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Mammalian Target of Rapamycin (mTOR) Regulates Transforming Growth Factor-β_1 (TGF-β_1)-Induced Epithelial-Mesenchymal Transition via Decreased Pyruvate Kinase M2 (PKM2) Expression in Cervical Cancer Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Epithelial-mesenchymal transition (EMT) plays an important role in cancer tumorigenesis. Transforming growth factor β_1 (TGF-β_1) can induced EMT, which could increase tumor migration and invasion. Moreover, recent studies have been proven that mammalian target of rapamycin (mTOR) is a critical regulator of EMT. We investigated the mechanisms of mTOR in transforming growth factor β_1 (TGF-β_1)-induced EMT in cervical cancer cells.

Material/Methods: HeLa and SiHa cells were treated with 10 ng/ml TGF-β_1 to induce EMT. Then, they were treated with or without rapamycin. CCK8 assay was performed to determine cell proliferation. Cell migration was detected by wound-healing assay; apoptosis was analyzed by flow cytometry; mTOR inhibitors inhibited mTOR pathway to assess the expression of E-cadherin, Vimentin STAT3, Snail2, p-p70s6k, and PKM2 expression.

Results: TGF-β_1 promoted proliferation and migration, and attenuated apoptosis in cervical carcinoma cells. Rapamycin abolished TGF-β_1-induced EMT cell proliferation and migration and reversed TGF-β_1-induced EMT. E-cadherin were suppressed, whereas Vimentin and PKM2 were increased in HeLa and SiHa cells after stimulation with TGF-β_1. Moreover, mTOR was activated in the process of TGF-β_1-induced EMT. Rapamycin inhibited the phosphorylation of p70s6k. Furthermore, inhibition of the mTOR pathway decreased PKM2 expression.

Conclusions: Inhibition of the mTOR pathway abolished TGF-β_1-induced EMT and reduced mTOR/p70s6k signaling, which downregulated PKM2 expression. Our results provide novel mechanistic insight into the anti-tumor effects of inhibition of mTOR.

MeSH Keywords: Uterine Cervical Neoplasms • Substance Abuse Treatment Centers • Biosynthetic Pathways

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Background

Epithelial-mesenchymal transition (EMT) plays an important role in cancer tumorigenesis. During the EMT, epithelial cells change their polarized epithelial phenotype to a spindle-shaped, myofibroblast-like phenotype with high motility [1], and cancer cells increased cell migration and invasion, which are crucial to cancer prognosis [2]. The partial loss of E-cadherin is generally accepted as a hallmark of the EMT [3], which reduces cell-cell adhesion and destabilizes the epithelial architecture. Moreover, Vimentin bestows a motile phenotype to cancer cells through changes in cellular architecture and cell-matrix interactions [4,5]. Further, many transcription factors, such as SNAIL, act as repressors of E-cadherin [6] and have been linked to the induction of the EMT under different cellular contexts. Signal transducer and activator of transcription 3 (STAT3) is also involved in EMT by regulating the transcriptional regulators of E-cadherin [7].

Large studies indicated that the EMT was induced by transforming growth factor β_1 (TGF-β_1) in various cancer cells [8,9]. Yin et al. reported that TGF-β_1 was overexpressed in anaplastic thyroid cancer, and they found that knockdown of TGF-β_1 could inhibited cell proliferation and colony formation, and promoted apoptosis in anaplastic thyroid cells [10]. Park et al. found that TGF-β_1 promotes cancer immune escape, and that it promoted cell proliferation, colony formation, and inhibited apoptosis [11].

TGF-β is known to activate Akt through PI3K, which in turn activates the mTOR complex 1 (mTORC1) [12]. The activation of mTORC1 affects cell growth, proliferation, and invasion by modulating protein synthesis through its downstream effector, eukaryotic translation initiation factor p70 S6 kinase [13]. mTORC1 consists of mTOR, while rapamycin was acutely able to inhibit it [14,15]. Moreover, mutations or overactivation of mTOR lead to persistent proliferation and tumor growth [16]. Recently, studies reported that sorafenib combined with a mammalian target of rapamycin (mTOR) inhibitor was a more effective and tolerable treatment strategy for advanced HCC [17]. Inhibition of the mTOR pathway reduced migration and invasion ability, and knockdown of mTORC1 induced mesenchymal-epithelial transition [18]. However, how mTOR signaling modulates EMT is unclear.

Recent studies showed that PKM2 (pyruvate kinase M2) is needed to induce EMT [19]. PKM2 is normally not expressed in adult tissues, but is reactivated in tumor tissues. PKM2 controls the final rate-limiting step of glycolysis and is an alternatively spliced variant of the PKM gene [20,21]. Moreover, PKM2 is a crucial glycolytic enzyme in the oncogenic mTOR-induced Warburg effect, in which hypoxia inducible factor-1α (HIF-1α) and c-Myc-hnRNP cascades are the transducers of mTOR regulation of PKM2 [22]. In addition, a study reported that loss of SNAIL inhibits cellular growth and metabolism through the miR-128-mediated p70s6k/PKM2 signaling pathway [23]. These studies showed there has always been a close association between mTOR and PKM2. Furthermore, Atsushi et al. found that PKM2 knockdown failed to induce spindle-shaped morphological changes, and hindered E-cadherin reduction and VIM increase compared with the control group [19]. We infer that the mTOR/p70s6k/PKM2 pathway can regulate the EMT state.

In this study, we hypothesized that the mTOR/p70s6k/PKM2 pathway is involved in regulating TGF-β_1-induced EMT. We investigated how the mTOR inhibitor regulated EMT, and explored the mechanisms of tumor suppression. Our data show that EMT was reversed by rapamycin, an mTOR inhibitor. mTOR inhibition decreased phosphorylation of p70s6k and reduced PKM2 expression. Our findings suggest that inhibition of mTOR/p70s6k/PKM2 signaling promotes cervical tumor suppression.

Material and Methods

Cell culture

Cervical cancer cell lines (HeLa, SiHa) (American Type Culture Collection, Manassas, VA) were grown in DMEM, supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C with 5% CO_2. Cells were treated with or without 10 ng/ml TGF-β_1 (PeproTech, USA) in a serum-free medium for 48 h. Cells were incubated with 0, 25, 50, and 100 nM of rapamycin (CST, Danvers, MA).

Cell viability assay

Cell viability was assessed by use of a cell counting kit-8 (CCK-8) assay. Cells were plated in a 96-well plate at 3×10^4 cells/well in triplicates and treated with or without rapamycin and TGF-β_1 for 24, 48, and 72 h. Ten µL CCK-8 was added to each well and incubated for 1 h at 37°C. The absorbance was measured at 450 nm. Cell viability was calculated by the following formula: Cell viability (%) = (OD treatment – OD blank)/(OD control – ODblank) ×100%.

