Apelin-13 induces MCF-7 cell proliferation and invasion via phosphorylation of ERK_{1/2}

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Received January 1, 2015; Accepted June 22, 2015

DOI: 10.3892/ijmm.2015.2265

Abstract. Apelin-13 is extensively expressed in various tissues, particularly breast tissue. Apelin-13 has been shown to promote tumor proliferation in various types of cancer, including hepatocellular, lung and ovarian cancer. However, the effect and molecular mechanism of apelin-13 in breast cancer cells remains unclear. The present study investigated the effect of apelin-13 on MCF-7. Therefore, cell proliferation was determined by MTT and flow cytometry analysis. The results revealed that apelin-13 markedly increased cell proliferation. Transwell assays demonstrated that apelin-13 increased MCF-7 cell invasion. Apelin-13 also markedly increased the expression of cyclin D1, extracellular matrix metalloproteinase-1 and amplified in breast cancer 1 (AIB1) in a dose-dependent manner by polymerase chain reaction assays. To study the molecular mechanism, cell proliferation, invasion and cyclin D1 were inhibited by pre-treatment with $10 \,\mu\text{M}$ of PD98059 (ERK_{1/2} inhibitor). Western blotting results suggested that apelin-13 significantly enhances the expression of p-ERK_{1/2} in a concentration-dependent manner. In conclusion, the results suggest that apelin-13 promoted MCF-7 cell proliferation and invasion via the ERK_{1/2}/AIB1 signaling pathway.

Introduction

Apelin was first identified as an endogenous ligand for the orphan G protein-coupled receptor APJ from bovine stomach extracts in 1998 (1). Apelin/APJ are widely expressed in various tissues, including the heart, lung, liver, brain and bone (2). They have multiple biological activities, including regulation of food intake, blood pressure and endoplasmic reticulum (ER) stress (3-6). Several studies have shown that

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Key words: apelin-13, proliferation, invasion, amplified in breast cancer 1, extracellular signal-regulated kinase

apelin was abundantly expressed in malignant carcinoma tissues, where it promoted the occurrence, proliferation and metastasis of cancer cells. Li *et al* found that apelin-13 increased vascular smooth muscle cell proliferation by upregulating the expression of cyclin D1, which was involved in an ERK-dependent activation of the Jagged-1/Notch3 signaling pathway (7). Apelin is associated with tumor cell proliferation, invasion and tube formation *in vitro* (8-10). Apelin-13 could induce lung adenocarcinoma cell proliferation and autophagy via ERK_{1/2} pathway (9). Apelin may be associated with angiogenesis and tumorigenesis. Recent studies demonstrated that a high level of apelin mRNA is expressed in a human breast carcinoma cell line (11). However, the mechanism of apelin-13 in breast cancer remains unknown.

The cell cycle is one of the most important activities in cell life processes; the G₁ phase activation is the critical process. Cyclin D1 is responsible for cell cycle progression in the transition from G₀/G₁ to S phase. The overexpression of cyclin D1 was identified in lung and breast cancer. Cyclin D1 may have an important role in the generation and development of human breast cancer. Extracellular matrix metalloproteinases (MMPs) are a family of Zn²⁺-dependent enzymes. MMP-1 (collagenase I) specifically degrades collagen I, a major component of the extracellular matrix (ECM). In a previous study, overexpression of MMP-1 was associated with advanced stages of breast cancer and may be a predictive marker for invasive disease (12). In the present study, the correlation of apelin-13, cyclin D1 and MMP-1 in MCF-7 cells was evaluated.

The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator that is overexpressed in breast cancer. AIB1 has been identified as amplified in 2-10% of human breast cancer tumors and overexpressed in 30-60% (13-17). The correlation between AIB1 and proliferation has also been shown for prostate (18), esophageal squamous cell (19) and urothelial cancer (20). AIB1 can interact with other signaling pathways (21-23). Therefore, the association between AIB1 and apelin-13 was investigated, as well as their effect on proliferation and invasion with or without PD98059 treatment.

The present study reported that apelin-13 stimulated the proliferation of MCF-7 and promoted the expression of AIB1 and cyclin D1. The $ERK_{1/2}$ inhibitor PD98059 attenuated the expression of cyclin D1, AIB1 and MMP-1 and prevented apelin-13-induced MCF-7 proliferation and invasion. The

study provided evidence that the effects of apelin-13 on MCF-7 proliferation and invasion were partly mediated by the ERK/AIB1/cyclin D1/MMP-1 signaling pathways.

