h or 24 h with control and M3RKO group cultured in the same time. ($^{***}P < 0.001$, $n = 3$, three-way factorial design ANOVA).

cells were mainly through M3R.

Our previous work demonstrated that M3R and M1R antagonist R2HBJJ, the analog of R2-8018, arrested the cell cycle in G_0/G_1 [10]. To explore whether R2-8018 restrains the cell cycle of H1299 cells, the effects of R2-8018 on cell cycle progression were observed using flow cytometry. The percentages of cells in the G_0/G_1 phase were increased by 8% ($n = 3$, $P < 0.05$), 16% (n = 3, $P < 0.001$) and 30% (n = 3, $P < 0.001$) after treatment with 5, 10 and 20 μM R2-8018, respectively, for 48 h (Fig. 2C), in the same time, R2-8018 induced G_0/G_1 arrest in a time-dependent manner (Fig. 2D). Moreover, 10 and 20 μM R2-8018 caused a significant arrest of the H1299 cell migration rate, from 66% to 44% (n = 3, $P < 0.001$) and 8% (n = 3, $P < 0.001$), respectively (Fig. 2 E).In addition, we previously observed R2-8018 causing an invasion arrest in a Transwell invasion assay [24]. Collectively, these results indicated that R2-8018 suppresses the proliferation and migration of H1299 cells, and induces cell-cycle arrest in G_0/G_1 phase.

3.2. M3R knockout (KO) inhibited the proliferation and migration of H1299 cells

To further evaluate whether changes in the proliferation and migration of H1299 cells were due to M3R inhibition, the CRISPR/Cas9 system was employed to knock out M3R gene in H1299 cells. H1299 M3R-KO cells proliferation was significantly inhibited compared with H1299 wild-type cells (Fig. 3A), with the percentage of cells in the G_0/G_1 phase increased by 18% (Fig. 3C, $P < 0.001$, $n = 4$). The results also showed that treatment with 20 μM R2-8018 had no effects on the proliferation of H1299 M3R-KO cells (Fig. 3B), which indicated that R2-8018 exerts its function on H1299 through M3R, and knocking out of M3R decreased cell migration by 9% (Fig. 3D, $P < 0.001$, $n = 3$). Taken together, these results suggested that M3R is indispensable for H1299 cells, and M3R KO produces marked effects on cell proliferation and migration. Moreover, the results indicated that R2-8018 inhibits the proliferation and migration of H1299 predominantly through M3R.

3.3. Co-treatment with M3R and EGFR antagonists suppressed H1299 cells proliferation and migration

To further assess how M3R participates in H1299 cell proliferation and migration, an antibody array was performed to detect changes in the expression levels of various signaling pathway molecules. The administration of 20 μM R2-8018 and knockout of M3R in H1299 cells significantly decreased EGFR activity and reduced downstream molecular expression (Supplementary materials, Table S1).

Cross-talk between GPCRs and EGFR is responsible for the proliferation and migration of cancer cells [12]. Subsequently, the effects of an EGFR antagonist, AG-1478, were assessed in H1299 cells. AG-1478 had a similar effect with R2-8018 on H1299 cells proliferation (Fig. 3A). However, neither R2-8018 nor AG-1478 influenced the proliferation of H1299 M3R-KO cells. Similar results were obtained for H1299 cell migration following the administration of R2-8018 or AG-1478. Statistically, coincubation of AG-1478 and R2-8018 produce the best results for whatever proliferation or migration, and three-way