Annexin V-FITC apoptosis assay

Cells were seeded in 6-well plates at 4×10^5 cells/well and then treated with 50 nM rapamycin with or without 10ng/ml TGF-β_1 for 24 h. Apoptotic cells were detected by flow cytometry using an Annexin V-FITC kit according to the kit instructions.

Migration assay

Cells were seeded in 6-well plates, and were cultured with fresh serum-free medium containing TGF-β_1 with or without

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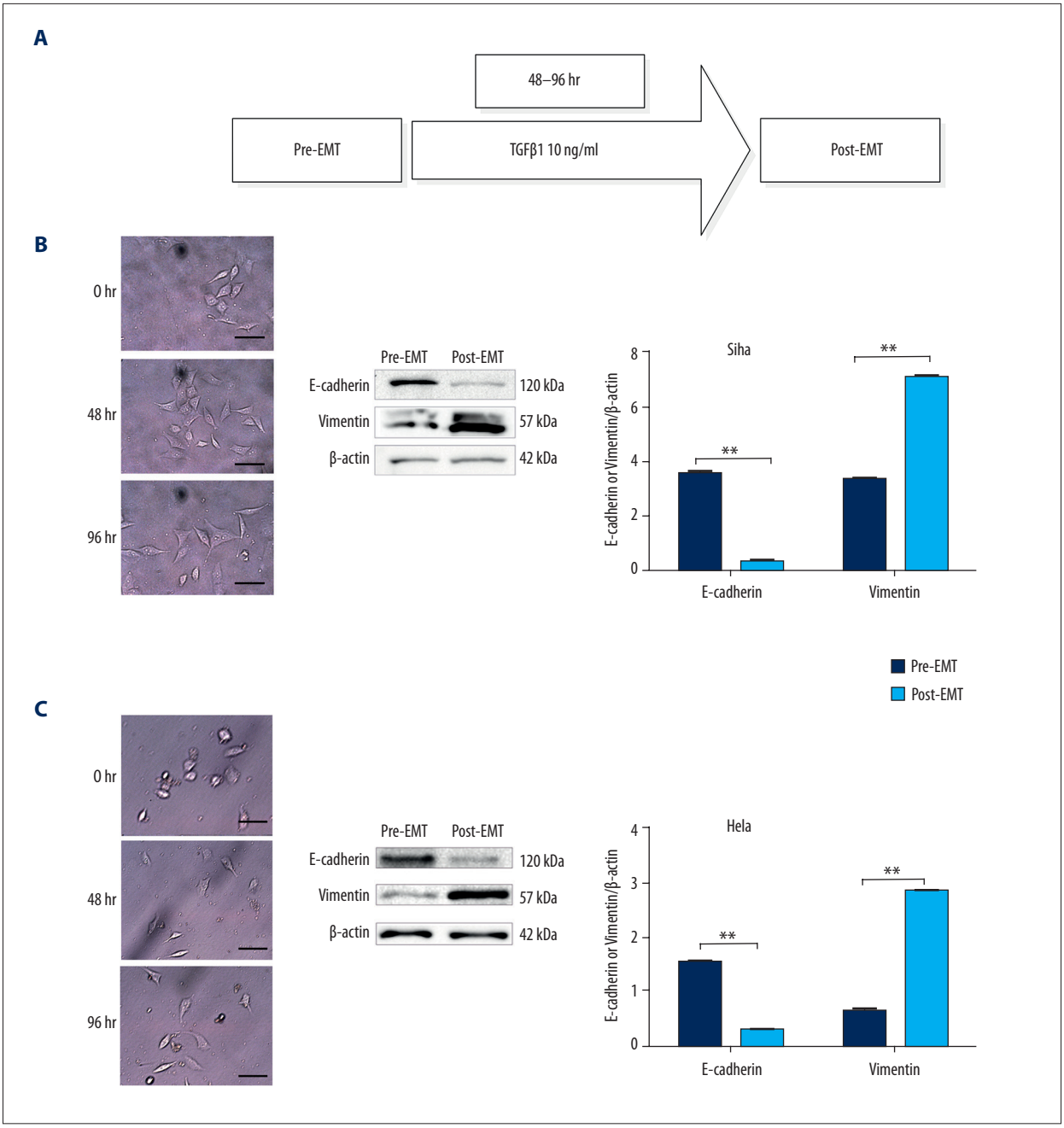

Figure 1. TGF-β_1 induces EMT in cervical cancer cells. (**A**) Schematic representation of the procedure for EMT induction. The cells incubated for 48 h after seeding are defined as pre-EMT, and the cells treated with 10 ng/mL TGF-β_1 are defined as post-EMT. (**B, C**) cell morphology and marker proteins were change in SiHa (**B**) and HeLa (**C**) cells. (Scale bar, 50 μm.) E-cadherin, Vimentin, and β-actin are shown by Western blot between the pre-EMT and post-EMT condition.

the indicated concentration of rapamycin for 24 h. Cell monolayers were wounded by scratching with sterile plastic 200 μl micropipette tips and photographed using phase-contrast microscopy. The migration distance of each cell was measured after the photographs were converted to Photoshop files. The migration index was quantified by (scratch distance at 0 h – scratch distance at 24 h)/scratch distance at 0 h.

Western blot analysis

The cells were harvested by centrifugation and washed with PBS, and then they were lysed in RIPA buffer containing protease inhibitors. Equal amounts of the protein lysates were electrophoretically separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked

