
Negative feedback regulation of AXL by miR-34a modulates apoptosis in lung cancer cells

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ABSTRACT

The AXL receptor tyrosine kinase is frequently overexpressed in cancers and is important in cancer invasion/metastasis and chemoresistance. Here, we demonstrate a regulatory feedback loop between AXL and microRNA (miRNA) at the post-transcriptional level. Both the GAS6-binding domain and the kinase domain of AXL, particularly the Y779 tyrosine phosphorylation site, are shown to be crucial for this autoregulation. To clarify the role of miRNAs in this regulation loop, approaches using bioinformatics and molecular techniques were applied, revealing that miR-34a may target the 3' UTR of AXL mRNA to inhibit AXL expression. Interestingly and importantly, AXL overexpression may induce miR-34a expression by activating the transcription factor ELK1 via the JNK signaling pathway. In addition, ectopic overexpression of ELK1 promotes apoptosis through, in part, down-regulation of AXL. Therefore, we propose that AXL is autoregulated by miR-34a in a feedback loop; this may provide a novel opportunity for developing AXL-targeted anticancer therapies.

Keywords: feedback loop regulation; AXL; miR-34a; JNK; ELK1; apoptosis

INTRODUCTION

MicroRNAs (miRNAs) are an abundant class of noncoding RNAs 18–25 nt in length that are involved in post-transcriptional regulation of gene expression and control a wide range of biological processes that represent the hallmarks of cancer, such as apoptosis, invasion, and metastasis (Lujambio and Lowe 2012). MiRNAs post-transcriptionally regulate gene expression to either inhibit mRNA translation or promote its degradation by targeting 3' UTR (Bartel 2004). Many miRNAs play roles as either oncogenes or tumor suppressors, depending on the nature of their targets, affecting biological functions during cancer initiation and progression. Aberrant expression of miRNAs has been reported in multiple human cancer types (Esquela-Kerscher and Slack 2006).

Abbreviations: RTK, receptor tyrosine kinase; miRNA, microRNA; UTR, untranslated region; EMT, epithelial–mesenchymal transition; JNK, c-Jun amino-terminal kinases; ELK1, ETS-Like Gene 1; Gas6, growth arrest-specific 6; PI3K, phosphoinositide 3-kinase; PARP, poly-ADP-ribose polymerase

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Receptor tyrosine kinases (RTKs) (e.g., EGFR and MET) may further modulate the strength of their downstream signaling pathways by microRNA-mediated mechanisms in a regulatory feedback manner. For example, miR-7 may down-regulate EGFR signaling (Kefas et al. 2008; Webster et al. 2009) and p21-activated kinase 1 (Reddy et al. 2008) to modulate cell proliferation and invasiveness. On the other hand, EGFR can activate miR-7 expression in lung oncogenesis (Chou et al. 2010). Additionally, MET forms a direct feedback loop with miR-27a in lung cancer (Acunzo et al. 2013). Therefore, RTK expression may be regulated by miRNAs via a feedback mechanism.

AXL, belonging to the TAM family (Tyro3, Mer, AXL) of receptor tyrosine kinases, regulates several aspects of cancer biology. Studies have shown that AXL is highly expressed in many human cancers and it has been implicated in the prognosis of cancer patients, including those with lung (Wimmel et al. 2001; Shieh et al. 2005), renal (Chung et al. 2003),

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myeloid leukemia (O'Bryan et al. 1991; Rochlitz et al. 1999), breast (Meric et al. 2002), gastric (Wu et al. 2002), prostatic (Wu et al. 2004; Sainaghi et al. 2005), and colon (Craven et al. 1995) cancers. Several pathways have been reported to be downstream from AXL signaling, including PI3K-AKT (Lee et al. 2002; Hasanbasic et al. 2004; Hafizi et al. 2005), MEK-ERK (Fridell et al. 1996), p38 (Wu et al. 2004; Sainaghi et al. 2005), and NF- κ B (Georgescu et al. 1999; Demarchi et al. 2001). Moreover, AXL is activated by both ligand-dependent (Demarchi et al. 2001; Gallicchio et al. 2005; Hafizi et al. 2005) and ligand-independent (Bellosta et al. 1995; Heiring et al. 2004) mechanisms. Recent studies have demonstrated that overexpression and/or activation of AXL play a pivotal role in cancer's resistance to chemotherapeutic regimens, in the phenotype of epithelial-to-mesenchymal transition (EMT), and in the promotion of increased metastasis, highlighting the importance of AXL as a therapeutic target (Wu et al. 2014). In addition to its known regulation by some transcription factors, such as EZH2 and MZF1, post-transcriptional regulation of AXL remains largely unknown (Mudduluru et al. 2010; Ott et al. 2012). Therefore, an understanding of the regulation mechanisms related to AXL expression appears important.

The miR-34 family consists of three processed miRNAs that are encoded by two different genes: miR-34a, which is encoded by its own transcript, and miR-34b and miR-34c, which share a common primary transcript (Hermeking 2010). Ectopic expression of miR-34a and miR-34b/c induces apoptosis, senescence, and cell-cycle arrest, as well as inhibits migration and invasion (Hermeking 2010). Their promoters are often silenced by CpG methylation in numerous types of cancers during tumorigenesis and may therefore represent tumor suppressor genes (Lodygin et al. 2008; Vogt et al. 2011). Several studies have reported relationships between AXL and miR-34a in colon carcinomas (Kaller et al. 2011), lung cancer (Lee et al. 2011), breast cancer (Mackiewicz et al. 2011), and leukemia (Boysen et al. 2014). However, how the microRNAs are modulated by the AXL signaling pathway is largely unclear, and the mechanisms underlying how the miR-34 family regulates AXL expression are in need of further study.

In this article, we clarify the involvement of miRNA in the post-transcriptional feedback autoregulation of AXL. We illustrate the negative feedback loop of AXL/ELK1/miR-34a wherein AXL up-regulates ELK1 via the JNK pathway, ELK1 in turn up-regulates miR-34a expression via direct promoter activation, and miR-34a returns to down-regulate AXL mRNA. Indeed, ectopic overexpression of ELK1 significantly decreases AXL expression and induces G₁ arrest and apoptosis. In addition, we found that overexpression of miR-34c may down-regulate AXL expression transcriptionally via promoter modulation. This post-transcriptional feedback autoregulation loop of AXL by miR-34a may shed light on how AXL is overexpressed in various cancers and have great therapeutic potential in cancer treatment.

RESULTS

AXL is negatively regulated in a post-transcriptional feedback loop

We first found that the expression levels of AXL protein were inversely correlated with its 3'-UTR reporter activity in eight different cancer cell lines (Fig. 1A,B; Supplemental Fig. 1A, B). Expression levels of AXL in different stable clones of CL1-0 were also found to possess an inverse correlation with the AXL 3'-UTR reporter activity (Fig. 1C). These results demonstrate that AXL may regulate its own expression by a previously unknown feedback mechanism; this was further confirmed by performing AXL 3'-UTR reporter assays in CL1-0 cells transiently transfected with AXL expression vector and in CL1-5 cells transfected with AXL shRNA. The results were consistent with expectations (Fig. 1D,E). Next, we designed the AXL 3'-UTR primers specifically to detect endogenous AXL mRNA level and found that it was also down-regulated by overexpression of AXL. Neither the kinase-dead (K567R) nor the intracellular domain deletion (del-ICD) AXL had this effect (Fig. 1F). A similar result was obtained from microarray analysis with the probe targeting the AXL 3'-UTR 4461–4520 region (Supplemental Table 1). Therefore, this feedback regulation may involve AXL kinase activity in suppressing AXL 3'-UTR reporter activity.

