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AKT drives sustained motility following MEK inhibition via promoting SNAIL and AXL in MDA-MB-231 LM2

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ABSTRACT

The adaptive activation of alternative signaling pathways contributes to acquired resistance against targeted cancer therapies. Our previous research has shown that blocking Ras/ERK signaling promotes PI3K/AKT signaling in the lung metastatic derivative of MDA-MB-231 (LM2). Because AKT activation was required to drive sustained cell motility following MEK suppression, we extend our research to elucidate how activation of the PI3K/AKT signaling drives sustained motility following MEK inhibition. Reverse phase protein array (RPPA) revealed that SNAIL (*SNAI1*) was upregulated in U0126 (MEK inhibitor)-treated LM2 cells. Importantly, LM2 cells simultaneously treated with U0126 and PI3K inhibitor LY294002 exhibited reduced expression of SNAIL. Furthermore, depletion of *SNAIL* led to reduced cell motility in U0126-treated LM2 cells. In addition, we identified AXL as another downstream effector of AKT. These results suggest that SNAIL and AXL are key factors mediating sustained motility of LM2 cells following MEK suppression. Because AKT mediates motile behavior under MEK suppression, our results suggest that AKT and AXL may be targeted to overcome resistance against drugs targeting the Ras/ERK pathway.

1. Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that does not express estrogen receptor (ER), progesterone receptor (PR), and lacks HER2/neu overexpression [1]. Because TNBC is unresponsive to drugs targeting ER, PR, and HER2, conventional chemotherapy is currently used to treat TNBC. Approximately 15% of breast cancer patients were diagnosed with TNBC. TNBC displays more aggressive behavior and metastasis than other subtypes of breast cancer, resulting in a higher recurrence rate and poor prognosis [2].

Because targeted therapy for TNBC is limited, researchers have tried to identify candidate genes to develop targeted therapy for TNBC. Targeting signaling pathways is one of the strategies for developing targeted therapy against TNBC. Systematic analyses have revealed that TNBC displays elevated Ras/Raf/MEK/ERK and PI3K/AKT pathways compared with other subtypes of breast cancer [3,4]. Therefore, targeting these signaling pathways may provide a clue to develop effective therapy against TNBC.

However, cancer cells can acquire resistance against targeted therapy by bypassing the inhibitory effect of these drugs via activation of alternative signaling pathways [5]. It has been reported that blocking the Ras/ERK pathway can promote the PI3K/AKT signaling, thereby desensitizing cancer cells to MEK inhibitor [6,7]. Because activation of the PI3K/AKT pathway may mediate resistance against drugs targeting Ras/ERK signaling, combinatory treatment targeting the Ras/ERK and PI3K/AKT signaling may overcome drug resistance.

We previously reported that metastatic derivatives of the MDA-MB-231 cell line that preferentially metastasize to lung (LM2) [8] express elevated Ras/ERK pathway. Genes that mediate metastasis to the lungs were downregulated after blocking the Ras/ERK pathway [9,10]. However, MEK inhibition did not block motility of LM2 cells because the PI3K/AKT signaling was activated in response to MEK inhibition. Combinatory treatment using the MEK inhibitor U0126 with PI3K inhibitor LY294002 reduced motility of LM2 cells, whereas LY294002 did not affect cell motility without MEK inhibition [9]. Thus, we found that the PI3K/AKT pathway is a key

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signaling pathway driving motility of LM2 in response to MEK suppression.

Here, we extend these studies to elucidate how activation of the PI3K/AKT pathway drives sustained motility in LM2 under MEK suppression. We found that SNAIL was upregulated in LM2 cells after MEK inhibition. The expression level of SNAIL was dependent on AKT activity. Knockdown of *SNAIL* resulted in reduced motility in LM2 cells in the presence of MEK inhibitor. We identified a receptor tyrosine kinase AXL as a downstream target of AKT. These findings suggest that AKT plays a critical role in motility by promoting SNAIL expression and AXL activation following MEK inhibition in lung metastatic derivatives.