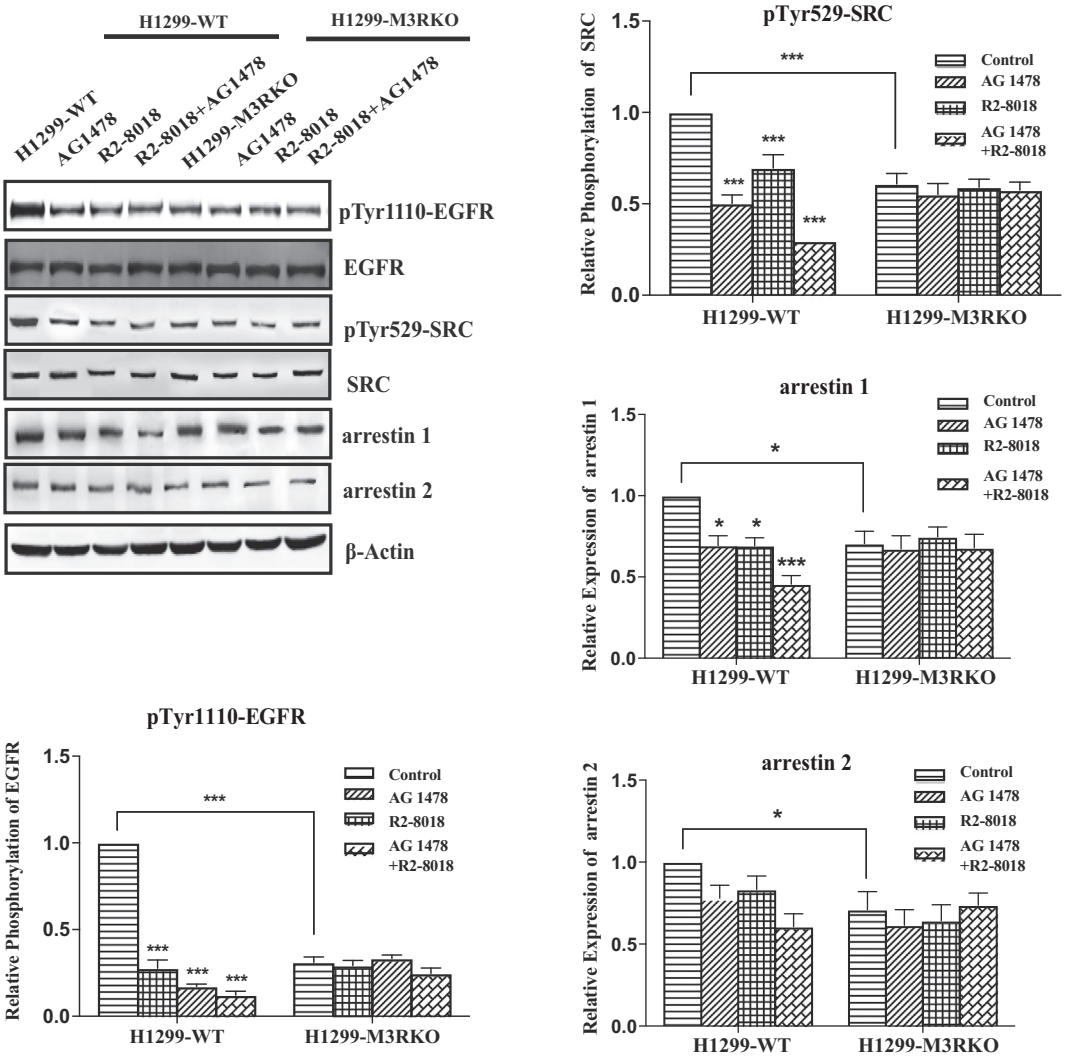

Fig. 5. R2-8018 and M3R knockout down-regulated the phosphorylation of EGFR, SRC and β-arrestin-1. In wild type H1299 cell line, both R2-8018 and AG-1478 reduced the phosphorylation of Tyr1110 EGFR and Try529 SRC, and coincubation of R2-8018 and AG-1478 showed the maximum inhibition in the phosphorylation of these two proteins. Although M3R knockout could suppress the phosphorylation of Tyr1110 EGFR and Try529 SRC, R2-8018 and AG-1478 could not improve the effects anymore. R2-8018 and AG-1478 only showed the inhibition of β-arrestin-1 in wild type H1299 cell line, but had no effects on β-arrestin-2. M3R knockout decreased the expression of β-arrestin-1 but not β-arrestin-2, and R2-8018 or AG-1478 or both was of no effects on β-arrestin-1 and -2 in M3RKO cells. The cells of administration group were treated with 20 μM R2-8018 or 10 μM AG-1478 or both for 72 h with control and M3RKO group cultured in the same time. After that, cell lysates were subjected to western blot analysis. β-Actin was used as the loading control. ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$ or 6, three-way factorial design ANOVA).

ANOVA showed interaction effects between the two were significant ($P < 0.05$, not shown) (Fig. 3B). Taken together, the present findings suggested the activation of both M3R and EGFR and the possible cross-talk between M3R and EGFR in H1299 cells. What seems somewhat strange is that AG-1478 failed to further suppress the proliferation and migration of H1299 after knocking out M3R. It could be because the activity of EGFR is mostly suppressed by M3R KO. This further indicated the importance of M3R and in keeping the activation of EGFR in H1299 cells.

