LukS-PV Induced HOXA9 Degradation in Acute Myeloid Leukemia Cells via Autophagy

Ping Qiang
Anhui Provincial Hospital

Chao Fang
Anhui Provincial Hospital

Kaidi Song
Anhui Provincial Hospital

Lan Shi
Anhui Provincial Hospital

Yuanyuan Dai
Anhui Provincial Hospital

Wenjiao Chang
Anhui Provincial Hospital

Liangfei Xu
Anhui Provincial Hospital

Xiaoling Ma (maxiaoling@ustc.edu.cn)
Anhui Provincial Hospital

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Abstract

Aberrant over-expression of HOXA9 is a prominent feature of AML driven by multiple oncogenes, thus therapeutic degradation of HOXA9 by autophagy may be an effective treatment strategy for AML. PVL is a pore forming cytotoxin secreted by Staphylococcus aureus, and it is composed of two subunits - LukS-PV and LukF-PV. Here, we show that LukS-PV can stimulate the conversion of LC3-I to LC3-II in AML cells in a concentration-dependent manner, and autophagic vacuoles can be found in LukS-PV-treated THP-1 cells. Furthermore, we find that an accumulation of LC3-positive structures in AML cells exposed to LukS-PV, indicating that an increased autophagic flux were formed. Therefore, LukS-PV induced autophagy of AML cells. We also demonstrated that LukS-PV could regulate the expression of HOXA9 at the protein level. HOXA9 molecules were detected in autophagosomes after LukS-PV treatment, indicating that autophagy induction accounted for the degradation of HOXA9.

Background

The HOX family is one of the most important homeodomain-containing transcription factors and the dysregulation of HOX genes results in numerous developmental malignancies 1. HOXA9 is a well-known transcription factor that plays a key role in blood cell development, proliferation, differentiation, and increased malignancy of leukemic cells2,3. Aberrant over-expression of HOXA9 is a prominent feature of AML driven by multiple oncogenes, and it is a predictor of poor outcomes. Previous studies have confirmed that continuous HOXA9 expression is required for AML cell survival, especially in human MLL-rearranged leukemia4,5. When the lentiviral-based shRNA approach was used to inhibit the HOXA9 expression in human AML cells, an almost immediate reduction of proliferative capacity occurred, followed by a progressive induction of apoptosis2,3. The effect of induced apoptosis is more pronounced in AML cells carrying an MLL-fusion than with MLL germline karyotype2. HOXA9 was necessary for maintaining leukemic transformation, thus therapeutic degradation of HOXA9 may be an effective treatment strategy for AML2,3,6-9. To date, HOXA9 inhibitors have not been developed because there are no binding pockets for direct targeting. The degradation of HOXA9 oncoproteins has recently created new pathways for therapeutic options in AML.

PVL is a pore forming cytotoxin secreted by Staphylococcus aureus, and it is composed of two subunits - LukS-PV and LukF-PV10. Previous studies have established the anti-leukemic activity of LukS-PV in AML11,12, but the role of LukS-PV in the degradation of oncoproteins has not been determined. In this study, we demonstrated that LukS-PV activated autophagy. Moreover, we also demonstrated that LukS-PV induced HOXA9 degradation through autophagy, and that the removal of HOXA9 was related to the increased autophagic activity. To our knowledge, this study was the first time where a link between LukS-PV and HOXA9 degradation of leukemic cells was functionally demonstrated.

Methods
Ethical approval and consent to participate

Ethical approval to undertake this study was examined by the Research Ethics Committee. A written informed consent for publication was obtained from each patient.

Recombinant LukS-PV production and purification

Recombinant LukS-PV production and purification were described previously by Ma et al.\textsuperscript{13}

AML patient selection and cell lines

THP-1, NB4, HL60 and OCI-AML3 cell lines were purchased from the Shanghai Institute for Biological Sciences (SIBS, Shanghai, PR, China). From January, 2015 through January, 2020 at The First Affiliated Hospital of University of Science and Technology of China, a total of 18 newly diagnosed and untreated adult AML patients [age range 16 - 68 years; male, 55.6\%] were recruited, together with 8 control subjects [age range 26 - 70 years; male, 62.5\%]. BM specimens were obtained before chemotherapy. The cells were separated using a lymphocyte separation medium (TBD, China), and then purified by anti-CD33 and anti-CD34 magnetic-activated cell sorting (MACS) separation columns (MiltenyiBiotec, Bisley, United Kingdom). The Trypan Blue dye exclusion test was used to determine the viability of the cells. Cells with >95\% viability were used in the study.