Materials and methods

Cell culture. MCF-7 human breast adenocarcinoma cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units penicillin and 100 µg streptomycin (all from Gibco-BRL, Gaithersburg, MD, USA). The cells were maintained in the growing medium in a humidified 5% CO₂ atmosphere at 37°C.

MTT assay. MCF-7 cells were cultured in 96-well plates $(5x10^4 \text{ cells})$ and were synchronized for 24 h in DMEM containing 0.1% FBS. After treatment with (0.01, 0.1 and 10 μ M) apelin-13 or pre-treatment with PD98059 (Sigma, St. Louis, MO, USA) for 1 h followed by the addition of 10 μ M apelin-13 for 24 h, the cells were incubated with MTT solution (Invitrogen Life Technologies, Carlsbad, CA, USA) for 4 h at 37°C. Non-reduced MTT was removed by aspiration and the formazan crystals were dissolved in dimethyl sulfoxide (150 μ l/well) for 30 min at 37°C. The formazan was spectroscopically quantified at 490 nm using a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay. MCF-7 cells in 3 ml of DMEM with 10% FBS were plated in a 6-well plate at a density of $1x10^6$ cells/well and incubated at 37° C with 5% CO₂. After 24 h, the cells were treated with apelin-13 (0.01, 0.1 and $10~\mu$ M) in serum-free medium and incubated for 12 h under similar conditions. BrdU (Amresco LLC, Solon, OH, USA) was added to the culture medium 1 h before the end of the 24-h incubation period, cells were collected and each cell sample was subsequently analyzed by flow cytometry.

Tumor invasion assay. The invasion of MCF-7 cells was measured using Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). After 12 h of serum depletion, MCF-7 cells were detached and resuspended with DMEM containing 0.1% bovine serum albumin (BSA) and added to the upper compartment with 2x10⁵ cells per each Transwell insert. The lower compartment of each well contained 0.5 ml of DMEM with 0.1% BSA with apelin-13 (0.01, 0.1 and 10 μ M) or pre-treatment with PD98059 for 1 h followed by the addition of 10 μ M apelin-13. After 6 h of incubation, the upper compartment was fixed and stained with 0.1% crystal violet (Zhuo Kang Biological Technology Co., Ltd., Shanghai, China) for 10 min. The number of invasion cells in 10 fields per filter was counted using a microscope (magnification, x100). Crystal violet was eluted with 33% acetic acid, and the eluent optical density value was measured at 570 nm using the Bio-Rad Microplate Reader.

RNA extraction. Total RNA from MCF-7 cells was extracted with TRIzol reagent (Invitrogen Japan K.K., Tokyo, Japan) following the manufacturer's instructions, and subsequently frozen at -20°C in RNase-free water [Takara Biotechnology (Dalian) Co., Ltd., Liaoning, China]. Single-stranded cDNA was reverse transcribed from total RNA (500 ng) using a First

Strand cDNA Synthesis kit for RT-PCR [Takara Biotechnology (Dalian) Co., Ltd.] and a random primer. Reverse transcription conditions were 1 cycle of 30°C for 10 min, 45°C for 30 min, 95°C for 5 min, and finally 5°C for 5 min.

Reverse transcription polymerase chain reaction (RT-PCR). cDNA (1 µg) was reverse-transcribed according to the manufacturer's instructions [Takara Biotechnology (Dalian) Co., Ltd.]. The sequences for the sense and antisense primers respectively were: Cyclin D1, 5'-GATGCCAACCTCCTC AACGAC-3' and 5'-CTCCTCGCACTTCTGTTCCTC-3' (171 bp); MMP-1, 5'-CCTTCTACCCGGAAGTTGAG-3' and 5'-TCCGTGTAGCACATTCTGTC-3' (158 bp); AIB1, 5'-CCA GCTGCACACTCTTCTCA-3' and 5'-TAAGAAAACCCT GCTGGGGC-3' (488 bp); and GAPDH, 5'-TCGGAGTCA ACGGATTTGGTCGTA-3' and 5'-TGGCATGGACTGTGG TCATGAGTC-3' (525 bp). The cyclin D1 and MMP-1 amplification program consisted of 5 min for initial denaturation at 95°C followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and 7 min for the final extension at 72°C. The AIB1 and GAPDH amplification program consisted of 5 min for initial denaturation at 95°C followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec and 7 min for the final extension at 72°C. The PCR products underwent electrophoresis in a 1.5% (w/v) agarose gel (Biowest, Barcelona, Spain), with a thickness ~0.5 cm. The gel imaging was scanned and the optical density of the electrophoretic bands was determined using BandScan 5.0 software (Glyko, Novato, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). MCF-7 cells were plated at a density of 2x10⁵ cells/well in 96-well plates. The concentrations of MMP-1 and cyclin D1 were quantified by ELISA kits (Invitrogen Life Technologies). The colorimetric reaction was measured using a Microplate Reader (Bio-Rad) at 450 nm wavelength.