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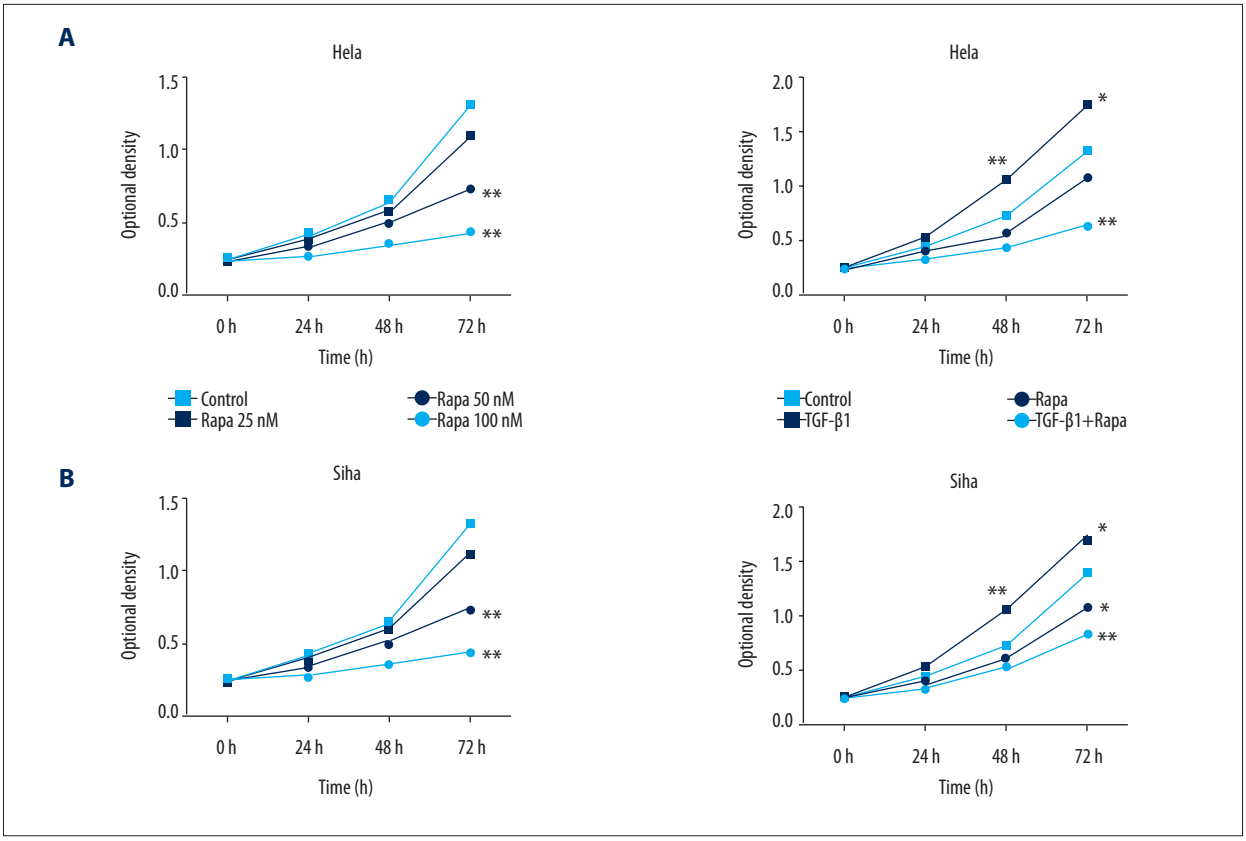

Figure 2. Rapamycin inhibits cell viability. HeLa (**A**) and SiHa (**B**) cells were treated with rapamycin (0-50 nM) for 24, 48, or 72 h, with or without 10 ng/ml TGF-β_1. Cell numbers were measured by CCK-8 assays at indicated times. Data are means±S.D. Rapa: rapamycin; * p<0.05, ** p<0.01 *vs.* untreated cells.

with 5% nonfat milk and then incubated overnight at 4°C with the primary antibodies: Sanil2, STAT3, phosphor-p70s6k, E-cadherin, Vimentin, and β-actin, which were purchased from CST. After incubation with the secondary antibody for 1 h at room temperature, the protein bands were detected using the ECL detection system (BD Biosciences). β-actin was used as the loading control.

Statistics

The statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL). The values are expressed as the means ±SD. The differences between the 2 groups were determined by the 2-tailed *t* test. A *p* value <0.05 was considered statistically significant.

Result

TGF-β_1 induces EMT in cervical cancer

To detect whether TGF-β_1 induced EMT, we used 10 ng/ml TGF-β_1 to stimulate HeLa and SiHa cells for 48 h. We observed that SiHa (Figure 1B) and HeLa cells (Figure 1C) cells became scattered, acquired a spindle-shaped morphology, and lost cell-cell contacts, which are characteristics of a mesenchymal-like morphology. Moreover, E-cadherin expression was abundant in the absence of TGF-β_1 (pre-EMT). When stimulated with 10 ng/ml TGF-β_1, E-cadherin was markedly decreased (post-EMT). In contrast, when stimulated by TGF-β_1, Vimentin increased (post-EMT) compared with the pre-EMT state. These changes in cell morphology and marker proteins indicate that EMT was induced when SiHa and HeLa cells were stimulated with 10 ng/ml TGF-β_1.

Rapamycin inhibits TGF-β_1-induced proliferation and migration, and induces apoptosis in cervical carcinoma cells

We evaluated the effect of inhibition of mTOR pathway on suppressing proliferation of cervical carcinoma cells. We used Cell Counting Kit-8 (CCK-8) assays to determine the effects of rapamycin (an mTOR inhibitor) with or without TGF-β_1 on cell proliferation. Cells were treated with 0, 25, 50, and 100 nM rapamycin, and 50 nM rapamycin with or without 10 ng/ml TGF-β_1. As shown in Figure 2, rapamycin inhibited the

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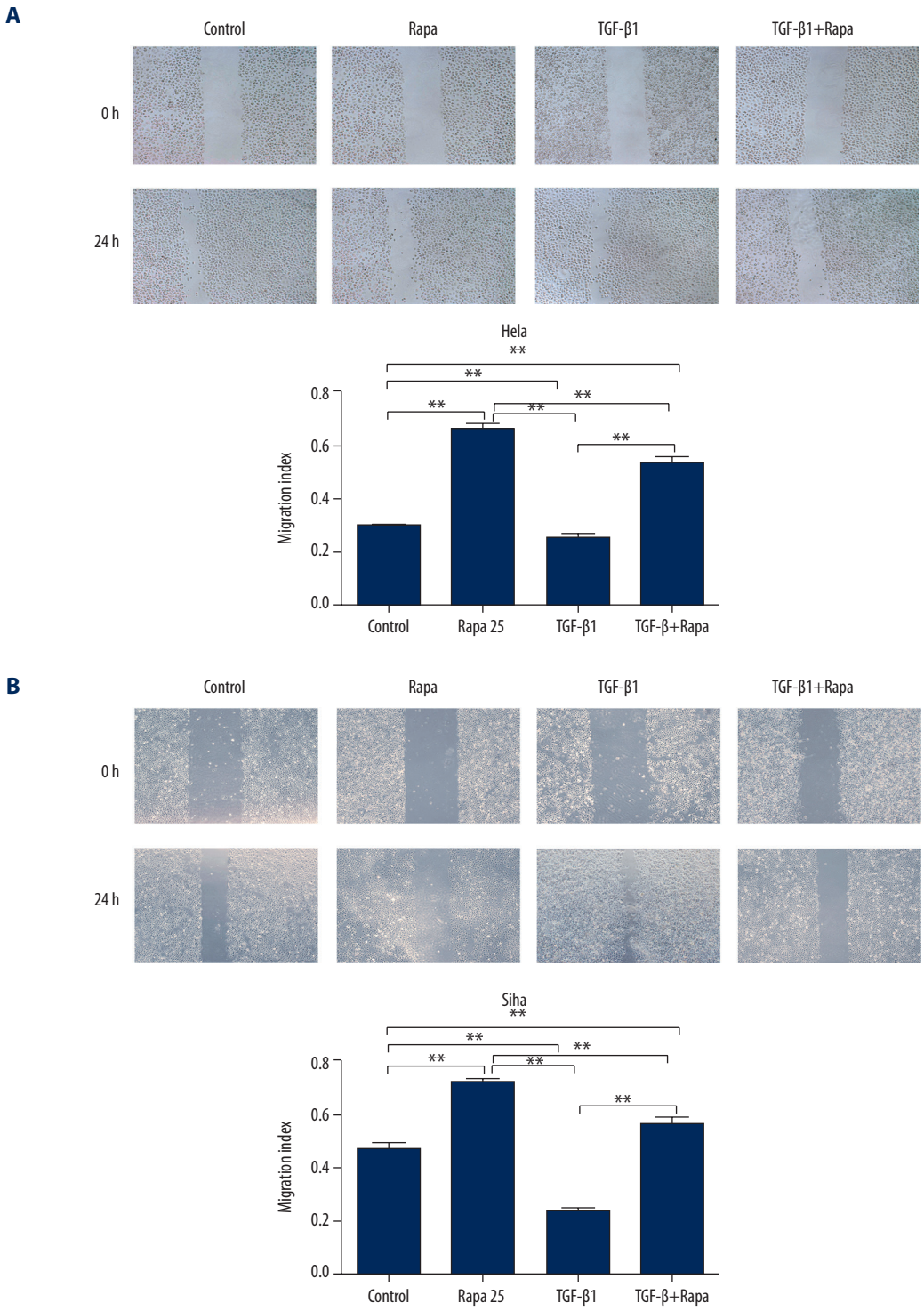