Antibodies and reagents

LC3B (D11, \# 3868S), HOXA9 (\# ab140631), anti-HOXA9/AF 350 (\# bs-6667R-AF350), 3-Methyladenin (3-MA, \#M9281) and Bafilomycin A1 (Baf-A1, \#S1413) were obtained from Cell Signaling Technology, Abcam, Bioss, Sigma and Selleck, respectively.

RNA extraction, real-time PCR and HOXA9 mRNA expression analysis

Total RNA was extracted from cell lines and human BM specimens using Trizol (Invitrogen). Reverse transcription and quantitative real-time PCR were performed using the SYBR Green PCR Kit (GenePharma, Shanghai, PR China) to determine HOXA9 and GAPDH mRNA expression. The primer sequences were as follows: HOXA9 forward: 5'--CACCAGACGAACAGTGAGGA-3'; reverse: 5'-TGGTCAGTAGGCCTTGAGGT-3'. The relative mRNA expression was analyzed using the 2$^{-\Delta\Delta C_T}$ method.

Apoptosis Assay

All of the cells treated with or without LukS-PV were harvested and analyzed for apoptosis using the Apoptosis Assay Kit (keyGEN, China). All of the cells were treated with Annexin V-PE and 7AAD(eBioscience, CA, USA) according to the manufacturer's instructions. Finally, the data were analyzed on aBD FACSCalibur.

mRFP-GFP-LC3 transfection and immunofluorescence staining
Adenoviruses encoding both mRFP-GFP and LC3 proteins were obtained from HanBio Technology (Shanghai, China). GFP was shown as red puncta while mRFP was shown as green puncta, based on the principle that GFP and mRFP are stable at different pH levels. At an acidic pH, the fluorescence of mRFP is stable, while the fluorescence of GFP is decreased. The fluorescence of mRFP was stable at an acidic pH inside the lysosome, while the fluorescence of GFP decreased. Therefore, in images where the green and red puncta merged, the merged yellow colored puncta indicated mRFP-GFP-LC3 (i.e., RFP + GFP +) in autophagosomes, while red puncta indicated mRFP-GFP-LC3 (i.e., RFP + GFP -) in autolysosomes. When the yellow and red puncta were simultaneously increased in cytoplasm, autophagic flux was increased.

THP-1 and primary AML cells were first seeded into 96-well plates at a final density of 5×10^4 cells per well, followed by transfection with adenoviruses according to the manufacturers' instructions. Autophagosomes were examined with a Zeiss LSM710 confocal microscope (Zeiss).

**Western blotting**

Proteins were resolved using 10% SDS-polyacrylamide gels and electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in Tris-buffered saline 0.1% Tween-20 solution with 10% milk, the membranes were probed with an appropriate amount of primary antibodies and horseradish peroxidase-conjugated secondary antibodies, and then visualized with an enhanced chemoluminescence detection system.

**EDU assay**

Cell proliferation was assessed by 5-ethynyl-2'-deoxyuridine (EdU) assay (KeyGenBioTECH, JiangSu, China) according to the manufacturer's instructions. The cells were analyzed by fluorescence activated cell sorter Calibur Flow Cytometer (BD Pharmingen, San Diego, CA).

**Cell counting kit-8 assay**

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo), following the manufacturer's instructions. Cells were first grown in 96-well plates at a final density of 5000 cells (200μL) per well, and then incubated with different reagents under different conditions. Afterwards, 20μl of CCK-8 solution was added to each well to culture the cells for 1-2 hours, and the absorbance was subsequently measured in a microplate reader at 450 nm (OD450nm). All CCK-8 measurements were performed in triplicate.

**Statistical analysis**

The data from different groups were analyzed with the unpaired two-tailed Student's t-test using SPSS (version 24), R language, and GraphPad Prism (version 7) software. A log-rank test was used for survival analysis and the Kaplan-Meier survival curve was applied to assess the overall survival (OS) in AML patients.