Western blotting. To assay the levels of protein expression and phosphorylation of p44/42 ERK $_{1/2}$ and β -actin, MCF-7 cells were cultured in 6-well plates and treated with 0.01, 0.1 and 10 μ M of recombinant apelin-13 or pre-treatment with PD98059 for 1 h followed by the addition of 10 μ M apelin-13 for 12 h. For immunoblotting, cells were washed twice with ice-cold phosphate-buffered saline and subsequently lysed in radioimmunoprecipitation assay buffer with a cocktail of protease inhibitors on ice for 15 min. Following centrifugation at 13,000 x g for 20 min at 4°C, the quantity of protein in the supernatants was detected using the bicinchoninic acid Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd., Shanghai, China). The protein samples were separated by SDS-PAGE and were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies specific for phospho-p44/42 ERK_{1/2} (1:2,000; #4370) and β -actin (1:2,000; #4970) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Blots underwent three 8 min washes with Tris-buffered saline with 0.1% Tween-20 and were incubated with secondary antibodies IgG (1:8,000; #14708; Cell Signaling Technology, Inc.) for 1 h. The membranes were washed as above and the

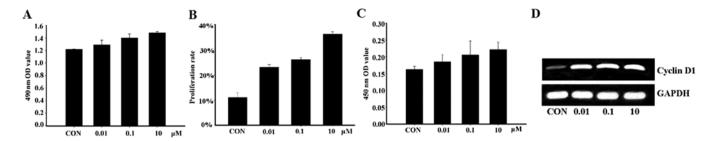


Figure 1. Effects of apelin-13 on MCF-7 cell proliferation and cyclin D1. (A) The MCF-7 cell proliferation was measured by the MTT assay. The optical density (OD) value was measured at 490 nm following treatment with apelin-13 for 24 h. (B) The effects of apelin-13 on MCF-7 proliferation rate. (C) Determination of expression by human cyclin D1 ELISA kit following treatment with exogenous apelin-13 for 12 h. Values are presented as mean ± standard error of the mean of triplicates. (D) Apelin-13 promotes cyclin D1 mRNA expression. The mRNA levels of cyclin D1 and GAPDH were determined using reverse transcription-polymerase chain reaction following treatment with exogenous apelin-13 for 12 h. Data are expressed as the mean ± standard deviation of six independent experiments.

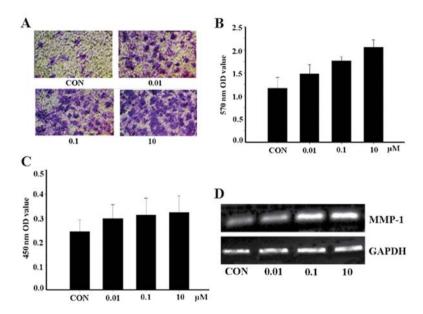


Figure 2. Apelin-13 promotes invasion of MCF-7 and expression of extracellular matrix metalloproteinase (MMP)-1. (A) MCF-7 cells were plated in the upper chamber. Apelin $(0.01, 0.1 \text{ and } 10 \,\mu\text{M})$ was added to the lower chamber, and the cells were allowed to invade for 6 h. The cells were fixed and stained with crystal violet. The invasion cells were counted by an investigator blinded to the sample (magnification, x100). (B) Crystal violet 570 nm optical density (OD) value was measured by elution with 33% acetic acid. (C) Determination of cell culture supernatant expression by human MMP-1 ELISA kit following treatment with exogenous apelin-13 for 12 h. Values are presented as mean \pm standard error of the mean of triplicates. (D) Apelin-13 promotes MMP-1 mRNA expression. The mRNA levels of MMP-1 and GAPDH were determined using reverse transcription-polymerase chain reaction following treatment with exogenous apelin-13 for 12 h. Data are expressed as the mean \pm standard deviation of six independent experiments.