Figure 3. Wound-healing assays. Rapamycin 50 nM with or without 10 ng/ml TGF-β_1 treated HeLa (**A**) and SiHa (**B**) cells for 48 h. Representative images were obtained at 40× magnification. Graphs show the relative migration distance after 24-h incubation. Data are means ±S.D. Rapa: rapamycin; * $p<0.05$, ** $p<0.01$ *vs.* untreated cells.

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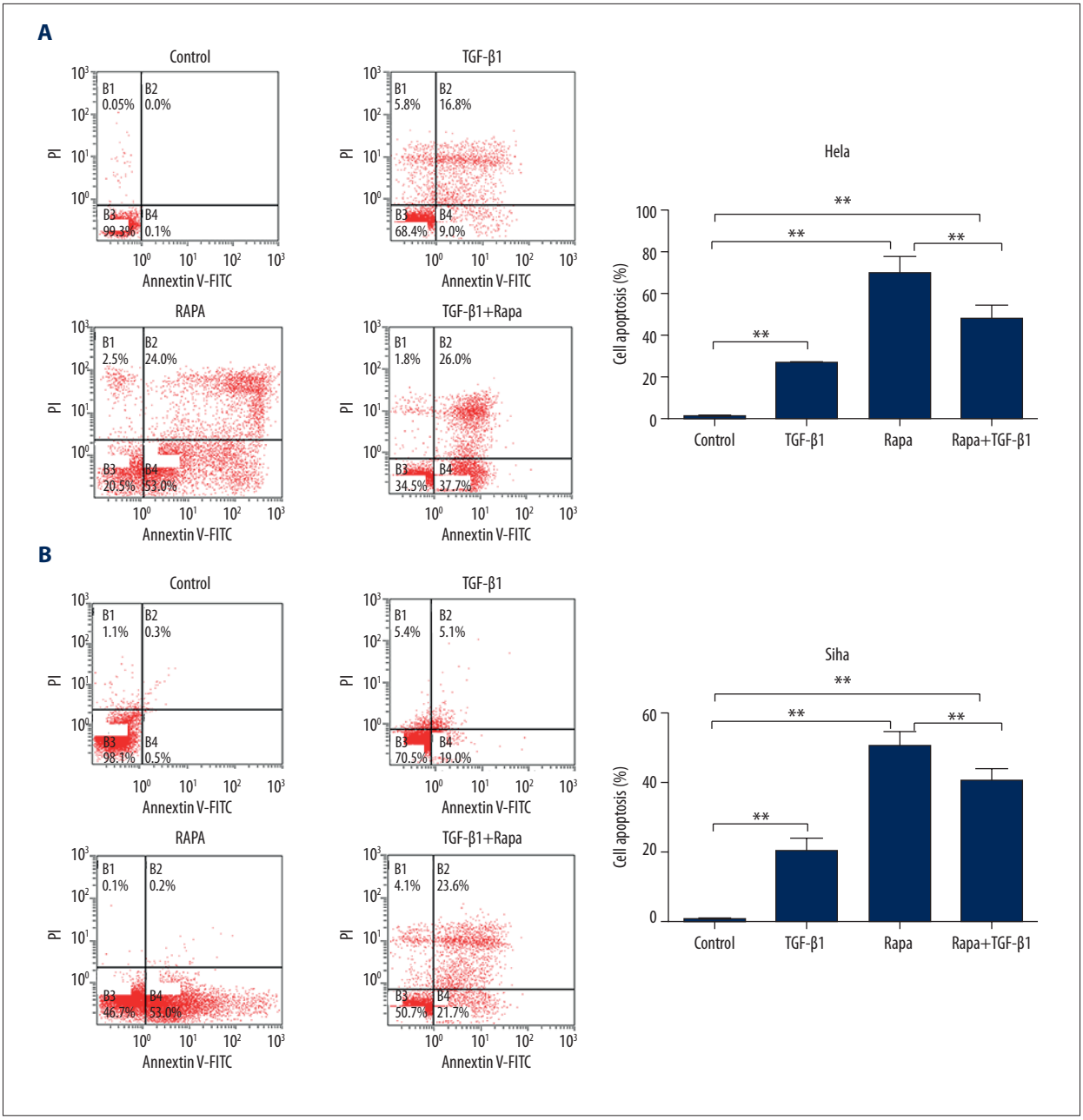

Figure 4. Rapamycin induces and apoptosis. Rapamycin 50 nM with or without 10 ng/ml TGF-β_1 treated HeLa (**A**) and SiHa (**B**) cells for 48 h. The cells were stained with PI and FITC-labelled Annexin V and subsequently underwent flow cytometry analysis to determine the percentage of apoptotic cells. Data are presented as the mean ± SD of 3 replicates per group. Rapa: rapamycin;* p<0.05, ** p<0.01 *vs.* untreated cells.

proliferation of in HeLa cells in a dose-dependent manner. Treatment with TGF-β_1 significantly increased the proliferation of both cell lines at 48 and 72 h, which was abolished by the addition of rapamycin (Figure 2A). The same was true in SiHa cells (Figure 2B).

The migration of cells was determined using wound-healing assays. Cells were treated with 50 nM rapamycin with or without 10 ng/ml TGF-β_1. Rapamycin significantly decreased the migration of HeLa cells. TGF-β_1 significantly increased cell migration in HeLa cell lines at 24 h, which was abolished by the addition of rapamycin (Figure 3A). Similar data were obtained from the wound-healing assays of SiHa cells (Figure 3B).