**Results**
1. HOXA9 was overexpressed in MLL-rearranged AML and acted as a predictor of poor outcomes

Previous studies have confirmed that continuous HOXA9 expression is required for AML cell survival, especially in human MLL-rearranged leukemia 4,5. In order to investigate the expression of HOXA9 in AML cell, we first used the human leukemia cell lines THP-1, NB4, HL60 and OCL-AML3. The results showed that HOXA9 was highly expressed in human leukemia cell lines THP-1, HL60 and OCL-AML3, especially in THP-1 cells (Fig 1A). Then, we investigate the expression of HOXA9 in 2 cases of 11q23/MLL gene rearrangement AML cells, and also found that HOXA9 was over-expressed (Fig 1A). In addition, 8 healthy controls and 18 newly diagnosed adult AML patients were recruited. The HOXA9 expression in healthy controls was compared with that of AML patients. It was discovered that the expression of HOXA9 was significantly higher in the AML patients than in the controls (P<0.0001, Fig 1B). Moreover, compared with AML without MLL rearranged, the expression of HOXA9 was significantly increased in AML patients bearing MLL rearrangement (P<0.01, Fig 1 C). Finally, publicly available clinical and RNA sequencing information of 135 AML samples were obtained from the TCGA database. We found that AML patients with higher HOXA9 expression had worse OS (P<0.05, Fig 1D). These results indicated that HOXA9 acted as a predictor of poor outcomes, and therapeutic degradation of HOXA9 may be an effective treatment strategy for AML.

Figure 1. HOXA9 expression in AML cells

(A) HOXA9 expression in primary AML cells. Whole-cell lysates from primary AML cells were analyzed for HOXA9 protein expression via western blotting. (B) The Kaplan-Meier analysis of the OS of AML patients. The HOXA9 RNA-sequencing data and survival information were obtained from the TCGA database. According to the expression of HOXA9, AML patients were dichotomized into high (above median expression levels, n=67) and low (below or at median expression levels, n= 68) groups. (C) Quantitative real-time PCR analysis of HOXA9 mRNA levels of AML patients and healthy controls. (D) The mean expression of HOXA9 was higher in primary AML patients bearing MLL rearrangement. The statistical significance calculated by unpaired Student's t-test.

2. LukS-PV induced HOXA9 down-regulation at the protein level

Though we have established the in vitro anti-leukemic activity of LukS-PV in AML cells, it has not been investigated in the genetic context of AML. Thus, we assessed the anti-leukemic activity of LukS-PV, according to the HOXA9 expression. THP-1 cell was associated with the monocytic AML-M5 cell line carrying t(9;11), and mimicked several clinical features of AML blast cells with the MLL/AF9 oncprotein. So, in order to investigate the role of LukS-PV in the degradation of HOXA9, we first tested the human leukemia cell lines THP-1. It was found that LukS-PV remarkably down-regulated the expression of the HOXA9 protein in THP-1 cells in a time- and dose-dependent manner (Fig 2A-B). It was further confirmed that LukS-PV affected HOXA9 protein expression in primary AML cells (Fig 2C). Taken together, LukS-PV induced HOXA9 down-regulation at the protein level.

Figure 2. HOXA9 protein expression is downregulated by LukS-PV
(A) HOXA9 expression in primary AML cells and a series of AML cell lines. Whole-cell lysates from primary AML cells and a series of AML cell lines were analyzed for HOXA9 protein expression via western blotting. Anti-actin and anti-HOXA9 were applied in Western blotting analysis. (B) THP-1 cells were treated with 1 μM LukS-PV for 8 to 24 hours. (C) THP-1 cells were treated 16 hours with LukS-PV at different concentrations. (C) Primary AML cells were treated with 1 μM LukS-PV for 16 hours.

3. HOXA9 sensitized AML cells to LukS-PV-induced autophagy

In order to assess the autophagy-promoting activity of LukS-PV in AML cells, the microtubule-associated protein 1 light chain 3 (LC3) in a series of AML cell lines and primary AML cells were studied by western blotting. During autophagy, cytosolic LC3 (LC3-I, upper band) was converted to autophagic membranes (LC3-II, lower band). The LC3-II level was specifically associated with autophagosomes, which presented a good evaluation method for autophagic activity. As can be seen in Fig 3A, LukS-PV increased the LC3-II levels in NB4, OCL-AML3, and THP-1 cells, indicating that LukS-PV activated autophagy. Interestingly, THP-1 cell was more sensitive than other cell lines, suggesting that the HOXA9 expression may determine susceptibility to LukS-PV-induced autophagy. Moreover, LukS-PV increased the LC3-II levels in THP-1 in a concentration-dependent manner and primary AML samples (Fig 3B). LukS-PV also increased the LC3-II levels in primary AML cells (Fig 3C). Furthermore, the addition of the lysosomal protease inhibitor bafilomycin A1 (BafA1) to LukS-PV treatment further increased the amount of LC3-II (Fig 3D). Since the cytoplasmic contents of autophagosomes are surrounded by double-membrane vacuolar structures, high-resolution TEM was used to identify autophagosomes. As indicated by Fig 3E, autophagic vacuoles can be found in LukS-PV-treated THP-1 cells but not in phosphate buffered saline (PBS) control cells.