The AXL kinase domain and autophosphorylation site Y779 of AXL are crucial to the feedback regulation

The CL1-3 cell line was originally derived from CL1-0 by selecting for increased invasiveness. Its AXL expression level is higher than CL1-0 and lower than CL1-5. CL1-3 is therefore an appropriate choice for conducting experiments in which both knockdown and overexpression of AXL are required. Among the constructs of various AXL mutations and deletions examined, we found that deletion of the intracellular domain and the kinase-dead point mutation (K567R) could both reverse the feedback regulation in the AXL 3'-UTR reporter assay (Fig. 2A). Among the three putative autophosphorylation sites (Y779, Y821, and Y866) predicted in AXL, only Y779 was required for this feedback regulation (Fig. 2B). Similar results were obtained using CL1-0 cells (Supplemental Fig. 1C,D).

The AXL ligand GAS6 is important for the negative feedback loop

Involvement of the AXL ligand GAS6 in the negative feedback mechanism was tested. Deletion of the immunoglobulin-like domain, which is the domain that contains the GAS6-binding site, significantly reversed the feedback regulation of AXL (Fig. 3A). Meanwhile, when the AXL 3'-UTR

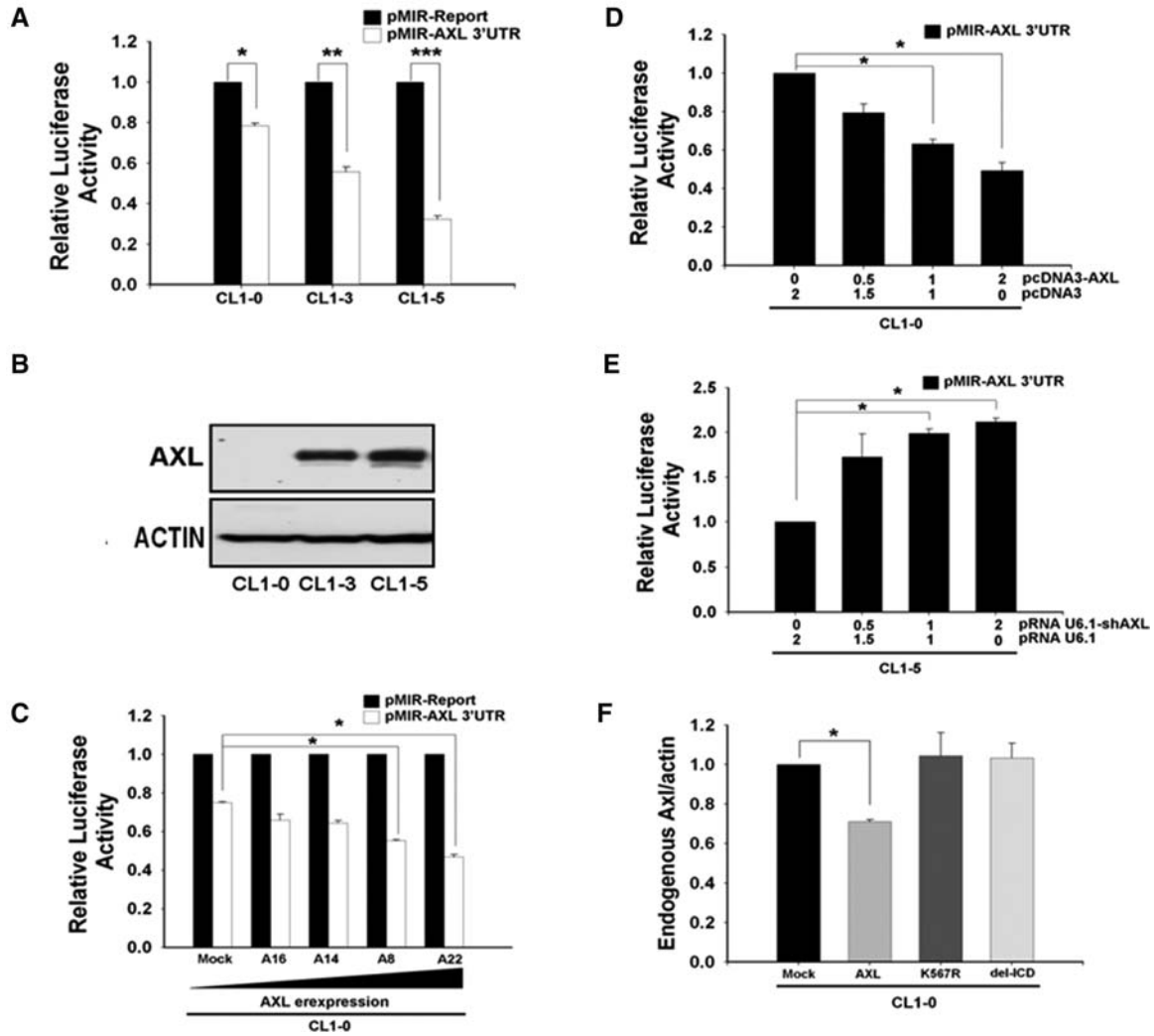


FIGURE 1. AXL negatively regulates its own expression through 3' UTR. (A) Inverse correlation between AXL protein level and AXL 3'-UTR reporter activity. The pMIR-Report-AXL 3'-UTR or p-MIR-Report (mock vector) plasmid was cotransfected with Renilla plasmid, serving as an internal control for transfection efficiency, into CL1-0, CL1-3, and CL1-5. After 24 h, luciferase activity of the cell lysates was measured. Relative fold change in luciferase activity for each cell line was plotted with respect to each pMIR-Report control. (B) AXL protein levels of three cell lines at steady state were analyzed by Western blot, using actin as an internal control. (C) AXL 3'-UTR reporter activity in stable transfectants of CL1-0 overexpressing various levels of AXL. CL1-0/Mock is vector alone control. (D) Ectopic expression of AXL suppresses AXL 3'-UTR reporter activity. The AXL 3'-UTR reporter constructs were cotransfected with various amounts of the AXL expression construct in CL1-0 cells, and luciferase activity was measured 24 h later. Equal amounts of DNA (2 μ g) were used for each transfection. (E) Knockdown of AXL expression increased the AXL 3'-UTR reporter activity. The AXL 3'-UTR reporter construct was cotransfected with various amounts of the AXL shRNA construct into CL1-5 cells, and reporter activity was measured as above. (F) Endogenous AXL mRNA levels were down-regulated by AXL in a kinase activity-dependent manner. CL1-0 cells were transfected with various AXL constructs. After 48 h, the endogenous AXL mRNA was measured by real-time RT-PCR using actin as an internal control. The ratios of endogenous AXL to actin were calculated. Mock, vector alone; AXL, full-length wild-type; AXL (K567R), kinase-dead; del-ICD, intracellular domain deletion. In A, C, D, E, and F, the values were derived from three independent experiments. Error bars represent SD (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

reporter plasmid was cotransfected with increasing amounts of GAS6 expression construct, the reporter activity was reduced in a dose-dependent manner in both CL1-3 and MDA-MB-231 cells (Fig. 3B; Supplemental Fig. 2A). In comparison, when GAS6 expression was knocked down by shRNA, increased reporter activity was observed (Supplemental Fig. 2B). Further, addition of recombinant human AXL-Fc, a soluble form of AXL that contains only its extracel-

lular domain that binds GAS6, could antagonize the GAS6-mediated down-regulation of AXL 3'-UTR reporter activity in both CL1-3 and MDA-MB-231 cells (Fig. 3C; Supplemental Fig. 2C). Additionally, R428, a selective small molecule inhibitor of AXL kinase, could block this feedback effect (Fig. 5A; Supplemental Fig. 2D). These results suggest the existence of a feedback loop that is activated by GAS6 to subtly balance the expression of AXL.