2. Materials & methods

2.1. Reagents and antibodies

Reagents. DMSO (Sigma-Aldrich, D8418), U0126 (Cell Signaling Technology, 9903S), PD0325901 (Selleck Chemicals, S1036), AZD8055 (Selleck, S1555), and LY294002 (Calbiochem, 440202).

Antibodies. p-AKT(S473) (#9271), AKT (#9272), p-ERK(T202/Y204) (#9101), and SNAIL (#3879) (all from Cell Signaling Technology); ERK1 (sc-94) and AXL (sc-166269) (from Santa Cruz Biotechnology); α-tubulin (Sigma-Aldrich, T5168), GAPDH (AbFrontier, LF-PA0212), and p-AXL(Y779) (R&D Systems, AF2228).

2.2. Cell culture

MDA-MB-231 parental cell line (Par) and LM2 cells were cultured in DMEM high glucose (Welgene) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Reverse phase protein array

We prepared duplicated protein lysates and request reverse-phase protein array to MD Anderson Cancer Center (Houston, TX, USA). The heatmap was drawn using web-based Morpheus software.

2.4. siRNA-mediated knockdown

siRNAs against *SNAI1* and *AXL* were purchased from Bioneer. Each siRNA was transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturers protocol. For each experiment, negative control siRNA (Bioneer, SN-1003) was transfected as control.

2.5. Immunoblotting

Whole cell extracts were prepared by lysing cells using RIPA lysis buffer. The protein lysates were separated by SDS-PAGE and transferred to PVDF membrane (Roche). Proteins were visualized with HRP substrate for ECL (Enzynomics). Images were taken by using ChemiDoc MP imaging system (Bio-Rad).

2.6. Wound healing motility assay

Cells were treated with 25 μM of U0126 or DMSO. After 24 h of incubation, cells were scraped with 200 μl plastic tip across the cell monolayer to make wounds. Images were taken by phase-contrast microscope (Nikon) during wound closure (0 h–20 h). The area of wound closure was measured using ImageJ software (NIH).

2.7. RT-PCR analysis

RNA was extracted using RiboEx (Geneall) and RNA isolation kit (Qiagen). RNA concentration was measured by Nanodrop spectrometer (Thermo). RT-PCR was performed using Primer ScriptTM one step RT-PCR kit Ver2 (Takara) and PCR machine (Bio-Rad). Differently expressed mRNA was separated by 1.2% agarose gel electrophoresis. Specific primer sequences are as follows:

SNAI1 fwd, 5-ACCCCACATCCTTCTCACTG-3;
SNAI1 rev, 5-CCGACAAGTGACAGCCATTA-3;
ACTB fwd, 5-GCTCGTCGTCGACAACGGCTC-3;
ACTB rev, 5-CAAACATGATCTGGGTCATCTTCTC-3

2.8. Statistical analysis

The significance of the experimental results was determined by an unpaired, two-tailed Student's t-test. All analyses were performed using IBM SPSS Statistics v25 (IBM). P-values < 0.05 were considered as statistically significant.

3. Results

3.1. MEK inhibition induces expression of SNAIL in MDA-MB-231 LM2 through activation of PI3K/AKT

Our previous research has revealed that MEK inhibition induces activation of PI3K/AKT signaling and this contributes to sustained cell motility in the MDA-MB-231 LM2 cell line [9]. To elucidate genes that are involved in sustained motility driven by the PI3K/AKT pathway upon MEK inhibition, we screened proteins that were specifically upregulated following treatment with MEK inhibitor U0126 in LM2 cells by the reverse phase protein array (RPPA). We found SNAIL to be the most prominently upregulated protein under this condition (Fig. 1A). Western blot analysis confirmed that phosphorylation of AKT was increased by treatment with MEK inhibitors (U0126 and PD0325901) in LM2, which is consistent with our previous results (Fig. 1B) [9,10]. Interestingly, SNAIL was upregulated in LM2 cells compared to Par at the basal level and showed a further increase upon MEK suppression only in LM2 cells (Fig. 1B). The increase in SNAIL was accompanied by upregulation of *SNAIL* mRNA level (Fig. 1C).