Finally, in order to verify the role of LukS-PV in controlling autophagic flux, the flux rate of autophagy was examined with a Zeiss LSM710 confocal microscope using an mRFP-GFP-LC3 reporter construct. THP-1 and primary AML cells were infected with mRFP-GFP-LC3 adenoviral particles. The effect of LukS-PV on the sub-cellular distribution of LC3 protein in THP-1 and primary AML cells was examined by immunofluorescence microscopy, which demonstrated an accumulation of LC3-positive structures in THP-1 and primary AML cells exposed to LukS-PV, indicating that an increased number of autophagosomes were formed (Fig 3F-G). Moreover, we detected more ectopically expressed mRFP-GFP-LC3 as red spots with occasional yellow spots in LukS-PV-treated cells than in untreated cells. The results suggested that LukS-PV induced autophagic flux. Taken together, LukS-PV induced autophagy of THP-1 and primary AML cells.

Figure 3. Activation of autophagy by LukS-PV in AML cells.

(A-D) LC3-II accumulation after LukS-PV exposure. (A) A series of AML cell lines cells were treated with 2.00 μM LukS-PV for 2 hours. Actin and LC3 antibodies were used as reagents in WB analysis. (B) THP-1 cells were treated with LukS-PV for 2 hours at different concentrations. (C) Primary AML cells were treated with 2.00 μM LukS-PV for 2 hours. (D) LukS-PV induced an active autophagic flux. THP-1 and primary AML cells were incubated for 16 hours with 2.00 μM of LukS-PV alone or in combination with...
100nM BafA1. (E) TEM of autophagosomes. THP-1 was treated with or without 2.00 μM of LukS-PV for 2 hours. (F-G) The autophagic flux rate of autophagy was examined with a Zeiss LSM710 confocal microscope using an mRFP-GFP-LC3 reporter construct, based on the principle that GFP and mRFP fluorescent proteins are stable at different pH levels. THP-1 and primary AML cells were incubated with adenoviruses encoding both mRFP-GFP and LC3 protein for 24h and then with 2.00 μM of LukS-PV for 16h. A Zeiss Apotome microscope was used to analyze the image. At least 100 cells were counted for quantification of LC3-positive dots and the experiment was repeated 3 times. * = P< 0.05.

4. LukS-PV induced HOXA9 degradation through autophagy

Previous studies have established that HOXA9 is required for the survival of AML cells with MLL gene rearrangement, and HOXA9 depletion by RNA interference immediately reduced the cell proliferative capacity in vivo and in vitro 2. Autophagy is a multistep lysosomal degradation pathway, we investigated the role of autophagy in the degradation of HOXA9 after LukS-PV treatment by first assessing the impact of autophagy on HOXA9 expression. It was confirmed that LukS-PV affected HOXA9 protein expression in THP-1 and primary AML cells. It can be seen from Fig 4A-B that the addition of the lysosomal protease inhibitor 3-Methyladenine partially restored HOXA9 expression in LukS-PV-treated cells. Moreover, according to immunofluorescence analysis, HOXA9 molecules colocalized with LC3 positive structures in THP-1 and primary AML cells treated with 2.00 μM LukS-PV (Fig 4C-D). The study revealed that HOXA9 molecules were detected within autophagosomes after LukS-PV treatment, indicating that HOXA9 could be degraded through LukS-PV-induced autophagy.

Figure 4. LukS-PV induced autophagy to reduce HOXA9 expression in AML cells.

(A-B) THP-1 cells or primary AML samples were incubated for 16 hours with 2.00 μM of LukS-PV alone or in combination with 5nM of 3-MA. Anti-actin and anti-HOXA9 were applied in Western blotting analysis. (C-D) Colocalization of HOXA9 and LC3-positive structures. THP-1 and primary AML cells were incubated first with adenoviruses encoding both mRFP-GFP and LC3 proteins for 24h and then with 2.00 μM of LukS-PV or PBS for 16h. The cells were analyzed by immunofluorescence with HOXA9 antibodies. A Zeiss Apotome microscope was used to analyze the images. * = P< 0.05.