To evaluate the effect of inhibition of mTOR for antagonizing the anti-apoptosis effect of TGF-β_1 on cervical carcinoma

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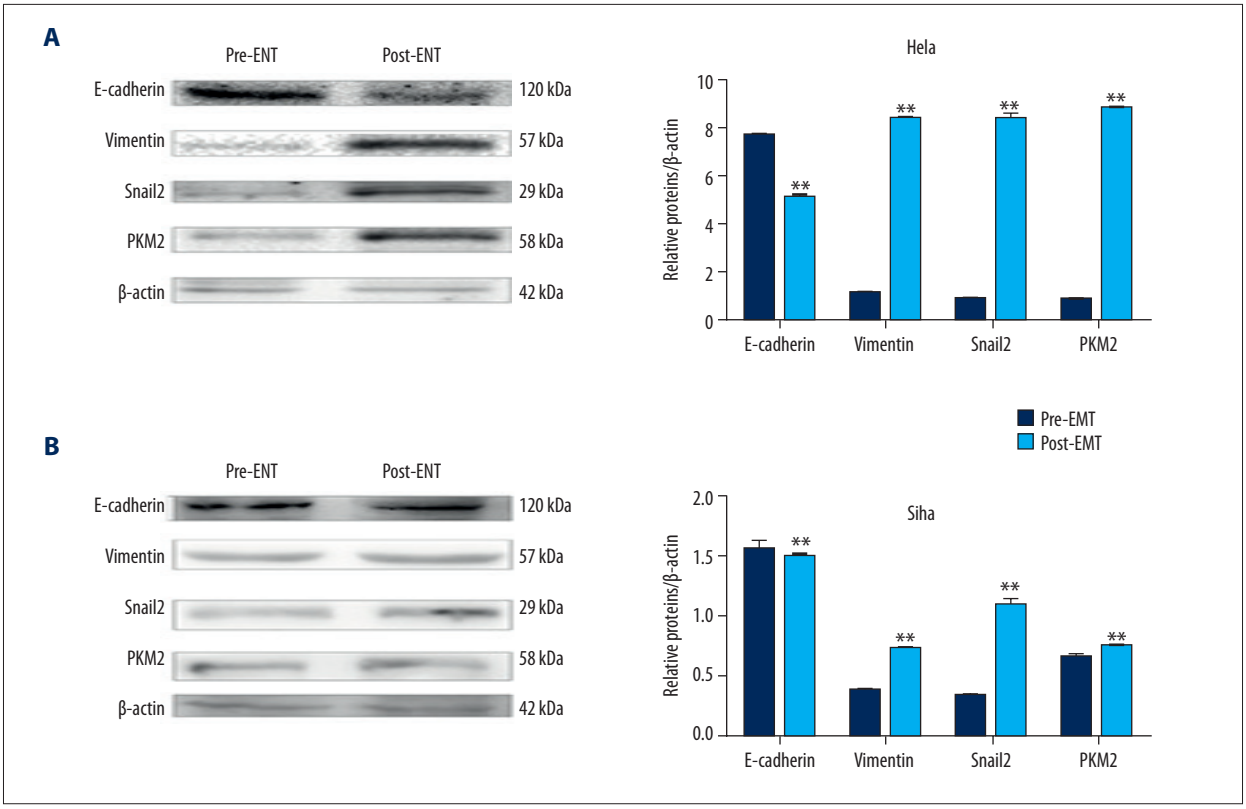

Figure 5. PKM2 expression was increased during the EMT condition. (**A**) HeLa and (**B**) SiHa cells were assessed for PKM2, E-cadherin, Vimentin, and Snail2 expression by Western blot in the pre- and post-EMT state. Post-EMT cells were harvested at 72 h. The data are presented as the mean ± SD of 3 replicates per group. * p<0.05, ** p<0.01 *vs.* untreated cells.

cells by Annexin V-FITC and PI staining, we assessed the effect of rapamycin with or without TGF-β_1 on the apoptosis of HeLa (Figure 4A) and SiHa (Figure 4B) cells. When cells were treated with TGF-β_1, the total number of apoptotic cells (early apoptotic+apoptotic) was significantly decreased compared to untreated cells in both cell lines. In cells treated with 50 nM rapamycin, the apoptosis rate significantly increased. Furthermore, the addition of rapamycin significantly abolished the TGF-β_1-induced anti-apoptosis effects in both cell lines.

The EMT condition induced an increase in PKM2

To determine whether the EMT condition induces an increase in PKM2, PKM2 expression was detected to compare levels pre- and post-EMT state. Cervical cancer cells were stimulated with 10 ng/ml TGF-β_1. As showed in Figure 1, SiHa (Figure 1B) and HeLa (Figure 1C) cells changed morphology from epithelial to fibroblastic-like and spindle-shaped and lost cell-cell contacts, which are characteristics of a mesenchymal-like morphology. Western blot was used to analyze the maker proteins. We found that the level of E-cadherin expression was suppressed compared with the pre-EMT state. Vimentin and SNAIL family zinc finger 2 (SNAI2) expressions were increased in the post-EMT state (Figure 5). PKM2 expression was stimulated to increase in the post-EMT condition (Figure 5). The data show that the induction of EMT resulted in a decreased level of E-cadherin, and increased VIM and PKM2 expression in HeLa (Figure 5A) and SiHa cells (Figure 5B). Moreover, Atsushiet et al. reported that they were able to knock down PKM2 under EMT conditions, but PKM2 knockdown failed to induce spindle-shaped morphological changes. Meanwhile, PKM2 knockdown hindered E-cadherin loss and VIM gain compared with the control [19]. Therefore, we confirmed that PKM2 expression was increased during the EMT condition.

mTOR is involved in regulation of PKM2 via stimulating mTOR/p70s6k/signaling

Next, we investigated whether the mTOR pathway affects PKM2 expression. We evaluated the effects of rapamycin (an mTOR inhibitor) on PKM2 (a critical glycolytic enzyme), and p70s6k (S6K1, a downstream effector of mTOR). To investigate the inhibitory effect on mTOR, we used 0, 12.5, 25, 50, and 100 nM rapamycin to treat HeLa (Figure 6A) and SiHa (Figure 6B) cells for 24 h. Ribosomal p70 S6 kinase (S6K1) is a main downstream mTOR effector. As shown in Figure 6, rapamycin, a specific mTOR inhibitor, inhibited the phosphorylation of p70s6k in a dose-dependent manner. Moreover, we investigated whether