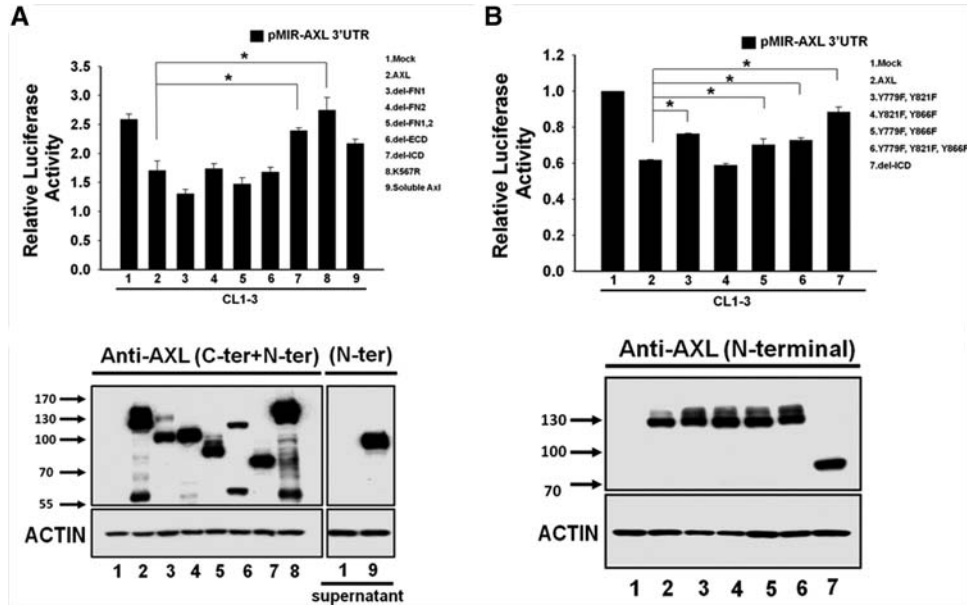


FIGURE 2. AXL kinase activity and the Y779 phosphorylation site are crucial for feedback regulation. (A) Effects of various AXL mutations on AXL 3'-UTR reporter activity. The AXL 3'-UTR reporter construct was cotransfected with various AXL mutants in CL1-3 cells, and luciferase activity was measured 24 h later. (B) Effects of double or triple mutants in AXL tyrosine phosphorylation sites on AXL 3'-UTR reporter activity. Luciferase reporter activity was assayed for CL-3 cells cotransfected with individual AXL mutants and the AXL 3'-UTR reporter construct. The lower panel shows the expression and molecular weights of various AXL mutant proteins verified by Western blot analysis using a mixture of antibodies against the carboxyl and amino terminal of AXL. Soluble AXL was concentrated from the conditioned medium and detected by Western blotting using AXL amino-terminal antibody. Actin was used as a loading control. The values were derived from three independent experiments. Error bars represent SD (*) $P < 0.05$.

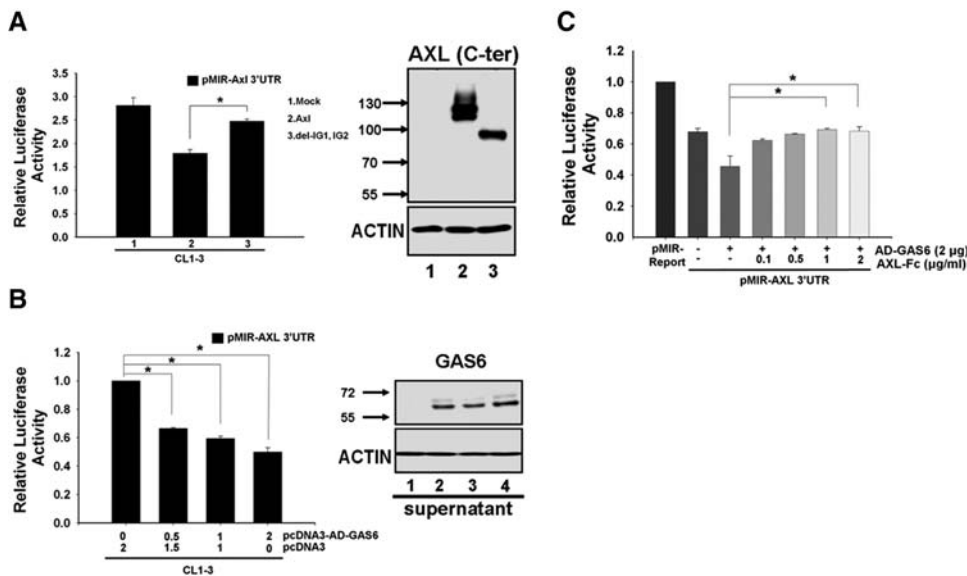


FIGURE 3. The AXL ligand GAS6 is crucial for the negative feedback loop. (A) Effects of AXL immunoglobulin domain deletion mutants on AXL 3'-UTR reporter activity. AXL 3'-UTR reporter was cotransfected with wild-type AXL or the immunoglobulin domain deletion mutant into CL1-3 cells, and luciferase activity was measured 24 h later. The expression and molecular weights of various truncated AXL proteins were verified by Western blot analysis (right panel). (B) GAS6 suppresses AXL 3'-UTR reporter activity in a dose-dependent manner. A fixed amount of AXL 3'-UTR reporter construct was cotransfected with various amounts of pcDNA3-AD-Gas6 into CL1-3 cells. The Renilla reporter was used as transfection efficiency control. Expression of GAS6 was concentrated from the conditioned medium and determined by Western blotting (right panel). Actin was used a loading control. (C) GAS6's inhibitory effect on the AXL 3'-UTR reporter activity was neutralized by AXL-Fc. Fixed amounts of the AXL 3'-UTR reporter and the pcDNA3-AD-Gas6 expression construct were cotransfected with various amounts of the AXL-Fc construct into CL1-3 cells. Luciferase activity was measured 24 h post-transfection. Equal amounts of DNA (2 µg) were transfected for each sample in B and C. The values were derived from three independent experiments. Error bars represent SD (*) $P < 0.05$.

Prediction of microRNAs that target AXL

To identify the regions potentially targeted by miRNAs that regulate *AXL* mRNA in a feedback manner, we used reporter constructs with various lengths of the *AXL* 3' UTR. Reporter assays revealed that two regions of the *AXL* 3' UTR, 1–846 and 846–1457, may contain putative target sites (Fig. 4A). Similar results were obtained using other cancer cell lines, including MDA-MB-231 and A549 (Supplemental Fig. 3A). By using bioinformatic approaches with four miRNA databases (TargetScan5.1, miRBase, PicTar, miRanda) according to both binding free energy and alignment scores, among the eight miRNAs predicted to have potential binding sites in the *AXL* 3'-UTR region, only miR-34a, miR-34c, and miR-367 have target sites located within the sequence of the 1–846 region (Supplemental Table 2). The MicroCosm Targets database shows that the 31–56 region was phylogenetically conserved among species (Supplemental Fig. 3C). The *AXL* 3'-UTR reporter lacking the miR-34 seed region had partially

recovered reporter activity in all three cell lines tested (CL1-5, A549, and MDA-MB-MB231) (Fig. 4B; Supplemental Fig. 4A). These results implicate that miR-34 may target this conserved sequence to mediate the feedback regulation of *AXL* expression.

miR-34a targets the 3' UTR of *AXL* mRNA and inhibits its expression

To identify the miR-34 family that regulates *AXL* mRNA, we examined the effects of miR-34a, miR-34b*, and miR-34c mimics on the *AXL* 3'-UTR reporter activity in CL1-5 cells (Supplemental Fig. 3B). We found that only miR-34a could down-regulate *AXL* 3'-UTR reporter activity (Fig. 4C). Similar results were obtained in MDA-MB-231 cells (Supplemental Fig. 4B). However, as shown, both miR-34a and miR-34c mimics could significantly down-regulate the expression of *AXL* mRNA and protein (Fig. 4D,E). When the putative miR-34 target site of the *AXL* 3' UTR was deleted