SNAIL (*SNAI1*) is a member of the SNAIL superfamily and known to drive the epithelial-mesenchymal transition (EMT) by upregulation of mesenchymal markers [11] and promote cell movement and invasion [12]. In addition, SNAIL is a downstream effector PI3K/AKT signaling [13]. As we observed that MEK suppression induces both activation of the PI3K/AKT signaling and upregulation of SNAIL, we tested whether expression of SNAIL is regulated by PI3K/AKT pathway upon MEK inhibition. LM2 cells were treated with PI3K inhibitor LY294002 or mTORC1/2 inhibitor AZD8055, both known to suppress AKT phosphorylation [14]. Blocking AKT activation resulted in suppressed MEK inhibition-induced upregulation of SNAIL (Fig. 1D). These results together suggest that upregulation of SNAIL following MEK suppression is dependent on AKT activity.

In addition to promoting transcription of *SNAIL* [15], PI3K/AKT is known to mediate upregulation of SNAIL by blocking its degradation through inactivation of glycogen synthase kinase-3 beta (GSK-3β) [16]. Because GSK-3β is inactivated when Ser9 residue is phosphorylated by upstream kinases such as AKT and ERK [17], activation of AKT may block the degradation of SNAIL in LM2 cells. However, RPPA analysis revealed that phosphorylation of GSK-3β did not show significant differences after MEK inhibition in LM2