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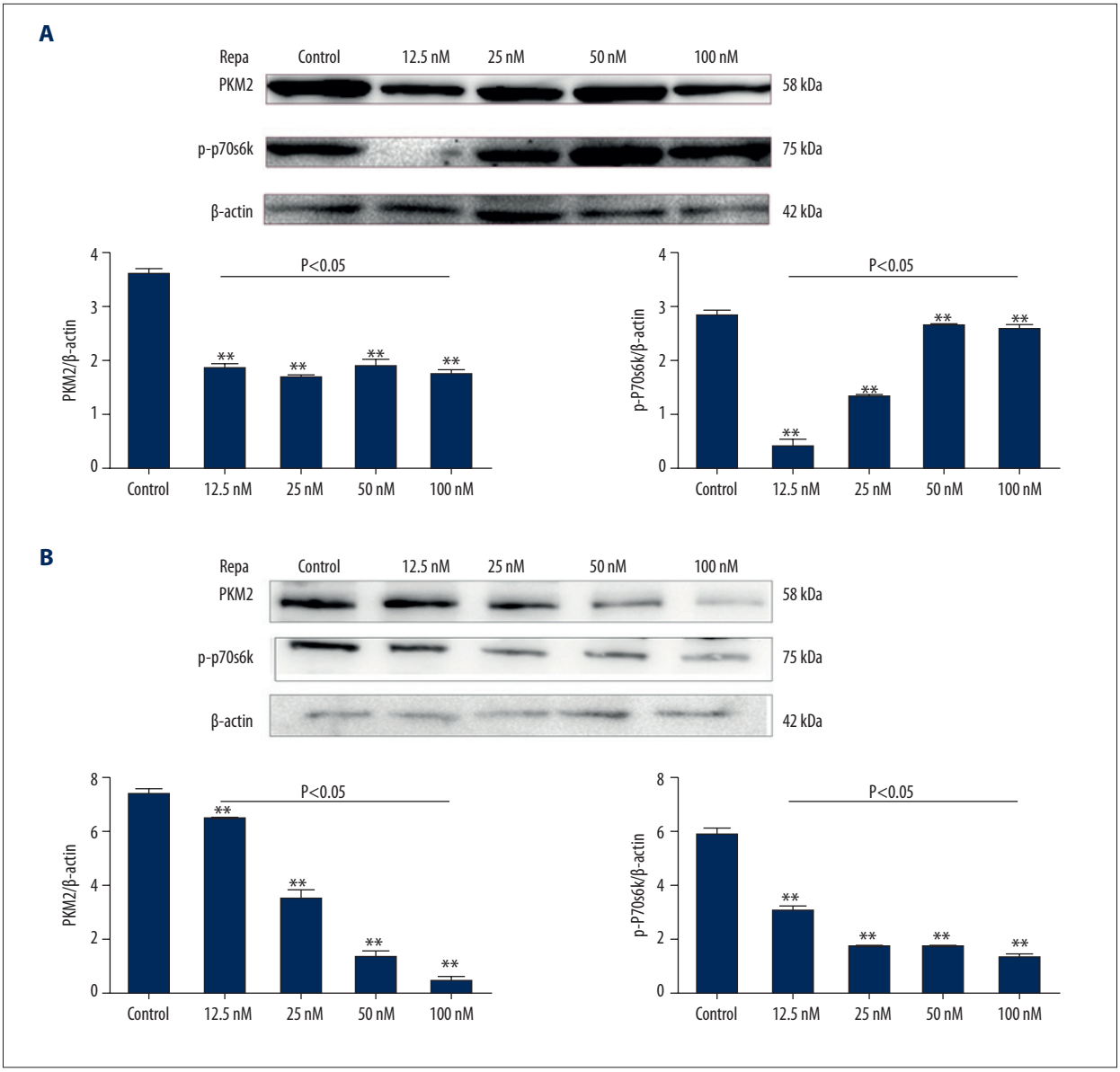

Figure 6. mTOR involving the regulation of PKM2 via stimulating mTOR/p70s6k/signaling. (**A**) Hela and (**B**) Siha cells were investigated p-p70s6k and PKM2 expression by Western blot, when SiHa and HeLa cells were treated with rapamycin.

mTOR/p70s6k signaling decreased PKM2 expression, which is a main downstream p70s6k effector. Rapamycin was added to cell cultures. We found that rapamycin treatment significantly decreased PKM2 in HeLa (Figure 6A) and SiHa (Figure 6B) cells. Based on these facts, inhibition of mTOR appears to affect cervical cancer through the mTOR/p70s6k/PKM2 pathway in cervical cancer cells.

Rapamycin reverses EMT in cervical carcinoma cells via regulation of mTOR/p70s6k/PKM2 signaling pathways

To determine whether rapamycin is involved in regulating EMT in cervical cancer, we examined the expression of EMT-related markers using Western blot analysis. TGF-β_1 significantly decreased the expression of E-cadherin and increased the expressions of Vimentin, STAT3 and Snail2 in HeLa (Figure 7A) and SiHa cells (Figure 8A). In addition, at concentration of 50 nM rapamycin reversed TGF-β_1-induced EMT-markers expression by repressing Vimentin, STAT3 and Snail2 expressions and restoring E-cadherin expression in in HeLa (Figure 7A) and SiHa cells (Figure 8A).

Next, we explored the possible that signaling pathways may be involved. As shown in Figures 7 and 8, we found that TGF-β_1 significantly increased the phosphorylation of p70s6k, which is the main downstream signaling intermediate of mTOR signaling. Simultaneously, TGF-β_1 significantly increased the expression

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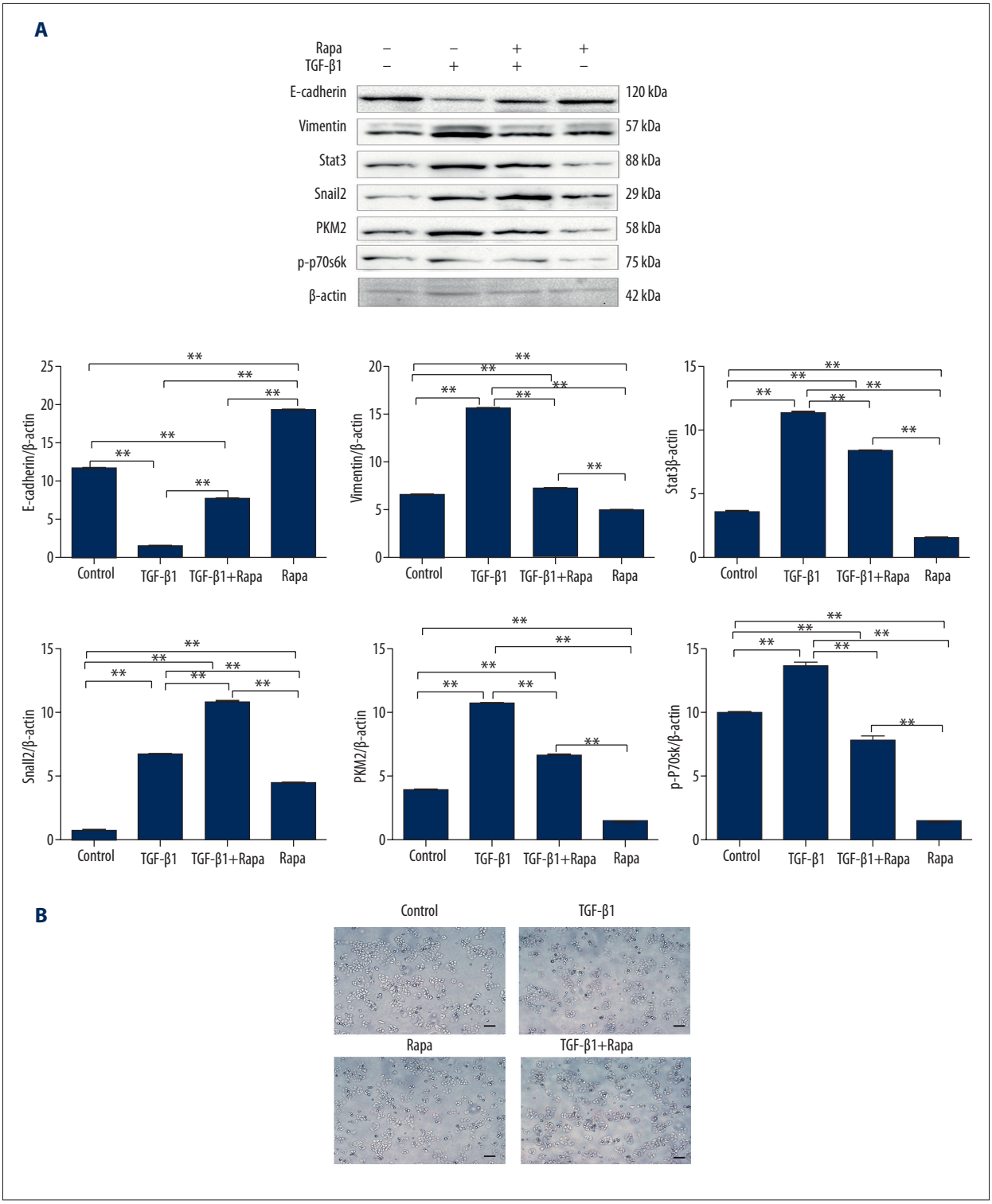