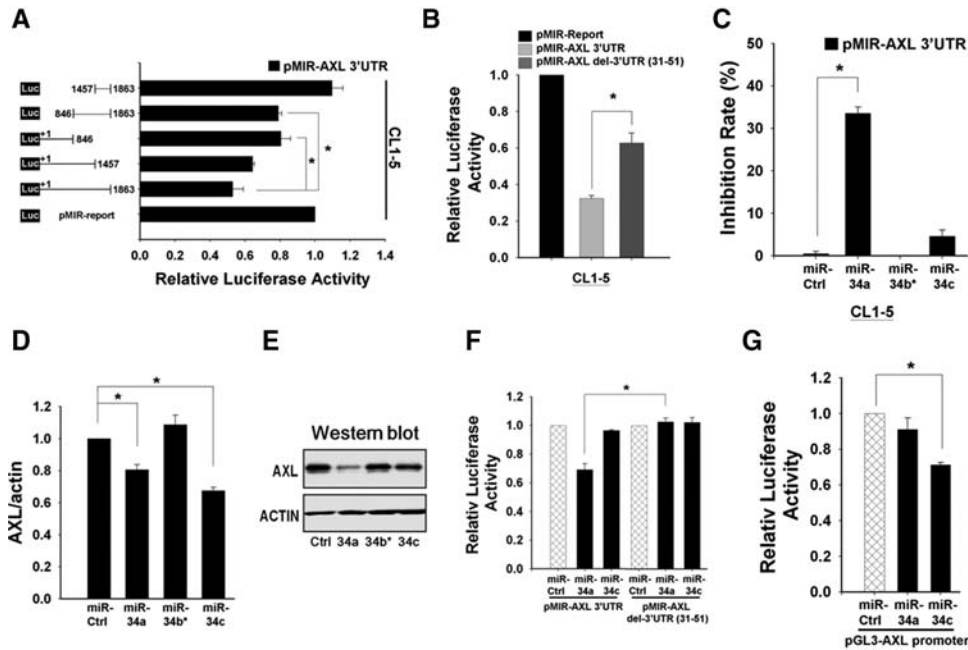


FIGURE 4. AXL expression is regulated by microRNAs and through different mechanisms. (A) Identification of the microRNA-binding region of *AXL* 3' UTR. The cotransfection reporter assay was performed in the CL1-5 cells using constructs of a series of *AXL* 3'-UTR deletion mutants (0.5 µg), with Renilla luciferase plasmid serving as an internal control for transfection efficiency. Then the luciferase activity was measured 48 h post-transfection. The schematic presentation on the left shows the deleted regions of various constructs in the *AXL* 3' UTR. Reporter activity obtained from the pMIR vector (bottom bar) was set to 1.0 and the activity of the other constructs was calculated and plotted. Relative luciferase activity is shown on the right and is presented as fold values relative to the basal activity. (B) Confirmation of target site (seed region) in *AXL* 3' UTR. Luciferase reporter vectors pMIR-Report-*AXL* 3' UTR (intact) or pMIR-Report-*AXL* 3' UTR (seed region deletion mutant) were transfected into CL1-5 cells, and luciferase activity was measured 24 h later. (C) Inhibition of *AXL* 3' UTR by miR-34a/b/c. The *AXL* 3'-UTR luciferase reporter activities of CL-5 cells transfected with miR-34a/b/c mimics or control oligos were determined. Luciferase activity of control oligos (siRNA) was set as 100% for normalization. An asterisk indicates a significant difference between miR-34a and the control. (D) Expression of the *AXL* mRNA was determined, in triplicate, by real-time RT-PCR. Fold changes between cells transfected with miR-34a/b/c mimics compared with control oligos are shown. (E) Western blot shows the inhibition of *AXL* protein levels by miRNAs in CL1-5 cells. Actin serves as a loading control. (F) Discrimination of the target site for miR-34a, but not miR-34c, in *AXL* 3' UTR. CL1-5 cells were cotransfected with miR-34a or miR-34c mimic, along with pMIR-Report-*AXL* 3' UTR (intact) or pMIR-Report-*AXL* del 3' UTR (seed region deletion mutant). An asterisk indicates a significant difference between miR-34c and the control. (G) MiR-34c, but not miR-34a, affects *AXL* promoter activity. CL-5 cells were transfected with miR-34a or miR-34c mimic along with the pGL3-*AXL* promoter luciferase construct. Luciferase reporter activity was assayed 48 h later. An asterisk indicates a significant difference between miR-34c and the control. All reporter assays were performed in triplicate and the Renilla reporter activity was used to normalize the transfection efficiency. The error bars represent SD (*) $P < 0.05$.

from the reporter, miR-34a could no longer reduce the AXL 3'-UTR reporter activity (Fig. 4F). In contrast, miR-34c changed neither AXL reporters' activities (Fig. 4F). In examining these miRNAs' effects on the AXL promoter's reporter activity, we found that miR-34c significantly down-regulated the AXL promoter's reporter activity, but miR-34a did not (Fig. 4G). These findings implicate that, in contrast to miR-34a, miR-34c may regulate AXL expression through an indirect mechanism, probably by targeting other molecules that modulate AXL promoter activity.

JNK-ELK1 signal pathway is involved in the regulation of miR-34a by AXL

To investigate what pathway is involved in this AXL feedback regulation, we used various inhibitors in the AXL 3'-UTR reporter assay, including PD98059 and U0126 (MEK-ERK), LY294002 (PI3K-AKT), SP600125 (JNK), PP2 (SRC), AG490 (JAK), BAY-11-7086 (NF- κ B), and SB203580 (P38). Only JNK inhibitor could significantly recover the AXL 3'-UTR reporter activity suppressed by AXL overexpression in CL1-0 and CL1-5 cells (Fig. 5A; Supplemental Fig. 5A,B). The AXL kinase inhibitor R428 had a similar effect (Fig. 5A; Supplemental Fig. 2D). Because ELK1 is downstream from the JNK pathway (Cavigelli et al. 1995; Whitmarsh et al. 1995), we wondered whether AXL kinase activity is required to activate ELK1. We examined the effects of overexpression of various AXL mutants in CL1-0 cells and observed that wild-type AXL could up-regulate p-AXL, p-JNK, and p-ELK1 expression but not the AXL kinase-dead (K567R) or the intracellular domain deletion (del-ICD) mutants of AXL (Fig. 5B). These results demonstrate that AXL kinase may activate ELK1 via the JNK pathway. To identify the AXL-inducible miRNAs, we compared the miRNA expression arrays between the CL1-0 cells and cells stably overexpressing AXL and found that miR-34a was among the AXL-inducible miRNAs (Supplemental Table 3). To confirm that AXL may up-regulate miR-34a, we transfected the constructs of AXL into CL1-0 cells and analyzed the primary transcript and mature form of miR-34a using quantitative real-time PCR. As a result, expression of miR-34a was indeed found to be up-regulated by AXL overexpression (Fig. 5C). The reporter activity of *miR-34a* promoter was also increased by AXL in a dose-dependent manner (Supplemental Fig. 5C). These results indicate that activation of AXL is essential for up-regulating the expression of miR-34a.

To clarify whether AXL decreases its own 3'-UTR activity in the presence of a miR-34 inhibitor, we designed two sets of miRNA sponges for anti-miR-34a usage. We used the miR-34a targeting sequence of AXL to synthesize a single-strand RNA oligonucleotide with 2'-Ome modification (SP-A34). The second miR-34a sponge has a sequence perfectly complementary to that of miR-34a (CM-A34). Delivery of the miRNA sponge can specifically neutralize and inhibit mature miRNA from target recognition and translational repression.