3.4. Cross-talk between M3R and EGFR in H1299 cells

GPCRs-induced transactivation of EGFR is one of the most important mechanism for extracellular GPCRs signals transduction. In various tumor cells, β-arrestins and c-Src are involved in the transactivation of EGFR by GPCRs wherein β-arrestins function as a transductor/adaptor linking GPCRs activation to post-EGFR signaling pathways through c-Src [26,27].

However, scarcely any researches are done in the M3R induced EGFR transactivation in NSCLC. The current work evaluated the effects of R2-8018 and AG-1478 on the most important molecules involved in this signaling process in H1299 cells. AG-1478 is the specific inhibitor of EGFR, and can block the EGFR phosphorylation induced by EGFR ligand. Results in Fig. 5 showed that the level of phosphorylated (p) Tyr1110-EGFR was inhibited in wild type cells following treatment with AG-1478, possibly indicating the overactivation of EGFR in H1299 cells. And R2-8018 alone also declined the phosphorylation level of EGFR, which implying the interplay between M3R and EGFR. Meanwhile, the results showed that pTyr1110 was reduced sharply by the combined of AG-1478 and R2-8018, of which the interaction effects were statistically significant. This further suggested the crosstalk between M3R and EGFR in H1299 cells. Moreover, the level of pTyr1110-EGFR was inhibited in M3R-KO cells, but co-treatment with R2-8018 and AG-1478 had no effects on M3RKO cells. These results were in line with that of the proliferation and migration assay.

Similar results were obtained for the level of pTyr529-Src and β-arrestin-1 (Fig. 5). pTyr529-Src and β-arrestin-1 were suppressed after treatment with R2-8018 or AG-1478 alone and after co-treatment with