bands were scanned by the Tanon 5500 fully automatic digital gel image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical analysis. The results are expressed as the mean ± standard deviation (SD). The mean and SD of each dataset were calculated using the SigmaPlot 10.0 software (Systat Software, San Jose, CA, USA). The differences between groups were assessed using the t-test to determine P-values (P<0.05 was considered to indicate a clear difference, and P<0.01 was considered to indicate a statistically significant difference).

Results

Apelin-13 promotes the proliferation of MCF-7 cells and cyclin D1 expression. Previous studies have suggested that apelin stimulates cell proliferation and invasion in several

cell lines, including endothelial, lung and oral squamous cells (7,9,10). The present study examined the effects of apelin-13 on MCF-7 proliferation and invasion. Using the MTT and flow cytometry assay, the treatment of MCF-7 with apelin-13 was demonstrated to significantly increase cell proliferation (Fig. 1A and B). In order to explore the effect of apelin-13 on cyclins, the expression level of cyclin D1 was detected by ELISA and RT-PCR analysis. The results showed that the protein and mRNA expression levels of cyclin D1 were significantly increased after MCF-7 cells were incubated with apelin-13 for 12 h, suggesting that apelin-13 affects the cell cycle through increasing cyclin D1 expression, and subsequently induces MCF-7 cell proliferation (Fig. 1C and D).

Apelin-13 promotes the invasion of MCF-7 cells and MMP-1 expression. Degradation of the stromal connective tissue and basement membrane components are critical elements in tumor invasion. This is particularly true regarding the

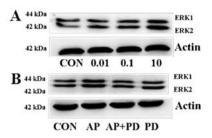


Figure 3. Effect of apelin-13 on ERK $_{1/2}$ phosphorylation in MCF-7 cells. (A) After MCF-7 cells were treated with apelin-13 (0.01, 0.1 and 10 μ M) for 12 h, cell lysates were subjected to western blotting and incubated with antibodies against ERK $_{1/2}$ (upper panel). (B) MCF-7 cells were treated with 10 μ M apelin-13 for 12 h, pre-treated with 10 μ M of PD98059 for 1 h, and subsequently the phosphorylation of ERK $_{1/2}$ (upper panel) was analyzed. β -actin was used as the loading control (lower panel). Data are expressed as the mean \pm standard deviation of three independent experiments.

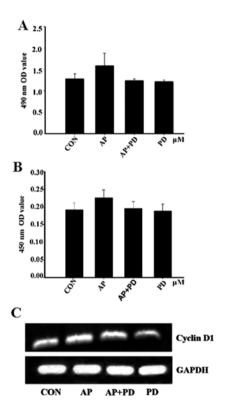


Figure 4. Effects of the specific ERK $_{1/2}$ inhibitor PD98059 on the proliferation and expression of cyclin D1 of MCF-7 cells induced by apelin-13. (A-C) MCF-7 cells were pre-treated with 10 μ M PD98059 for 1 h followed by treatment with 10 μ M apelin-13. (A) MCF-7 proliferation was measured with the MTT assay for 24 h. (B) The protein levels of cyclin D1 were measured using the ELISA kit for 12 h. (C) The mRNA levels of cyclin D1 and GAPDH were determined using reverse transcription-polymerase chain reaction following treatment with exogenous apelin-13 for 12 h. Data are expressed as the mean \pm standard deviation of six independent experiments.

interstitial collagens, which were degraded by MMPs. MMP-1 is a member of a family of enzymes that can degrade the majority of ECM macromolecules, particularly types I and III. Therefore, whether apelin-13 could promote MCF-7 cell invasion and MMP-1 expression was investigated. As hypothesized, the results revealed that the quantity of apelin-13-treated cells passing through the basement membrane into the lower compartment was significantly greater compared with that

of the control group (Fig. 2A and B). This finding suggests that apelin-13 can promote the invasion of MCF-7 cells. Subsequently, the expression of MMP-1 was examined and the expression of the proteins was closely correlated with apelin-13 (Fig. 2C and D). The results showed that MMP-1 may be critically involved in the metastasis of tumors.