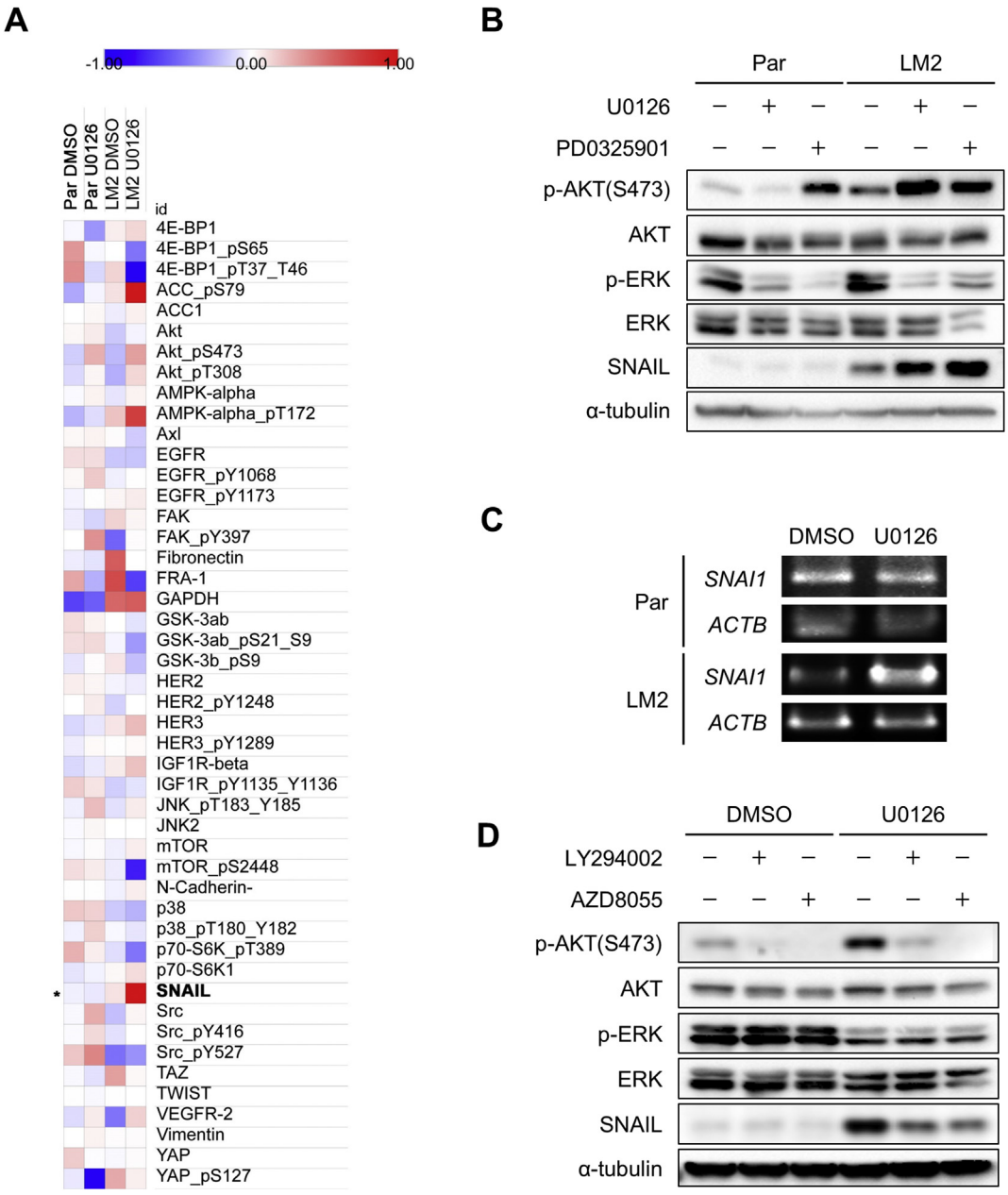

Fig. 1. MEK inhibition induces expression of SNAIL in MDA-MB-231 LM2 via AKT activation.
(A) MDA-MB-231 Par and LM2 cells were treated either DMSO or 25 μM of U0126 and incubated for 48hr. Cell extracts were analyzed by RPPA. Heatmap of indicated proteins was drawn based on normalized RPPA data. SNAIL is marked with an asterisk.
(B) MDA-MB-231 Par and LM2 cells were treated with DMSO, 25 μM of U0126, or 1 μM of PD0325901 for 48hr, respectively. Cell lysates were immunoblotted to measure the level of indicated proteins (n = 3).
(C) MDA-MB-231 Par and LM2 cells were treated with either DMSO or 25 μM of U0126 for 48hr. mRNA was analyzed through RT-PCR using specific primer for *SNAI1* (SNAIL). *ACTB* (β-actin) was used as a loading control.
(D) LM2 cells were treated with either DMSO or 25 μM of U0126 for 24hr, then 10 μM of LY294002 or 1 μM of AZD8055 is added for 24hr respectively. Cell lysates were immunoblotted to measure the level of indicated proteins (n = 3).

cells (Fig. S1). These results suggest upregulation of SNAIL upon MEK inhibition is due to regulation of mRNA levels rather than protein stability.

3.2. PI3K/AKT signaling drives sustained motility by inducing SNAIL expression

We previously reported that the PI3K/AKT signaling drives motile behavior of LM2 cells after MEK inhibition [9]. To test whether activation of the PI3K/AKT signaling drives sustained motility by promoting SNAIL expression, *SNAIL* was depleted by siRNA and cell motility was measured by wound healing assay. Knockdown of *SNAIL* resulted in retardation of motility in LM2 cells under MEK inhibition, while LM2 cells transfected with control siRNA did not display any significant changes in cell motility (Fig. 2A and B). Thus, SNAIL plays a critical role in sustained motility