Discussion

In this study, we focused on the LukS-PV-regulating autophagy pathway, aiming to investigate the role of LukS-PV in HOXA9-dependent leukemia. The biochemical and microscopy results from our study suggested that LukS-PV induced cytotoxic autophagy in AML cells. HOXA9 molecules were detected in autophagosomes after LukS-PV treatment, indicating that autophagy induction accounted for the degradation of HOXA9. In conclusion, our in vitro results showed that LukS-PV could activate autophagy and induce HOXA9 degradation.

One of the most difficult challenges in cancer treatment is the occurrence of chemoresistance, which necessitates the development of newer therapies and small-molecule targeting drugs for AML patients.
Bacterial toxins play an important role in either altering the tumor's cellular biological processes or inducing disease regression by directly killing tumor cells\textsuperscript{15,16}. Several bacterial toxins have been approved by the Food and Drug Administration (FDA) as cancer therapies due to their safety and anticancer effects\textsuperscript{17}. For example, diptheria toxin-related interleukin 2 fusion protein, also called DenileukinDiftitox, was the first bacterial immunotoxin approved by FDA for the treatment of adult T-cell leukemia/lymphoma (ATL)\textsuperscript{18}. PVL is a pore forming cytotoxin secreted by Staphylococcus aureus, which is composed of LukS-PV and LukF-PV subunits\textsuperscript{10}. Low concentrations of PVL resulted in AML cell apoptosis, while high concentrations resulted in cell lysis\textsuperscript{19}. PVL activated caspase-9 and caspase-3, and induced human neutrophil apoptosis by the Bax-independent mitochondrial pathway\textsuperscript{20}. Previous studies have established the anti-leukemic activity of LukS-PV in AML\textsuperscript{11,12}. LukS-PV induced cell-cycle arrest through changes in cell-cycle regulatory proteins, and inhibited cell viability in HL-60 cells. When treated with LukS-PV, a significant S phase arrest was observed, and the expression of CDK2 and cyclin-A was increased\textsuperscript{11}. In vitro, LukS-PV could induce apoptosis in AML cells in a dose- and time-dependent manner, through a tumor necrosis factor (TNF) receptor-independent and caspase-8-dependent mechanism. LukS-PV could also up-regulate microRNA-125a3p and promote apoptosis by down-regulating the expression of NF1 and Bcl-2 in vivo and in vitro\textsuperscript{21}. The human complement receptor C5aR is high in myeloid cells with low expression in lymphocytes, and it acts as a host target for LukS-PV\textsuperscript{22}. Therefore, LukS-PV is expected to be a potential C5aR-mediated small-molecule targeting drug for AML.

Previous studies have demonstrated that LukS-PV has a good anti-leukemia effect and it could potentially be a promising therapy for adult acute myeloid leukemia (AML). Furthermore, aberrant over-expression of HOXA9 is a prominent feature of AML driven by multiple oncogenes, and it is a predictor of poor outcomes. HOXA9 was necessary for maintaining leukemic transformation, thus therapeutic degradation of HOXA9 may be an effective treatment strategy for AML. Autophagy is one of the two major clearance mechanisms that govern the regulation of intracellular proteolysis and it may be useful to investigate if autophagy can play a role in cancer treatment, such as by investigating a connection between autophagy and HOXA9. However, a connection between autophagy and HOXA9 has not yet been reported in the research literature.

Autophagy is a multistep lysosomal degradation pathway that supports metabolic adaptation and nutrient recycling and it can affect the tumor immune microenvironment. It is increasingly clear that autophagy induction may restrain the growth of tumors and improve the response to tumor therapy\textsuperscript{23}. For example, the drug Bortezomib killed AML cells by inducing the degradation of FLT3-ITD oncoprotein through an autophagy-dependent mechanism\textsuperscript{14}. ATO and ATRA induced autophagy via the mTOR pathway in PML/RARα-rearranged APL cells, and autophagic degradation contributed significantly to the therapy-induced proteolysis of PML/RARA oncoprotein\textsuperscript{24}.