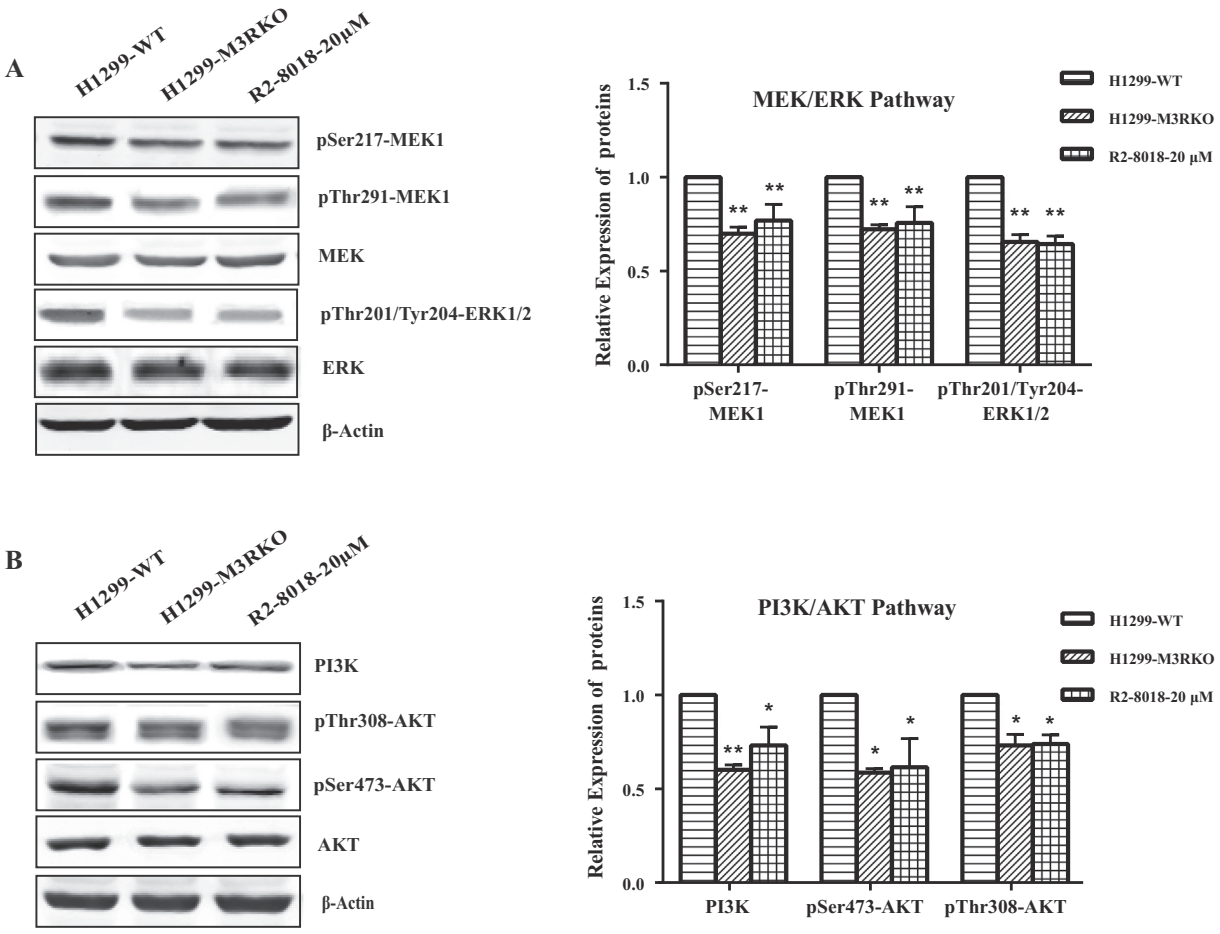

Fig. 6. R2-8018 and M3R knockout down-regulated the phosphorylation of MAPK and PI3K-AKT signal pathway. **A.** R2-8018 treatment and M3R KO reduced the phosphorylation of MEK1 and ERK1. **B.** R2-8018 and M3R KO decreased the level of PI3K and phosphorylation of AKT. The cells of administration group were treated with 20 μM R2-8018 for 72 h with M3RKO group cultured in the same time. Cell lysates were then subjected to western blot analysis. β-Actin was used as the loading control ($^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$, one-way ANOVA).

these two compounds in wild-type H1299 cells. However, the results showed no significant effects on β-arrestin-2 (Fig. 5). The expression of pTyr529-Src and β-arrestin-1 were declined after knockout of M3R, and in M3R-KO cells, R2-8018 treatment and co-treatment with AG-1478 or AG-1478 alone showed no effects on neither c-Src nor β-arrestin-1. These results indicated the participation of c-Src and β-arrestin-1 in EGFR transactivation by M3R. Additionally, AG-1478 also caused a significant drop of the expression of c-Src and β-arrrestin-1, suggesting that the inhibition of EGFR influence the activation of c-Src and the expression of β-arrestin-1 in some way. In H1299 M3RKO cells, what echoed with the migration and proliferation results described previous was that AG-1478 failed to inhibit the EGFR phosphorylation at Tyr1110, the expression of pTyr529-Src and the β-arrrestin-1, indicating that knocking out M3R gene deactivate the general activity of EGFR, which further prove the importance of M3R in H1299 cells. In conclusion, the present results suggested that in H1299 cells, with the help of β-arrestin-1, c-Src can probably link M3R activation to EGFR through phosphorylation of Tyr1110.

3.5. Effects of R2-8018 and M3R KO on MAPK and PI3K/AKT signaling