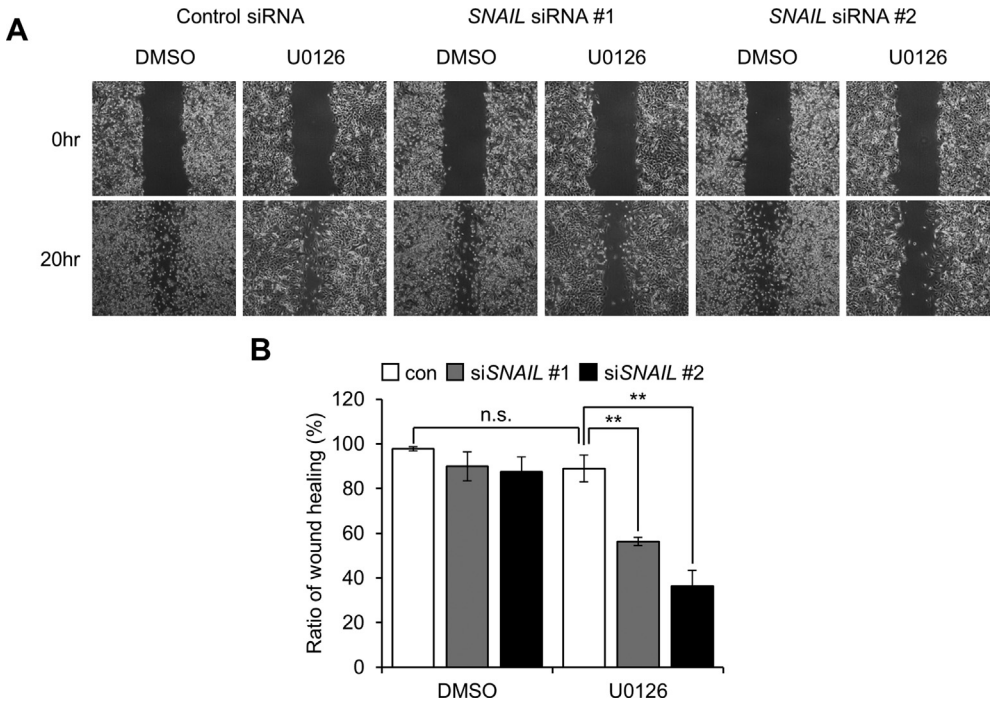

Fig. 2. The PI3K/AKT signaling drives sustained cell motility by inducing SNAIL expression. LM2 cells were transfected with 25 nM of siRNA against *SNAIL*. At 24hr following transfection of siRNAs, cells were treated with either DMSO or 25 μM of U0126 for 48hr. Monolayer of cells was scraped to make wound (n = 3).
(A) Representative images of wounds taken at 0hr and 20hr.
(B) Bar graph representing relative covered area of wounds (Mean ± SD. **P < 0.01, n.s. nonsignificant).

following MEK inhibition in LM2 cells.

3.3. AXL function as a downstream effector of AKT

It has been reported that MEK suppression drives compensatory activation of epidermal growth factor receptor (EGFR) or HER2 (*ERBB2*) [7,18]. However, we previously reported that AKT activation under MEK inhibition is independent of EGFR and HER2 [10]. RPPA analysis indicated that there were no significant differences on activation of EGFR, HER2, and HER3 (*ERBB3*) after MEK suppression in LM2, which is consistent with our previous results (Fig. S2). Although there was no significant difference among other RTKs, we found that AXL was activated following MEK suppression (Figs. 1A and 3A). Because it has been reported that AXL activation contributes to resistance against drug targeting the Ras/ERK pathway [19], we hypothesized that AXL may play a key role in sustained motility following MEK suppression in LM2 cells.

AXL receptor tyrosine kinase is a member of the TAM family and prominently activated in response to inhibitors targeting Ras/ERK signaling [19,20]. To test whether AXL mediates cell motility when MEK is suppressed, we performed wound healing assay after silencing *AXL*. Depletion of *AXL* in the presence of MEK inhibitor U0126 reduced cell motility in LM2 cells (Fig. 3B and C). Western blot analysis showed that depletion of *AXL* does not affect AKT activation and SNAIL expression (Fig. 3D). To test whether AKT mediates activation of AXL, LM2 cells were treated with PI3K inhibitor LY294002. Blocking PI3K/AKT signaling with MEK inhibitor resulted in reduced phosphorylation of AXL (Fig. 3E). These data suggest that AXL may contribute to the motile behavior of LM2 cells as another downstream effector of AKT.

4. Discussion

Because cancer metastasis is responsible for about 90% of mortality among cancer patients [21], identifying genes and signaling pathways that are involved in metastasis is necessary for developing targeted drugs to prevent metastasis. However, despite achievements in developing targeted therapies, cancer cells often acquire adaptive drug resistance against targeted therapies [5]. Thus, we need to elucidate underlying mechanism of adaptive drug resistance. In this paper, we demonstrate that activation of AKT drives sustained cell motility under MEK suppression by upregulation of SNAIL in LM2 cells. Furthermore, we identify AXL as a downstream effector of AKT.