Figure 7. Rapamycin reverses TGF-β_1-induced EMT in HeLa cells involved in mTOR/p70s6k/PKM2 signaling pathways. (**A**) Cells were treated with TGF-β_1, rapamycin, or both agents for 48 h. The protein expression of E-cadherin, SNAIL, STAT3, Vimentin, PKM2, p-p70s6k, and β-actin were assessed by Western blot. β-actin was used as a loading control. (**B**) The morphology of HeLa cells treated with TGF-β_1, rapamycin, or both agents for 48 h. The cells were observed using phase-contrast microscopy at 200× magnification. Scale bar: 100 μm. The data are presented as the mean ±SD of 3 replicates per group. Rapa: rapamycin. * p<0.05, ** p<0.01 *vs.* untreated cells.

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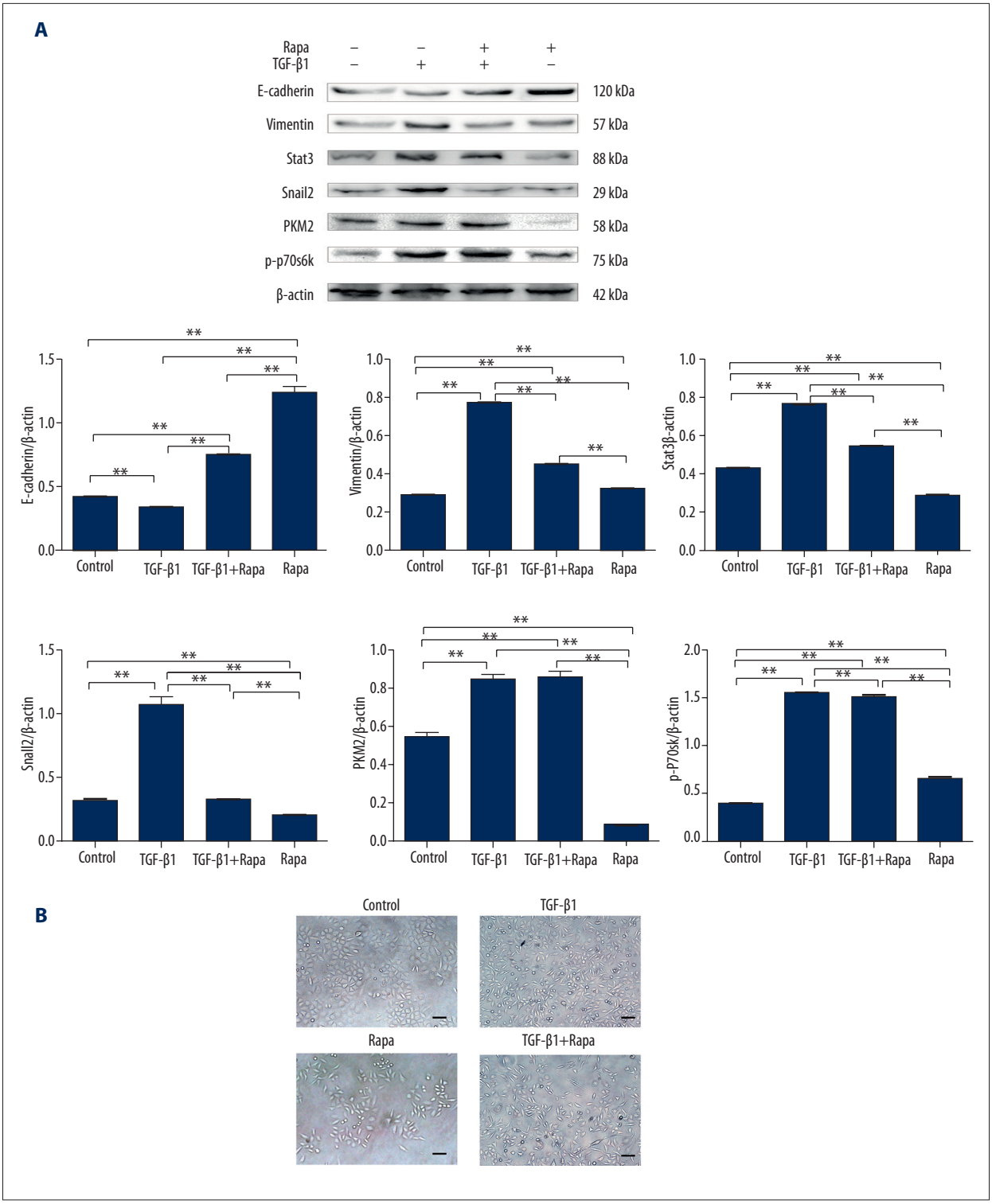

Figure 8. Rapamycin reverses TGF-β_1-induced EMT in SiHa cells involved in mTOR/p70s6k/PKM2 signaling pathways. (**A**) Cells were treated with TGF-β_1, rapamycin, or both agents for 48 h. The protein expression levels of E-cadherin, SNAIL, STAT3, PKM2, p-p70s6k, Vimentin, and β-actin were assessed by Western blot. β-actin was used as a loading control. (**B**) The morphology of SiHa cells treated with TGF-β_1, rapamycin, or both agents for 48 h. The cells were observed using phase-contrast microscopy at 200× magnification. Scale bar: 100 μm. The data are presented as the mean ±SD of 3 replicates per group. Rapa: rapamycin. *p<0.05, ** p<0.01 *vs.* untreated cells.