Mitogen-activated protein kinase (MAPK) family play an important role in the extracellular signal transduction to cellular response, three MAPKs families are clearly characterized, namely classical MAPK (also known as extracellular regulated protein kinases (ERK), important downstream target of EGFR), C-Jun N-terminal kinase stress activated protein kinase (JNK/SAPK) and p38 kinase [28]. Phosphatidylinositol 3-kinase (PI3K)/AKT is another ubiquitous post-EGFR signaling pathway involved in cancer, and is testified a crosstalk with MAPK [29,30].The antibody arrays indicated that the levels of molecules involved in the MAPK and PI3K/AKT signaling pathways exhibited changes following treatment of R2-8018 (Supplementary materials, Table S1). To confirm these results, Western blot analyses were conducted to measure the levels of specific proteins associated with MAPK and PI3K/AKT signaling pathways. In terms of the MAPK signaling pathway, R2-8018 and M3R KO both reduced the levels of pSer217-MEK1 (mitogen-activated protein kinase kinase, MEK), pThr291-MEK1, and pThr201/Try204-ERK1/2 (Fig. 6A). In terms of the PI3K/AKT signaling pathway, R2-8018 and M3R KO reduced the levels of PI3K, pThr308-AKT, and pSer473-AKT (Fig. 6B). And previously we identified the down-regulated expression of pSer473-AKT in H460 cells in a concentration dependent manner following R2-8018 treatment [24]. Taken together, these results suggested that R2-8018 and M3R KO depress M3R-induced transactivation of EGFR, then influence downstream MAPK and PI3K/AKT signaling molecules to inhibit the proliferation and migration of NSCLC cells. Moreover, the reduced expression of phosphorylated Glycogen synthase kinase 3beta (GSK-3β) was also observed in H1299 and H460 cells [24]. GSK-3β can be inhibited by AKT through phosphorylation, while hypo-phosphorylated of GSK-3β controls the cell cycle process in a way of degrading the cyclins.