Because the Ras/Raf/MEK/ERK pathway is frequently elevated in various types of tumors [22,23], components of Ras/ERK signaling have been targeted to develop drugs such as Trametinib [24]. However, cancer cells can acquire resistance against Ras/ERK inhibitors via promoting alternative signaling pathways [7,25]. We previously reported that MEK inhibition did not reduce cell motility of LM2 cells due to activation of the PI3K/AKT pathway [9]. In this paper, we demonstrate that activation of the PI3K/AKT signaling promotes SNAIL expression when the Ras-ERK pathway is suppressed in LM2 cells.

It has been reported that the Ras/ERK signaling axis is known to contribute to EMT [26–28]. ERK is known to be required for TGF-β1-induced EMT [29] and inactivation of GSK-3β [30] thereby contributing EMT. We found that MEK inhibition promotes expression of SNAIL in the lung-metastatic subline whereas parental MDA-MB-231 cells display reduced expression of SNAIL (Fig. 1). Expression of TWIST did not show any significant change (Fig. 1A). Because AKT is activated in U0126-treated LM2 cells, elevated PI3K/AKT signaling may compensate MEK suppression. Because the PI3K/AKT signaling promotes EMT [31] and stabilizes SNAIL by inactivating GSK-3β [17,32], AKT activation may promote SNAIL expression when the Ras/ERK pathway is suppressed. As described above, we demonstrate that expression of SNAIL depends on AKT activity.