AXL, MET, and BCL2 are known targets of miR-34a. Our results demonstrate that treatment of CM-A34 or SP-A34 resulted in a significant up-regulation of AXL, MET and BCL2 protein expression in CL1-0, CL1-5, and A549 cells, especially SP-A34 (Supplemental Fig. 6A). We also found that AXL-mediated down-regulation of its 3'-UTR reporter activity and the endogenous AXL mRNA level was significantly increased upon SP-A34 treatment (Fig. 5D,E).

A recent study found that ELK1 may regulate miR-34a expression via promoter targeting during oncogene-induced senescence (Christoffersen et al. 2010). To investigate the effect of ELK1-S383 phosphorylation on its ability to function as an activator for the *miR-34a* gene promoter, the S383 residue was mutated to aspartate (S383D) to mimic phosphorylation status or to alanine (S383A) to mimic non-phosphorylation status as a control. The *miR-34a* promoter activity and primary transcript expression were both increased by transfection with wild-type ELK1 and ELK1-S383D, but not ELK1-S383A (Fig. 5F,G; Supplemental Fig. 6B). To further validate the existence of an ELK1-mediated feedback loop for AXL regulation, we transfected ELK1 or ELK1 siRNA into CL1-5 cells and found that overexpression and knockdown of ELK1 resulted in down- and up-regulation of AXL protein expression, respectively (Fig. 5H; Supplemental Fig. 6C). CHIP assay was performed to determine whether ELK1 directly binds to the *miR-34a* promoter when AXL expression was knocked down by siRNA in CL1-5 and when AXL was ectopically overexpressed in CL1-0 cells. The results show that there is a direct binding between ELK1 protein and the *miR-34a* promoter (Fig. 5I), demonstrating that ELK1 is a transcription activator of the *miR-34a* promoter. Indeed, ELK1-mediated down-regulation of AXL 3'-UTR reporter activity was significantly reversed when cells were treated with miR-34a sponge SP-A34 (Supplemental Fig. 6D). Furthermore, AXL-mediated up-regulation of miR-34a and down-regulation of its own mRNA expression could also be reversed by ELK1 siRNA (Supplemental Fig. 6E,F). In summary, this feedback loop is activated by AXL overexpression through JNK-mediated ELK1 activation, and this subsequently leads to an up-regulated expression of miR-34a that in turn down-regulates AXL protein expression.

ELK1 promotes apoptosis in part through inhibition of AXL kinase activity

To explore the effect of ELK1 on the cell cycle, we analyzed the cell-cycle distribution in ELK1-transfected cells by flow cytometry. ELK1 caused significant G₀/G₁ arrest in CL1-5 cells (Fig. 6A). To further elucidate the mechanism underlying this cell-cycle arrest effect, we analyzed apoptosis of transiently ELK1-transfected cells by flow cytometry and found that overexpression of ELK1 induced apoptosis (Fig. 6B). Similar results were obtained using AXL shRNA (Fig. 6C). Double transfection experiments were conducted to clarify that AXL is one of the main mediators of ELK1-induced