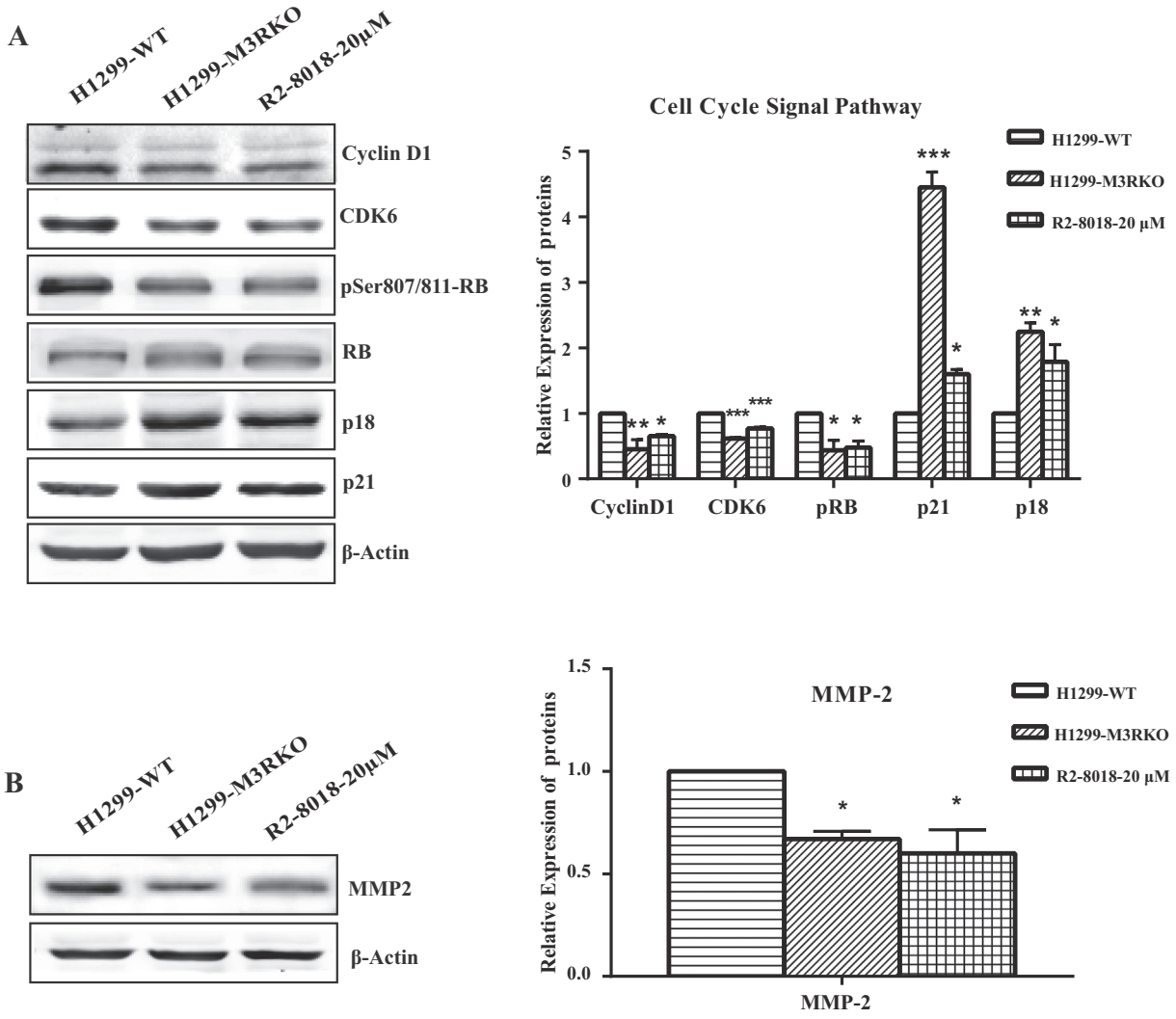

Fig. 7. R2-8018 and M3R knockout affected the level of cell cycle regulatory proteins and MMP2. **A** R2-8018 treatment and M3R KO reduced the level of Cyclin D1, CDK6, and phosphorylation of Rb. Meanwhile, R2-8018 and M3R KO also promoted the level of p18, p21. **B** R2-8018 and M3R KO reduced the expression of MMP2. The cells of administration group were treated with 20 μM R2-8018 for 72 h with M3RKO group cultured in the same time. Cell lysates were then subjected to western blot analysis. β-Actin was used as the loading control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$, one-way ANOVA).

3.6. R2-8018 and M3R KO altered the activity of signaling molecules involved in H1299 cells proliferation and migration

Cell cycle arrest is a major mechanism repressing tumor growth. The most important regulators in the cell cycle progression are the cyclins and the cyclin-dependent kinases (CDKs), they form complexes whose activities are regulated by the cellular CDK inhibitors (CKIs) (p21 or p18 etc.). Cyclin D1-CDK4/6 complexes initiate or regulate the early G1 phase progression through phosphorylation of the downstream retinoblastoma (Rb) protein. Hypo-phosphorylated of Rb suppresses the release of the transcription factor E2F, which is responsible for transcription of DNA synthesis and transition of G1 to S phase. To the contrary, hyperphosphorylation of the Rb leads to the less repression of cell cycle thus causing malignant cell growth [31,32].

In this work, R2-8018 and M3R KO both decreased the levels of cyclinD1, CDK6, and pSer807/811-Rb, but promoted those of the CKIs p18 and p21(Fig. 7A). These results revealed that R2-8018 and M3R KO alter the levels of proteins involved in cell cycle progression, which may be the molecular interpretation for G_0/G_1 arrest.

Matrix metalloproteinase (MMP)-2 is an important molecule involved in cancer cell migration to enable metastasis from the primary tumor [33]. It was found that R2-8018 and M3R KO decreased the level of MMP-2 (Fig. 7B), demonstrating the repression of the migration in H1299 cells.

4. Discussion

Accumulated studies identified that ACh is a pivotal molecule not only as a neurotransmitter in the central and peripheral nervous systems but also as a ligand acts with acetylcholine receptor (AChR), leading to the activation of the downstream signaling pathways in tumor cells [11,34–36]. Our previous study showed that R2HBJJ, a novel muscarinic antagonist, inhibits NSCLC proliferation and arrests the cell cycle in G_0/G_1 [10]. In the present study, another novel muscarinic antagonist R2-8018, which was designed and synthesized by our institute, was used to further elucidate the mechanisms underlying the involvement of M3R in NSCLC proliferation and migration. The current work showed that R2-8018 significantly inhibited the cell viability (Fig. 2A), process of cell cycle (Fig. 2C, D), and the migration (Fig. 2E) of H1299 cells, all in a concentration-dependent manner. Moreover, M1R selective antagonist VU did not exert any effects on H1299 cells (Fig. 2B) and R2-8018 lost its ability to inhibit cell proliferation and migration of H1299 after knocking out of the M3R (Fig. 3), indicating that R2-8018 displays its function predominantly through M3R in