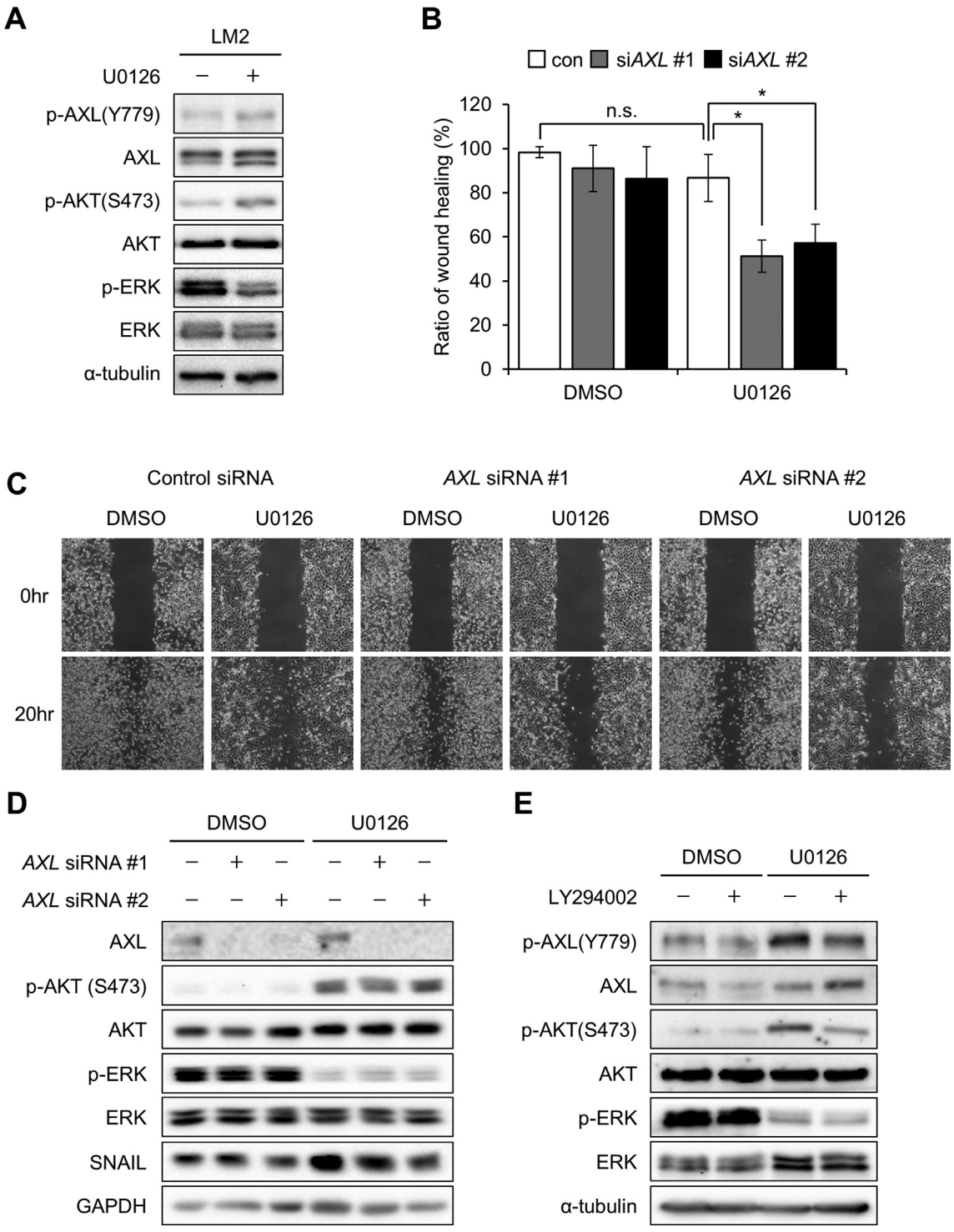

Fig. 3. AXL function as a downstream effector of AKT.
(A) LM2 cells were treated with either DMSO or 25 μM of U0126 for 48hr. Cell lysates were immunoblotted to measure the level of indicated proteins (n = 3).
(B) Prior to wound-healing assay, LM2 cells were transfected with 25 nM of siRNA targeting *AXL* or control siRNA for 24hr, then cells were treated with either DMSO or 25 μM of U0126 for 48hr. Monolayer of cells was scraped and visualized after 20 h (n = 3). (Mean ± SD. *P < 0.05, n.s. nonsignificant).
(C) Representative images of wound closure.
(D) Cell lysates of (B) were immunoblotted to measure the level of indicated proteins.
(E) LM2 cells were treated wither either DMSO or 25 μM of U0126 for 24hr. 10 μM of LY294002 was added to culture medium and incubated for 24hr. Cell lysates were immunoblotted to measure the level of indicated proteins (n = 3).

We found that SNAIL expression is dependent on AKT activity (Fig. 1D). SNAIL is known to be a transcription factor that mediates EMT [33], and its expression is correlated with cell motility [34–36]. SNAIL also promotes cancer cell survival, thereby mediating drug resistance. Thus, SNAIL may be a key factor that drives resistance against MEK inhibition. We observed that SNAIL is a critical factor in sustained cell motility under MEK suppression because depletion of *SNAIL* by siRNA reduced motility of U0126-treated LM2 cells (Fig. 2). Although SNAIL is upregulated after MEK inhibition in LM2 cells, there was no significant differences among other EMT markers such as TWIST and VIM (vimentin) (Fig. 1A). Thus, SNAIL can be considered as a key factor that drives motility of LM2 cells upon MEK inhibition.

It has been reported that a main cause of adaptive resistance of cancer cells treated with inhibitors of various signaling pathways is activation of alternative receptor tyrosine kinases. When a pathway is suppressed by drug treatment, some population of cancer cells may bypass the inhibitory effect of a drug by activating secondary RTK signaling [37]. For example, EGFR-mutant cancers can bypass drugs targeting EGFR signaling such as Gefitinib and Erlotinib by activating other RTKs such as IGF-1R, FGFR, and AXL. To test whether RTKs are involved in activation of the PI3K/AKT signaling following MEK suppression, RTKs were analyzed through RPPA. Although no significant differences were observed among RTKs, phosphorylation of AXL is increased after MEK inhibition (Fig. 3A), suggesting that AXL may contribute to activation of AKT and resistance against MEK inhibition. AXL expression can drive resistance to targeted inhibitory drugs of other signaling pathways. For example, in BRAF-mutant melanoma, AXL-high groups display resistance to drugs targeting the Ras/ERK pathway while AXL-low groups are sensitive to inhibitors [19].

In summary, our results demonstrate that SNAIL drives sustained cell motility in LM2 cells when the Ras/ERK pathway is suppressed. MEK inhibition results in upregulation of SNAIL by activation AKT. AKT also mediates motile behavior of LM2 cells upon MEK inhibition through activation of AXL. Understanding how AXL activation promotes cell motility may provide clues to overcome adaptive resistance targeting the Ras/ERK pathway.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.05.043.

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