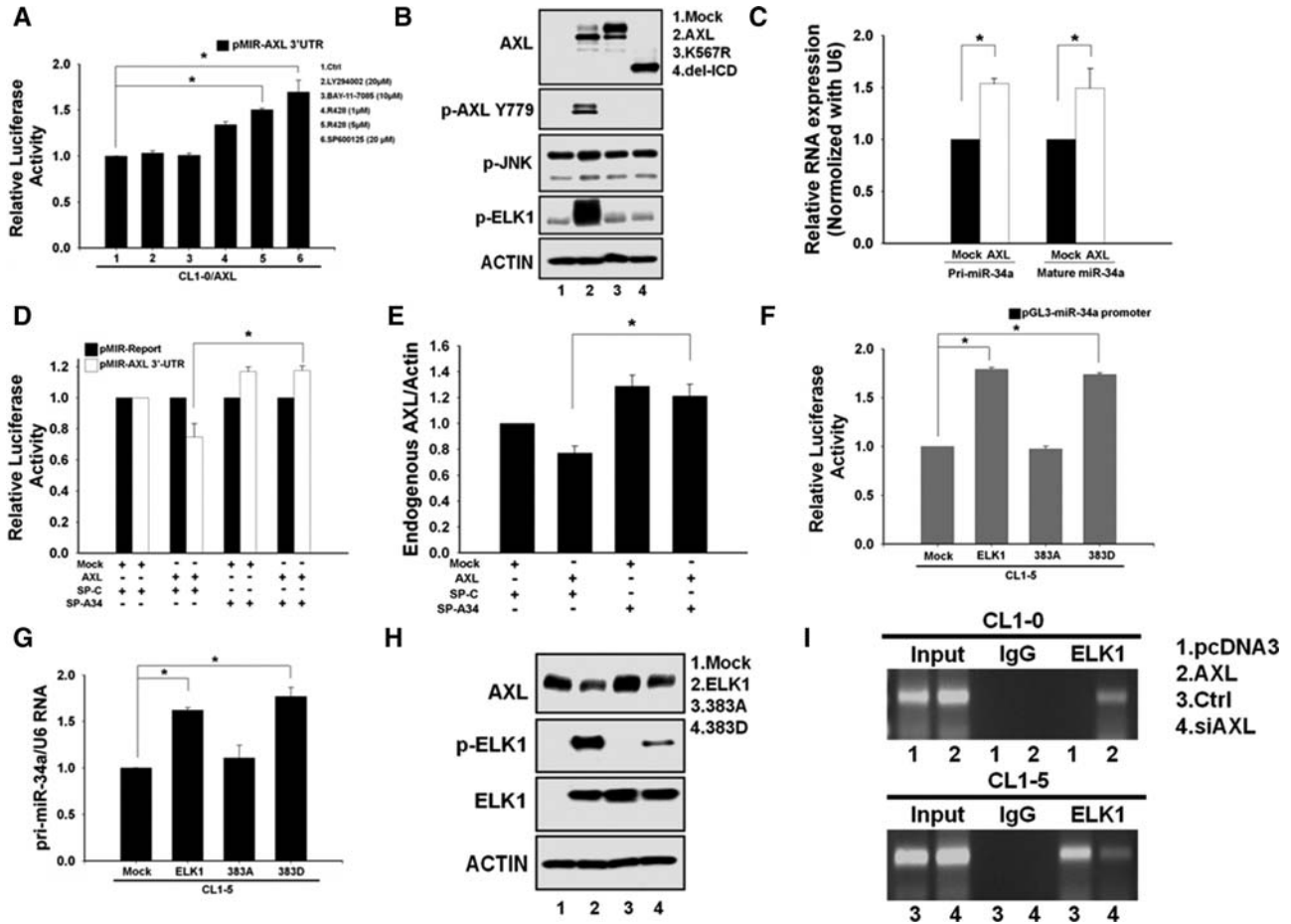


FIGURE 5. AXL regulates miR-34a via the JNK/ELK1 pathway. (A) The JNK pathway is involved in the negative feedback regulation. After transfection with the AXL 3'-UTR reporter for 24 h, CL1-0/AXL cells were treated with various inhibitors. The luciferase activity was measured 48 h later. An asterisk indicates a significant inhibitory effect on AXL 3'-UTR reporter activity. (B) AXL kinase activity is essential for ELK1 phosphorylation. CL1-0 cells were transfected with the full-length, kinase-dead mutant (K567R) or the intracellular domain deletion construct (del-ICD) of AXL. The protein levels of p-AXL, p-JNK, and p-ELK1 were determined by Western blot. (C) Expression of miR-34a is up-regulated by AXL. After CL1-0 cells were transfected with AXL or the vector-alone control plasmid, the primary transcript and mature forms of miR-34a were measured by real-time PCR. U6 small RNA was used as the internal control. (D) AXL-mediated down-regulation of the reporter activity can be rescued by blocking miR-34a. Luciferase reporter activity was determined in CL1-0 cells after cotransfection with pcDNA3-AXL plus miR-34a sponge (SP-A34) or pcDNA3-AXL plus control sponge (SP-C). The Renilla reporter activity was used to normalize the transfection efficiency. (E) Endogenous AXL expression is up-regulated by blocking miR-34a. CL1-0 cells were cotransfected as in D. The qRT-PCR assay was performed to determine the endogenous AXL mRNA level as described above. (F,G) The promoter activity and primary transcript expression of miR-34a are up-regulated by ELK1. CL1-5 cells were transfected with pcDNA3-ELK1, pcDNA3-ELK1 (S383D), or pcDNA3-ELK1 (S383A) plasmid along with the pGL3-AXL promoter reporter construct. The assay of luciferase reporter activity and primary transcript level of miR-34a were measured as described above. (H) Expression of AXL protein is up-regulated by ELK1. CL1-5 cells were transfected with pcDNA3-ELK1, pcDNA3-ELK1 (S383D), or pcDNA3-ELK1 (S383A) plasmid. The protein levels of AXL, ELK1 and p-ELK1 were determined by Western blot. (I) ELK1 binds to the *miR-34a* promoter in ChIP. CL1-5 cells were transfected with siAXL to knock down AXL expression. CL1-0 cells were transfected with AXL construct for AXL overexpression. ChIP assay was performed as described in the Materials and Methods section. At least three independent experiments were performed for each assay. Student's *t*-test was performed to demonstrate statistical significance. (*) $P < 0.05$.

apoptosis. CL1-5 cells were first transfected with full-length AXL construct, AXL kinase-dead mutant (K567R), or AXL intracellular domain deletion construct (del-ICD), the next day, all were transfected with an ELK1 expression vector. We found that only the wild-type AXL (full-length AXL), but not the kinase-dead AXL or the intracellular domain deletion mutant AXL, decreases poly-ADP-ribose polymerase (PARP) expression; this suggests that AXL kinase activity could attenuate ELK1-induced apoptosis (Fig. 6E). Under

conditions with knockdown expression of AXL, the protein level of cleaved PARP was drastically increased (Fig. 6D), further supporting that inhibition of AXL could trigger apoptosis.

DISCUSSION

Regulation of AXL is of great clinical interest because of AXL's important relationships with cancer invasion and

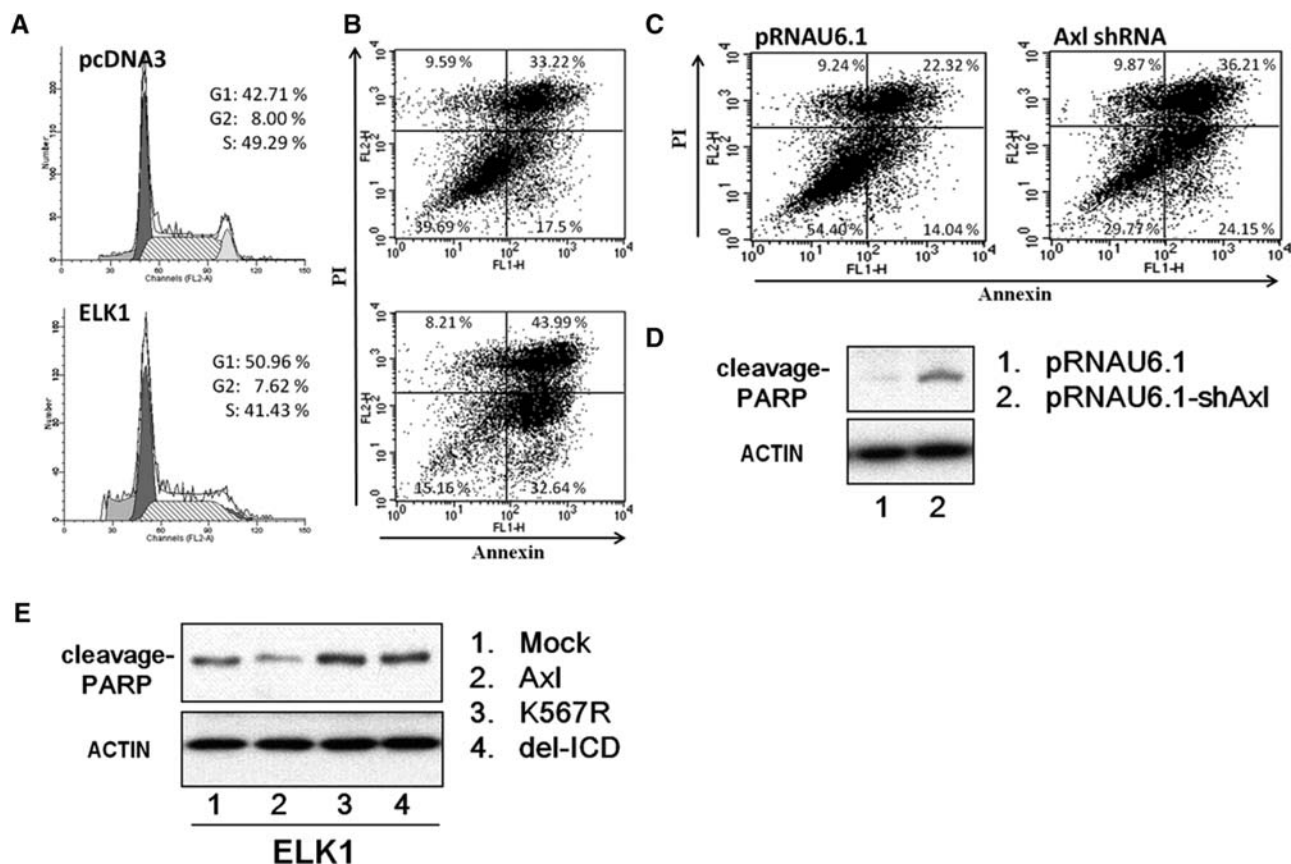


FIGURE 6. ELK1 protein induces G₁ arrest and apoptosis. (A) Cell-cycle progression of CL1-5 cells overexpressing ELK1. Cells were transfected with ELK1 or mock vector, harvested, fixed with ethanol, and stained with propidium iodide. DNA content was measured by flow cytometry. The percentage of the cell population in each of the G₁, G₂-M, and S phases was calculated. Representative plots of one set of triplicate experiments are shown. (B) Overexpression of ELK1 promotes apoptosis. Cells were transfected with ELK1 or mock vector for 48 h. Apoptosis was determined by annexin V-FITC/PI double staining and flow cytometry analysis. The percentage in the *lower right* quadrant indicates the percentage of early apoptotic cells. The percentage in the *upper right* quadrant indicates the percentage of late apoptotic cells. (C) Knockdown of AXL expression promotes apoptosis. CL1-5 cells were transfected with AXL shRNA or mock vector for 48 h. Apoptosis was determined by annexin V-FITC/PI double staining and flow cytometry analysis. The percentage in the *lower right* quadrant indicates the percentage of early apoptotic cells. The percentage in the *upper right* quadrant indicates the percentage of late apoptotic cells. (D) Western blot analysis of poly (ADP-ribose) polymerase (PARP) cleavage. Whole cell lysates were subjected to Western blotting using antibody against PARP. Actin was used as an internal control for protein loading. (E) Western blot analysis of poly (ADP-ribose) polymerase (PARP) cleavage. After transfection with various constructs of AXL for 24 h, cells were allowed to express ELK1 for 2 days prior to harvest. Total cell lysates were subjected to Western blot analysis for PARP protein. Actin was used as an internal control for protein loading. Mock, vector alone; AXL, full-length wild-type; K567R, kinase dead; delICD, deletion of the entire intracellular domain.

chemotherapy resistance. In this study, we found not only that AXL could up-regulate miR-34a expression but also that miR-34a could turn back to down-regulate AXL by directly targeting its 3' UTR. We further identified an AXL regulation feedback loop that involves ELK1-mediated up-regulation of miR-34a expression via the JNK pathway and that subsequently represses AXL expression to induce G₁ arrest and apoptosis. Although several studies have shown that miR-34a negatively regulates AXL expression (Mackiewicz et al. 2011; Mudduluru et al. 2011; Boysen et al. 2014), here we report that AXL can regulate miR-34a expression through the JNK/ELK1 pathway. Our findings suggest that the relationship between miR-34a and AXL may play a critical role in the maintaining of balanced AXL expression and may provide new insights into its role in tumor progression.