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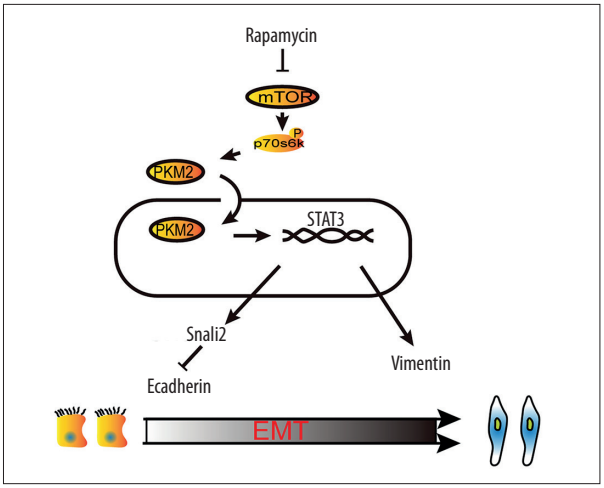

Figure 9. Schematic representation of roles of rapamycin in TGF-β_1-induced epithelial-to-mesenchymal transition in cervical carcinoma cells.

of PKM2 in HeLa (Figure 7A) and SiHa cells (Figure 8A), while rapamycin significantly decreased the expressions of p-p70s6k and PKM2. These results suggest that rapamycin reverses TGF-β_1-induced EMT via the mTOR/p70s6k/PKM2 pathway.

Further, we examined the effect of rapamycin with or without TGF-β_1 on the morphology of HeLa and SiHa cell lines. After stimulation with 10 ng/ml TGF-β_1 for 48 h, both HeLa (Figure 7B) and SiHa cells (Figure 8B) cells became scattered, acquired a spindle-shaped morphology, and lost cell-cell contact, which are characteristics of a mesenchymal-like morphology. Treatment with 50 nM rapamycin for 48 h abolished the TGF-β_1-induced morphological changes in SiHa and HeLa cell lines.

Discussion

We investigated whether the mechanisms of the mTOR pathway affect tumorigenesis in TGF-β_1-induced EMT in cervical cancer cells. Rapamycin, an mTOR inhibitor, can inhibit cell proliferation and migration, as well as promoting apoptosis and reversing TGF-β_1-induced EMT via the mTOR/P70s6k/PKM2 signaling pathway.

Previous studies have reported that the EMT can be triggered by TGF-β_1. Park et al. reported that TGF-β_1 induces lung cancer cell metastasis [24]. Moreover, E-cadherin plays a critical role in maintaining the cell surface and mediating normal epithelial tissue functions [25]. The partial loss of E-cadherin is a fundamental event in EMT [26]. Overexpression of Vimentin in cancer correlates well with accelerated tumor growth and invasion [27]. During EMT, cells start to exhibit a mesenchymal phenotype and show increased Vimentin expression, with high motility rates [28]. As shown in Figure 1, we established an EMT model in cervical cancer cells. Consistent with these observations, our data indicated that TGF-β_1-induced cells acquired a striking morphological change and promoted E-cadherin loss and VIM gain, as well as promoting proliferation and migration, and inhibiting apoptosis caused by EMT.

The mTOR signaling pathway plays an important role in integrating intracellular and extracellular signals, and is a central regulator of cell metabolism, growth, proliferation, and survival [14]. Recent studies reported that using rapamycin derivatives in therapy against liver cancer achieves positive outcomes, leading to the implementation of large clinical trials [29]. Pedro et al. showed that rapamycin (an mTOR inhibitor) inhibits tumor growth in PTEN-negative Ishikawa tumor cells [30]. Our data also showed that inhibition of mTOR significantly decreased cell proliferation, apoptosis, and migration, and reverses EMT in cervical carcinoma cells.

Previous studies found that PKM2 plays a crucial role in EMT development in cancer. When cancer cell stayed in the EMT state, PKM2 knockdown failed to induce spindle-shaped morphological changes, and hindered E-cadherin reduction and VIM increase compared with the control group [19]. Our data show that the induction of EMT resulted in a decrease level of E-cadherin, and increased VIM and PKM2 expression in cervical cancer cells (Figure 5), suggesting that PKM2 is involved in the EMT state.

RPS6KB1(p70s6k) is a key downstream target of mTOR, and an essential target for regulating translation rates and additional metabolic processes for cell growth [12,31,32]. PKM2 is a crucial glycolytic enzyme in the oncogenic mTOR-induced Warburg effect, in which hypoxia-inducible factor-1α (HIF-1α) and c-Myc-hnRNP cascades are the transducers of mTOR regulation of PKM2 [22]. Sun et al. reported that mTOR upregulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. PKM2 level was augmented in mouse kidney tumors and consequent mTOR activation, and was reduced by mTOR suppression [22]. Based on these studies on the interaction of mTOR, p70s6k, and PKM2, we investigated whether mTOR/p70s6k signaling regulates PKM2 in cells in cervical cancer. We treated cells with rapamycin, an mTOR inhibitor. The results showed that the phosphorylation level of p70s6k was inhibited and decreased PKM2 expression, which suggests that the mTOR/p70s6k/PKM2 pathway is involved in regulation of cervical cancer (Figure 6).

TGF-β activates the pathway that connects mTOR with p70s6k [12]. Our data show that TGF-β_1 significantly increases the expression of p70s6k and PKM2 (Figures 7, 8), suggesting the mTOR pathway was activated in the process of TGF-β_1-induced EMT, and PKM2 is involved in the process of TGF-β_1-induced EMT.

Further, we propose that rapamycin exerts its antitumorigenic effects and abolishes TGF-β_1-induced EMT through

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mTOR/p70s6k/PKM2 signaling in cervical carcinoma cells (Figure 9). Rapamycin, a specific mTOR inhibitor, was added to cell cultures to determine whether mTOR regulates TGF-β_1-induced EMT. Our data showed that rapamycin treatment significantly decreased phosphorylation of p70s6k and PKM2 in SiHa and HeLa cells. More importantly, we demonstrated that inhibition of mTOR/p70s6k/PKM2 signaling is the therapeutic target of cervical cancer. Inhibition of mTOR reversed TGF-β_1-induced EMT via the mTOR/p70s6k/PKM2 signaling pathway.

Conclusions

We believe this is the first study showing that inhibition of mTOR reverses TGF-β_1-induced EMT in tumor cells through mTOR/p70s6k/PKM2 pathways. Our data showed that TGF-β_1 induced proliferation and EMT, and rapamycin inhibits cell proliferation and reverses EMT. The mechanism involves suppression of p70s6k activation and reduced PKM2 expression, mediated by inhibiting mTOR/p70s6k signaling, which provides novel mechanistic insights into the anti-tumor effects of mTOR.

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Conflicts of interest

The authors declare no conflicts of interest.

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