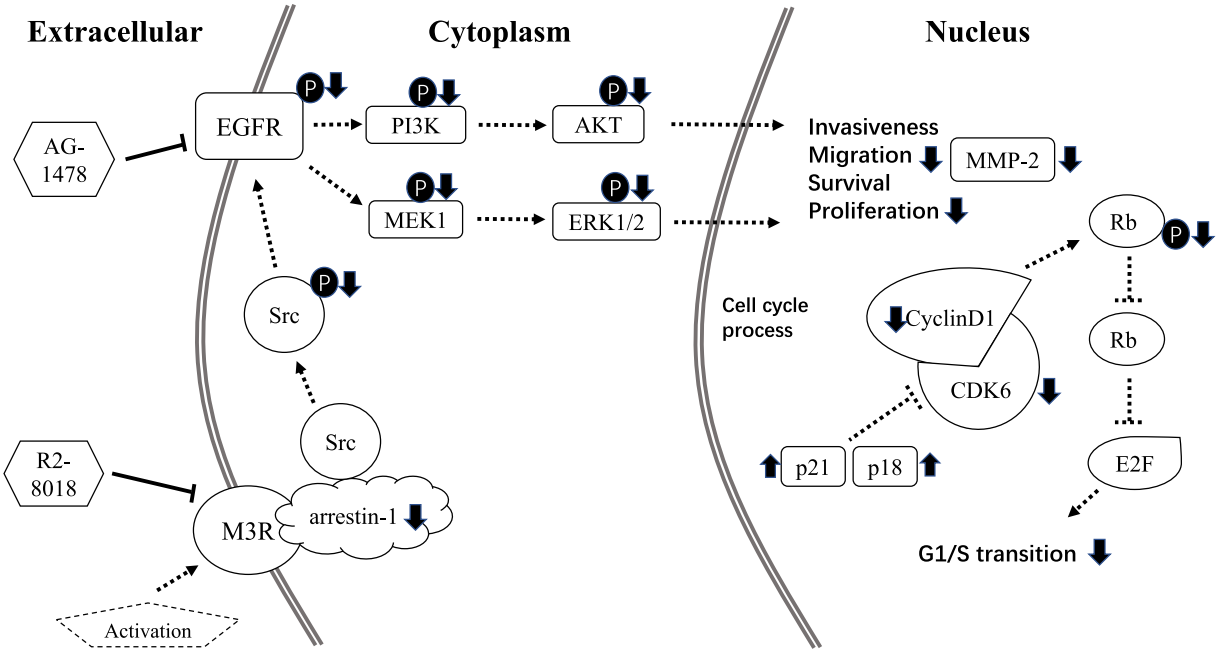

Fig. 8. Model depicting mechanism underlying M3R antagonized by R2-8018 (solid line, black filled arrow) and activation of M3R (dash line/arrow) on the proliferation and migration of NSCLC cells. In H1299 cells, M3R activation regulates the expression and activation of β-arrestin-1 and c-Src. The β-arrestin-1 and c-Src are able to link signals from M3R to EGFR. Phosphorylation of EGFR results in the activation of following mitogenic pathways, including MEK/ERK and PI3K/AKT signaling pathways. Finally impress the cell cycle progression and migration of NSCLC cells. This study demonstrated that the M3R antagonist R2-8018 can attenuate the biological process described above, ending up with the repression of cell proliferation and migration by which cell cycle transition (G1-to-S) and MMP-2 are both suppressed.

H1299 cells.

M3R is a type of GPCR that is also referred to as the seven-transmembrane spanning receptor, which has approximately 1000 members in humans and constitutes the largest family of cell surface proteins [37]. GPCR is one of the most important and most intensively studied drug targets. Historically, GPCRs signaling was thought to be activated exclusively by G proteins and then terminated by GPCRs kinase phosphorylation and β-arrestin binding [38]. However, it is now generally accepted that many GPCRs signals also transmit via other transduction mechanisms including biased activation, intracellular activation, dimerization activation, biphasic activation, and transactivation [39]. Increasing data demonstrated that GPCRs-induced transactivation of EGFR represents a major mechanism in various cancers [11,34,35,40]. EGFR is a critical receptor that controls a wide variety of biological responses, including cell proliferation, differentiation, migration, and apoptosis [41,42]. It is clear that EGFR family members are overexpressed in lung cancer [43]. GPCRs transactivation of EGFR amplified downstream signaling and the ensuing effects on cell function. Results in Fig. 5 showed that the phosphorylation of EGFR in H1299 cells is sharply decreased by R2-8018 antagonism or knocking out of M3R gene (H1299-M3RKO). Coincubation of AG-1478 and R2-8018 notably reduces the proliferation and migration of H1299 (Fig. 4) and the two has a synergistic interaction effect statistically, these results strongly identified the transactivation of EGFR.