In this paper, we showed that LukS-PV could regulate the expression of HOXA9 at the protein level through induction of autophagy. Our study is the first to functionally demonstrate a link between HOXA9 degradation and autophagy in leukemic cells. Previous research has established that inhibiting HOXA9 expression in MLL-rearranged acute leukemia cells by a lentiviral-based shRNA approach rapidly reduced
the proliferative capacity of the cells. Hence, RNA interference was not adopted to inhibit the expression of HOXA9 in THP-1 and primary AML cells in this study. Our study uncovered more details as to why AML cells with HOXA9 over-expression are sensitive to LukS-PV, and will provide a mechanism-based rationale for the study of LukS-PV in AML.

**Declarations**

**Funding**

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**Conflicts of interest**

The authors declare that they have no competing interests.

**Availability of data and materials**

All of the data are available from the corresponding author on reasonable request.

**Authors’ contributions**

PQ, CF, KS, LS, YD WC, and LX performed all of the experiments. XW, XL, HL, ZS, and XM designed the study. PQ analysed the data, and wrote the paper. XM provided financial support and final approval of the manuscript. All authors approved and commented on the manuscript.

**Consent for publication**

Not applicable.

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Figures
Figure 1

HOXA9 expression in AML cells (A) HOXA9 expression in primary AML cells. Whole-cell lysates from primary AML cells were analyzed for HOXA9 protein expression via western blotting. (B) The Kaplan-Meier analysis of the OS of AML patients. The HOXA9 RNA-sequencing data and survival information were obtained from the TCGA database. According to the expression of HOXA9, AML patients were dichotomized into high (above median expression levels, n=67) and low (below or at median expression levels, n=68) groups. (C) Quantitative real-time PCR analysis of HOXA9 mRNA levels of AML patients and healthy controls. (D) The mean expression of HOXA9 was higher in primary AML patients bearing MLL rearrangement. The statistical significance calculated by unpaired Student’s t-test.
HOXA9 protein expression is downregulated by LukS-PV (A) HOXA9 expression in primary AML cells and a series of AML cell lines. Whole-cell lysates from primary AML cells and a series of AML cell lines were analyzed for HOXA9 protein expression via western blotting. Anti-actin and anti-HOXA9 were applied in Western blotting analysis. (B) THP-1 cells were treated with 1 μM LukS-PV for 8 to 24 hours. (C) THP-1 cells were treated 16 hours with LukS-PV at different concentrations. (C) Primary AML cells were treated with 1 μM LukS-PV for 16 hours.
Figure 3

Activation of autophagy by LukS-PV in AML cells. (A-D) LC3-II accumulation after LukS-PV exposure. (A) A series of AML cell lines cells were treated with 2.00 μM LukS-PV for 2 hours. Actin and LC3 antibodies were used as reagents in WB analysis. (B) THP-1 cells were treated with LukS-PV for 2 hours at different concentrations. (C) Primary AML cells were treated with 2.00 μM LukS-PV for 2 hours. (D) LukS-PV induced an active autophagic flux. THP-1 and primary AML cells were incubated for 16 hours with 2.00 μM LukS-PV alone or in combination with 100nM BafA1. (E) TEM of autophagosomes. THP-1 was treated with or without 2.00 μM of LukS-PV for 2 hours. (F-G) The autophagic flux rate of autophagy was examined with a Zeiss LSM710 confocal microscope using an mRFP-GFP-LC3 reporter construct, based on the principle that GFP and mRFP fluorescent proteins are stable at different pH levels. THP-1 and
primary AML cells were incubated with adenoviruses encoding both mRFP-GFP and LC3 protein for 24h and then with 2.00 μM of LukS-PV for 16 h. A Zeiss Apotome microscope was used to analyze the image. At least 100 cells were counted for quantification of LC3-positive dots and the experiment was repeated 3 times. * = P< 0.05.

Figure 4
LukS-PV induced autophagy to reduce HOXA9 expression in AML cells. (A-B) THP-1 cells or primary AML samples were incubated for 16 hours with 2.00 μM of LukS-PV alone or in combination with 5nM of 3-MA. Anti-actin and anti-HOXA9 were applied in Western blotting analysis. (C-D) Colocalization of HOXA9 and LC3-positive structures. THP-1 and primary AML cells were incubated first with adenoviruses encoding both mRFP-GFP and LC3 proteins for 24h and then with 2.00 μM of LukS-PV or PBS for 16h. The cells were analyzed by immunofluorescence with HOXA9 antibodies. A Zeiss Apotome microscope was used to analyze the images. * = P< 0.05.