Apelin-13 activates the phosphorylation of ERK_{1/2} in MCF-7 cells. To evaluate the potential signaling pathways involved in apelin-13-induced MCF-7 cell proliferation and invasion, the role of apelin-13 on ERK_{1/2} was examined by western blotting. As is shown in Fig. 3A, dose-dependent effects of apelin-13 on ERK_{1/2} activation were determined. ERK_{1/2} reached maximal phosphorylation upon treatment with apelin-13 at 10 μ M. PD98059 (an inhibitor of ERK_{1/2}) significantly inhibited the overexpression of apelin-13-induced ERK_{1/2} phosphorylation (Fig. 3B).

PD98059 inhibits apelin-13-induced MCF-7 cell proliferation. To further evaluate whether ERK_{1/2} has any effect on apelin-13-induced MCF-7 cell proliferation, the protein and mRNA expression levels of cyclin D1 were detected. MTT analysis indicated that pre-treatment with PD98059 prevented apelin-13-induced MCF-7 proliferation (Fig. 4A). The data were confirmed by ELISA and RT-PCR. The levels of cyclin D1 expression were decreased following pre-incubation with PD98059 for 1 h (Fig. 4B and C). These results suggested that apelin-13 influenced MCF-7 cell proliferation, possibly via ERK_{1/2} signaling cascades.

PD98059 inhibits apelin-13-induced MCF-7 cell invasion. To address the functional significance of apelin-mediated MCF-7 cell invasion in the context of ERK_{1/2} activation, the effect of PD98059 upon MCF-7 cell invasion and MMP-1 was examined. MCF-7 cell invasion and the expression of MMP-1 were reduced by PD98059 (Fig. 5). These results suggested that apelin-13-induced invasion of MCF-7 cells and ERK_{1/2} may be involved in the signaling pathway.

Apelin-13 promotes AIB1 mRNA expression via $ERK_{1/2}$. AIB1 amplification and overexpression are associated with proliferation, invasion and poor prognosis in breast cancer. Therefore, AIB1 mRNA expression was detected by RT-PCR. Apelin-13 could promote the endogenous levels of AIB1 mRNA in a concentration-dependent manner. Additionally, the molecular mechanism of AIB1 overexpression was observed following treatment with the apelin-13. The result showed that PD98059 inhibited AIB1 expression. Together, these data suggested that apelin-13 promoted AIB1 expression via the $ERK_{1/2}$ signal pathway (Fig. 6).

Discussion

In the present study, the regulation and signaling of apelin on breast cancer cells were investigated. A previous finding identified the role for AIB1 in breast cancer and described a new mechanism of $ER\alpha/AIB1$ gene regulation, which could regulate cyclin D1 genes depending on the promoter context (24). The coactivator protein AIB1 has previously been associated with the initiation of breast cancer. The MMPs are indicated in the

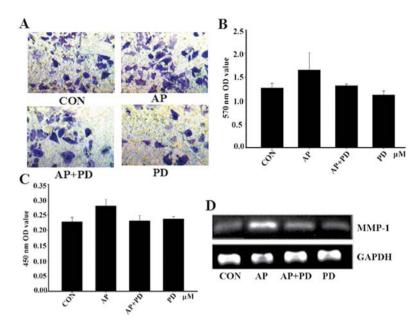


Figure 5. Effects of the specific $ERK_{1/2}$ inhibitor PD98059 on the invasion and expression of extracellular matrix metalloproteinase (MMP)-1 of MCF-7 cells induced by apelin-13. (A-D) MCF-7 cells were pre-treated with 10 μ M PD98059 for 1 h followed by treatment with 10 μ M apelin-13. (A) MCF-7 cell invasion was measured with the Transwell assay. (B) Crystal violet 570 nm optical density value was measured by elution with 33% acetic acid. (C) The protein levels of MMP-1 were measured using the ELISA kit. (D) The mRNA levels of MMP-1 and GAPDH were determined using reverse transcription-polymerase chain reaction. Data are expressed as the mean \pm standard deviation of six independent experiments.