It is now evident that β-arrestin binding, which terminates the GPCRs coupling, also initiates a positive signal transduction where β-arrestin functions as an adapter to connect GPCRs and Src [26,27,44,45]. In terms of arrestins, this study identified that β-arrestin-1, not β-arrestin-2, is involved in the process of M3R signals transduction. The level of β-arrestin-2 after R2-8018 treatment did not alter in this study, and Kallifatidis [46] proved that β-arrestin-2 is a negative regulator of mitogenic signaling in cancer. Previous data in tumor cells demonstrated that c-Src is activated by GPCRs with the help of β-arrestins, finally linking GPCRs activation to the EGFR downstream pathways [12,41,47,48]. c-Src is a non-receptor tyrosine protein kinase and a transducer of transmembrane signals eliciting from many different receptor proteins, including EGFR. c-Src is identified overexpressing in various tumors and has a strong link with EGFR. The current work demonstrated that M3R-mediated EGFR transactivation may probably by c-Src, in which scaffold protein β-arrestin-1 is involved. However, regrettably, in this part, what we have done still leaves much to be desired. Specifically, it remains to be elucidated that if β-arrestin-1/Src affects the transactivation of EGFR by combination with each other, and in which way c-Src influences the phosphorylation of EGFR [12]. Perfecting the undone work above will bring advantage to the clear understand of M3R-induced EGFR transactivation as well as the identification of new targets in NSCLC.

Efforts to explore signaling mechanism of ACh in promoting tumorigenesis and metastasis uncovered that it could transactivate the EGFR signaling via M3R to induce the phosphorylation of ERK1/2 and AKT in various tumor cells [11,40,49,50]. However, little is known about the role of ACh and M3R in NSCLC. It is found in this study that M3R antagonist R2-8018 inhibited both the MEK/ERK1/2 and PI3K/AKT pathways, and inhibition of AKT leading to the declined inhibition of GSK-3β in the previously study [24], finally caused a suppression of the cell cycle in NSCLC cells. It was in line with the results in the flow cytometric assay. These results indicated that MEK/ERK1/2 and PI3K/AKT signaling pathways post-EGFR activation are highly involved in the process of tumor growth (Fig. 8). In addition, since many other proteins are also detected changed in the antibody array (Supplementary materials, Table S1.), it can be sure that some of these other proteins, if not all, are participated in NSCLC progression. Therefore, wider researches into these proteins are worthy to be done to gain more comprehensive understanding of molecules involved in NSCLC.

Different from the previous work that demonstrated activation of M3R by ACh promotes non-small cell lung cancer cell proliferation and invasion via EGFR/PI3K/AKT pathway [11], present work demonstrated that M3R induced EGFR transactivation by a novel muscarinic antagonist R2-8018. Beyond our expectation, what is entirely contrary to [11] is that present work did not show the proliferation promotion

effects after exogenous Ach and Carbachol treated on NSCLC cells, merely induced an increase in intracellular calcium levels (Supplementary materials, Fig. S2). Different investigators in our lab tried many times using different methods, but turned out the same negative results (Supplementary materials, Fig. S2). There may be two possible reasons for this, one of which is that M3R is probably associated with constitutive activation [50]; the other is perhaps M3R is autocatalyzed by itself or by interactions with other proteins or receptors [39]. And in addition to PI3K/AKT pathway, current work also identified that another post EGFR pathway MEK/ERK is involved in the effects of M3R on NSCLC. Furthermore, we investigated the molecular mechanism of the cell cycle progression and migration of NSCLC cells. And it is particularly noteworthy that current work proved that β-arrestin-1 and c-Src is able to link signals from M3R to EGFR, which is of great importance because EGFR is a critical receptor that controls a wide variety of biological responses and is overexpressed and overactivated in NSCLC. These findings will definitely facilitate the design of therapeutic strategy in NSCLC.

It should be noted that this study only assessed the M3R on NSCLC in vitro, and nude mice experiment in vivo is required to validate the in vivo activity of M3R on NSCLC. Notwithstanding this limitation, this study does suggest that the novel M3R antagonist, R2-8018, can reduce the proliferation and migration of non-small cell lung cancer by disturbing transactivation between M3R and EGFR, with c-Src and β-arrestin-1 involved as the signaling intermediator, and then a series of postEGFR pathways are repressed.

In summary, current findings of specific signaling pathways involved in post-EGFR transactivation will facilitate the identification of new biomarkers for molecular targeting strategies in NSCLC. We anticipate subsequent work will focus on the internal activity of M3R and the role of M3R in NSCLC in vivo.

5. Conclusion

In conclusion, the present study suggests that R2-8018 can reverse the activation of the acetylcholinergic M3R system to deactivate EGFR thus suppressing downstream signaling pathways to ultimately retarding NSCLC cell proliferation and migration. These findings provide further insight into NSCLC carcinogenesis, and suggest combination treatment strategy simultaneously targeting M3R and EGFR may be needed for NSCLC therapy.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116742.

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