AXL affects multiple cellular behaviors including chemoresistance, epithelial-to-mesenchymal transition, and invasion/metastasis process of cancer cells via different signaling pathways (Wu et al. 2014). AXL can be activated either by Gas6, the biological ligand for AXL, or self-dimerization upon overexpression (Wu et al. 2014). Our results demonstrate that the AXL kinase domain is necessary for this feedback mechanism by using a series of AXL mutants in the reporter experiments (Fig. 2). There are three putative autophosphorylation sites; only one of them, Y779, is required for the feedback loop regulation (Fig. 2B; Supplemental Fig. 1D). In addition, residual reporter activities remained for the double and triple mutations of the Y779, Y821, and Y866 sites, when compared with that of the whole intracellular domain deletion mutation (ICD) (Fig. 2B). Therefore, we assume that there may be other

potential phosphorylation sites of AXL that regulate other factors involved in the feedback mechanism. Additionally, deletion of AXL's IG domain, which is required for Gas6 binding, significantly compromised its ability to inhibit AXL 3'-UTR reporter activity (Fig. 3A). Supporting this, the AXL ligand GAS6 was also found to be involved in the negative feedback regulation of AXL expression (Fig. 3B,C). Taken together, this feedback regulation of AXL may involve both ligand-dependent and ligand-independent pathways.

In this study, bioinformatic approaches were used to identify the miR-34 family as potential candidates that may target the AXL 3' UTR (Fig. 4C; Supplemental Table 2). Both miR-34a and miR-34c may down-regulate the AXL mRNA and protein levels (Fig. 4D,E). By cotransfecting the miR-34 family and the AXL 3'-UTR reporters containing or not containing the seed sequences targeted by the respective miRNAs, our results have shown that AXL is a real target of miR-34a (Fig. 4F), a finding consistent with the results of Mudduluru et al. (2011). However, miR-34c apparently suppressed AXL expression through its promoter rather than its 3' UTR (Fig. 4F,G), indicating that miR-34c may down-regulate AXL expression in an indirect manner. In mammalian cells, microRNAs primarily modulate gene expression by targeting 3' UTR of mRNAs. However, recent studies have indicated an alternative way of microRNAs in regulating gene expression through promoter targeting (Li et al. 2006; Place et al. 2008; Younger and Corey 2011). However, we identified no miR-34c targeting sequences in the AXL promoter region (+1 to -1726). We believe that miR-34c most likely may target a transcription factor crucial for the AXL promoter activity and thus deserves to be further investigated. Taken together, our results suggest that miR-34a directly binds on the seed region of AXL 3' UTR in controlling AXL expression, whereas miR-34c down-regulates AXL expression through indirect modulation of its promoter activity.

The results of our miRNA microarray analyses show that AXL-overexpressing cells have higher expression of miR-34a and miR-34b* (Supplemental Table 3). In addition, the *miR-34a* promoter reporter activity was also increased by AXL in a dose-dependent manner (Supplemental Fig. 5C). Although AXL kinase activity is required for up-regulation of ELK1 and expression of the primary transcript and mature miR-34a (Fig. 5B,C), ectopic overexpression of ELK1 in CL1-5 cells dramatically suppressed the reporter activity of AXL 3' UTR (Supplemental Fig. 6D). By overexpression or knockdown of ELK1, we demonstrate that ELK1 expression levels may influence ELK1's own phosphorylation, subsequently resulting in up- or down-regulation of AXL protein expression (Fig. 5H; Supplemental Fig. 6C). Moreover, down-regulation of both the AXL- and ELK1-mediated AXL 3'-UTR reporter activity could be rescued by blocking miR-34a (Fig. 5D,E; Supplemental Fig. 6D), suggesting the existence of an AXL/ELK1/miR-34a regulation loop.

It is clear that miR-34a affects cell proliferation, induces apoptosis (Chang et al. 2007; Yamakuchi et al. 2008; Lize

et al. 2010), and targets multiple oncogenes to down-regulate their expression and inhibit invasion ability in various cancers (Migliore et al. 2008; Li et al. 2009a,b; Yan et al. 2009; Corney et al. 2010; Mudduluru et al. 2011), acting like a tumor suppressor. More interestingly, a recent study reveals that miR-34a and miR-199a/b could inhibit AXL expression in solid tumors via epigenetic mechanisms (Mudduluru et al. 2011); the importance of this observation is further strengthened by several studies reporting the close relationships between AXL and miR-34a in colon carcinoma (Kaller et al. 2011), lung cancer (Lee et al. 2011), breast cancer (Mackiewicz et al. 2011), and leukemia (Boysen et al. 2014). However, recent studies also show that miR-34a is highly expressed in papillary thyroid carcinoma (PTC) and functions as an oncogenic miRNA by regulating GAS1 expression via the PI3K/Akt/Bad pathway to promote cell proliferation and prevent apoptosis (Cahill et al. 2006; Ma et al. 2013). Additionally, expression of miR-34a may also commonly be high in many human cancers (Dutta et al. 2007). As either an onco-molecule or as a tumor suppressor, miR-34a is important, and its mutual regulation with AXL as demonstrated in this study highlights its potential in cancer therapeutic opportunities. These results also implicate that the tumor-suppressive roles played by microRNAs are not as simple as they might seem. In different types of cancers, these microRNAs may be under the control of different yet complicated fine-tuning mechanisms and may target multiple genes; some may even possess opposite functions in certain different conditions. Therefore, the detailed regulatory mechanism of AXL modulated by miRNA remains to be further clarified.

Although p53 mutation rate is >50% in many human cancers, up-regulation of miR-34a may promote the cancer cells toward apoptosis regardless of the p53 status (Raver-Shapira et al. 2007; Christoffersen et al. 2010; Hermeking 2010). A recent study has shown that wild-type p53 activation negatively regulates AXL expression via miR-34a and may offer therapeutic opportunities for chronic lymphocytic leukemia B cells (Boysen et al. 2014). According to our data, phosphorylated ELK1 not only is essential for up-regulating miR-34a expression (Fig. 5F,G) but also directly binds to the *miR-34a* promoter in vivo (Fig. 5I). Because these CL1 sublines have a p53 status of R248W mutation, the regulatory mechanism described in this report is likely to be p53-independent (Tsai et al. 2014). In this study we emphasize that ELK-1 could also directly induce miR-34a expression via promoter targeting, a result consistent with that observed during oncogene-induced senescence (Christoffersen et al. 2010). Although overexpression of ELK1 led to significant G₁ arrest and apoptosis (Fig. 6A,B), knockdown of AXL expression with shRNA produced a similar effect (Fig. 6C,D). We further found that AXL kinase activity could overcome the ELK1-induced apoptosis (Fig. 6E). In line with these results, overexpression of ELK1 significantly decreased AXL expression and subsequently induced G₁ arrest and apoptosis via a miR-34a-mediated feedback loop. Recently, several reports

have indicated that miR-34a expression is silenced in various cancers because of aberrant CpG methylation on its promoter (Lodygin et al. 2008; Vogt et al. 2011). Once the *miR-34a* promoter is methylated, phosphorylated ELK1 may not bind to its promoter to inhibit AXL expression, and the feedback loop will be consequently interrupted. Dysfunction of the AXL/ELK1/miR-34a feedback loop may presumably shift the equilibrium of AXL expression from a proto-oncogene into a bona fide oncogene by overexpression during cancer progression. In the future, we plan to verify the correlation between AXL-mediated ELK1 phosphorylation and the methylation status of *miR-34a* promoter, especially the role of AXL phosphorylation at Y779 in this feedback regulation.