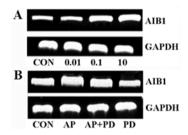


Figure 6. Apelin-13-induced amplified in breast cancer 1 (AIB1) expression via ERK_{1/2} signaling pathway. The total RNA was isolated from the cells, and the mRNA levels of AIB1 and GAPDH were determined using reverse transcription-polymerase chain reaction. (A) MCF-7 cells were treated with apelin-13 (0.01, 0.1 and 10 μ M) for 12 h. (B) MCF-7 cells were treated with 10 μ M apelin-13 for 12 h following pre-treatment with 10 μ M of PD98059 for 1 h. Data are expressed as the mean \pm standard deviation of six independent experiments.

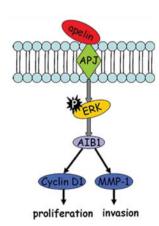


Figure 7. Schematic depiction of apelin-13 induction via the ERK $_{1/2}$ /AIB1 pathway. Apelin/APJ phosphorylates ERK $_{1/2}$ leading to activation of amplified in breast cancer 1 (AIB1) transcription. AIB1-mediated expression of cyclin D1 and extracellular matrix metalloproteinase (MMP)-1 positively promote MCF-7 proliferation and invasion.

basic processes of tumor progression, such as degradation of basement membrane and ECM, stimulation of cellular proliferation and invasion. In the present study, several methods by which apelin-13 regulated cell proliferation, invasion and the underlying mechanisms and signaling pathways were demonstrated as follows: i) MCF-7 cell proliferation was documented by the MTT and flow cytometry assays; ii) MCF-7 cell invasion was measured by the Transwell assay; iii) cyclin D1, MMP-1 and AIB1 were transcriptionally upregulated by apelin-13, induced in a dose-dependent manner; iv) apelin-13 enhanced the phosphorylation of ERK_{1/2} in a dose-dependent manner; and v) cyclin D1, MMP-1 and AIB1 were transcriptionally inhibited by PD98059. These results showed that apelin-13 treatment promoted human MCF-7 proliferation and invasion in a dose-dependent manner, which was consistent with our previously reported results (Fig. 1A and B; Fig. 2A and B). To further explore the role of apelin-13-induced proliferation and invasion of MCF-7 cells, the expression of cyclin D1 and MMP-1 were detected using ELISA and RT-PCR assays. The results revealed that the expression levels were upregulated in a concentration-dependent manner (Fig. 1C and D; Fig. 2C and D). Finally, the downstream pathways that contribute to proliferating MCF-7 cells were investigated. The apelin/APJ system has been shown to induce various signaling pathways, including the JNK, P38 MAPK and ERK_{1/2} pathways. The present results showed that apelin-13 promoted the expression of ERK_{1/2} phosphorylation and PD98059 (an ERK_{1/2} inhibitor) inhibited MCF-7 cell proliferation and invasion induced by apelin-13 (Figs. 3-5). These data also revealed that the activation of the $ERK_{1/2}$ pathway could regulate cyclin D1, MMP-1 and $ERK_{1/2}$ expression. PD98059 was able to repress cyclin D1 and MMP-1 expression (Fig. 4B and C; Fig. 5C and D). These results indicated that apelin-13 induced MCF-7 cell proliferation and invasion via the ERK signaling

pathway. Additionally, AIB1 has an important role that is relevant to breast cancer cell survival and proliferation. Activation of the ERK_{1/2} pathway upon growth factor or hormone stimulation led to the subsequent transactivation of AIB1 in MCF-7 breast cancer cells (25). ERK_{1/2} signaling was affected in mice with reduced AIB1 levels during Neu-induced tumorigenesis (26). Therefore, the effects of apelin-13 and PD98059 on AIB1 mRNA expression were analyzed. The results suggested that AIB1 expression was promoted by apelin-13 and reduced following pre-treatment with PD98059 (Fig. 6). These data suggested that AIB1 may have an important role in tumor maintenance by regulating cyclin D1 and MMP-1 in breast cancer cells, which may be due to the activation of the ERK cascade and thereby resulting in activation of the cyclin D1 promoter.

In conclusion, as shown in Fig. 7, apelin-13-induced MCF-7 proliferation and invasion was mediated through the ERK_{1/2}/AIB1 signaling pathway, further leading to transcriptional activation of downstream target proteins cyclin D1 and MMP-1. This novel finding not only revealed the mechanism of apelin-13-induced MCF-7 cell proliferation, but also provided certain potential targets for future treatment of breast cancer.

Acknowledgements

The present study was supported by the Natural Science Fund of Jilin (grant no. 201215001) and the Jilin Province Science and Technology Research Projects (grant no. 20140204059YY).

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