The present study shows that AXL up-regulates miR-34a expression via the JNK/ELK1 signal pathway and that miR-34a consequently returns to inhibit AXL expression via binding to AXL's mRNA 3'-UTR. These results demonstrate that this negative regulatory loop might play an important role in maintaining a balanced AXL expression and that malfunction of this regulatory loop may contribute to resisting apoptosis and promoting invasion and metastasis in cancer cells. For example, cancers may be more malignant because of AXL overexpression caused by miR-34a inactivation, whereas miR-34a activation by ELK1 may slow down cancer progression because of reduced AXL expression. Therefore, if we could better understand the molecular mechanism of this negative feedback loop regulation, we would be able to develop drugs that induce the expression of miR-34a and/or ELK1 to improve anticancer efficacy in clinical applications. Therefore, we believe that the insights into this feedback loop autoregulation of AXL may help to enhance the treatment efficacy of using AXL inhibitors along with chemotherapies for AXL-associated cancers.

MATERIALS AND METHODS

Cell lines, transfection, and siRNA

The CL1 sublines (i.e., CL1-0, CL1-3 and CL1-5) were established by selection for increasingly invasive cancer cells from a human lung adenocarcinoma cell line, CL1-0, as described previously (Lay et al. 2007). CL1 sublines, MCF-7, and MDA-MB-231 were cultured in RPMI; A549, HeLa, and PANC-1 were cultured in DMEM. Both media are supplemented with 10% fetal bovine serum. AXL 3'-UTR reporter (0.5 µg), AXL promoter, Renilla reporter (0.05 µg), with or without other plasmids or miRNA mimics, were transfected into 5×10^5 cells by using Lipofectamine 2000 (Invitrogen) for reporter assays. miRNA mimics (50 nM) (MDBio Inc.) and various plasmids (2 µg) were transfected into 1×10^6 cells for qRT-PCR or Western blot analysis. The siRNA sequence for ELK1: 5'-GCGGCCAGAA GUUCGUCUACAAGUU-3' (MDBio Inc.). The anti-miR-34a sequences were shown in Supplemental Table 4.

Identification of miRNA binding sites

We used miRBase, PicTar, TargetScan5.1 (Lewis et al. 2003), and miRanda (John et al. 2004) software for prediction of potential

AXL 3'-UTR sites targeted by micro-RNAs. The sequence of AXL 3' UTR is shown in Supplemental Table 2.

qRT-PCR for microRNAs quantification

To detect miR-34a expression, total RNA was isolated with TRIzol reagent (Invitrogen). The Mir-X miRNA First-Strand Synthesis Kit (BD Clontech) was used for cDNA synthesis from miRNAs. To detect endogenous AXL mRNA and primary miRNA expression, total RNA was prepared from CL1-0 cells transfected with various AXL constructs using the RNeasy Mini Kit (QIAGEN). All primer sequences used for AXL mRNA and miR-34a detection are listed in Supplemental Table 4. Actin and U6 RNA were used as the internal control for normalization of AXL and pri-miR-34a, respectively. PCR was performed in a 20 µL reaction mixture containing 10 µM forward and reverse primers, and 1× SYBER GREEN reaction mix (Takara). All reactions were performed in an ABI 7500 sequence detection system.

Plasmids construction

The AXL 3'-UTR reporters were constructed in the pMIR-Report vector by inserting full-length and various mutation or deletion forms of the AXL 3'-UTR sequences into the SpeI/SacI restriction site of the pMIR-Report vector. The *miR-34a* promoter reporters were constructed by insertion of the PCR-amplified sequences into the XhoI/HindIII site of the pGL3 vector. All primers used for these constructs are listed in Supplemental Table 4. AD-Gas6 is a constitutively active form of Gas6 with its amino-terminal Gla domain deleted. Constructs of AXL shRNA, AXL promoter, and wild-type and various mutants of AXL were as described previously (Lay et al. 2007; Hong et al. 2008; Huang et al. 2013).

The mutations of ELK1 on the S383 residue and the double/triple mutations of the AXL putative autophosphorylation sites were generated by site-directed mutagenesis. All primers used for these constructs are listed in Supplemental Table 4.

Microarray analysis of AXL-responsive miRNAs

CL1-0 cells and their stable transfectants overexpressing AXL were compared by using the Agilent human miRNA microarray of 723 human and 76 human viral miRNAs. Data were analyzed using the GeneSpring 7.3.1 program (Agilent Technologies).

Immunoblots

Primary antibodies to AXL (amino and carboxyl terminals), Gas6, ELK1, p-ELK1, and PARP1 were from Santa Cruz Biotechnology. HRP-conjugated anti-goat, anti-mouse, and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology. Anti-phospho-AXL (Y779) was made-to-order from Genetex. Anti-phospho-JNK was from Cell Signal, Inc. Antibody to actin was from Sigma-Aldrich.

Reagents

The MEK/ERK inhibitors (PD98059 and U0126) and PI3K/AKT inhibitors (Wortmannin and LY294002) were from Cell Signaling

Technology. The JNK inhibitor (SP600125), SRC inhibitor (PP2), JAK inhibitor (AG490), and P38 inhibitor (SB203580) were from Calbiochem. The NF- κ B inhibitor (BAY-11-7086) was from Santa Cruz. AXL-Fc was from R&D Systems. R428 was kindly provided by Rigel, Inc.

Chromatin immunoprecipitation (ChIP) assay

An EZ-ChIP kit and reagents from Millipore were used, according to the manufacturer's instructions, to detect the *in vivo* binding between DNA and proteins. Briefly, anti-ELK1 was incubated with the nuclear extract overnight at 4°C with rotation. After immunoprecipitation, DNA was recovered and subjected to PCR amplification by using the primers specific for detection of the +335/+125 region of the *miR-34a* promoter that contains the putative ELK1-binding site. The primer sequences used for PCR detection of the *ELK* promoter region are listed in Supplemental Table 4.

Cell-cycle analysis

At harvest, cells were collected and washed twice with PBS, resuspended in 1 mL of PBS, fixed by adding 4 mL of 100% ethanol at -20°C for 2–24 h, and then stained with a propidium iodide (PI) solution containing 50 μ g/mL PI and 50 μ g/mL RNase (Sigma-Aldrich) in PBS at room temperature for 30 min in the dark. Stained cells were passed through a nylon mesh sieve to remove cell clumps and were then analyzed by flow cytometry (BD). Data were collected and analyzed by CellQuest and ModFit LT software.

Apoptosis analysis by flow cytometry

Apoptosis was also determined using the Annexin V-FITC/propidium iodide (PI) detection kit from BD Pharmingen. At harvest, cells were washed in cold PBS, stained with Annexin V-FITC and PI, following the manufacturer's instructions. The cells were then analyzed by flow cytometry.

Statistical analysis

Quantitative PCR and reporter assays data are shown as mean \pm SD. Differences between control and treatment groups were analyzed by Student's *t*-test. All experiments were performed in triplicate and repeated at least three times and *P*-values <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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