Biochemical and Biophysical Research Communications 447 (2014) 271–277

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

EEN regulates the proliferation and survival of multiple myeloma cells by potentiating IGF-1 secretion

Er-Wen Huang [a,b,1], Sheng-Jiang Xue [a,1], Xiao-Yan Li [c,1], Suo-Wen Xu [d,1], Jian-Ding Cheng [a], Jin-Xiang Zheng [a], He Shi [b], Guo-Li Lv [b], Zhi-Gang Li [b], Yue Li [b], Chang-Hui Liu [b], Xiao-Hui Chen [b], Hong Liu [b], Jie Li [e,*], Chao Liu [b,*]

[a] Department of Forensic Pathology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China
[b] Guangzhou Institute of Forensic Science, Guangzhou, China
[c] Department of Pharmacy, The Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China
[d] Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, China
[e] Department of Anaesthesiology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China

ARTICLE INFO

Article history:
Received 22 March 2014
Available online 2 April 2014

Keywords:
Multiple myeloma
Proliferation
Apoptosis
EEN
IGF-1

ABSTRACT

The molecular mechanisms of multiple myeloma are not well defined. EEN is an endocytosis-regulating molecule. Here we report that EEN regulates the proliferation and survival of multiple myeloma cells, by regulating IGF-1 secretion. In the present study, we observed that EEN expression paralleled with cell proliferation, EEN accelerated cell proliferation, facilitated cell cycle transition from G1 to S phase by regulating cyclin-dependent kinases (CDKs) pathway, and delayed cell apoptosis via Bcl2/Bax-mitochondrial pathway. Mechanistically, we found that EEN was indispensable for insulin-like growth factor-1 (IGF-1) secretion and the activation of protein kinase B-mammalian target of rapamycin (Akt-mTOR) pathway. Exogenous IGF-1 overcame the phenotype of EEN depletion, while IGF-1 neutralization overcame that of EEN over-expression. Collectively, these data suggest that EEN may play a pivotal role in excessive cell proliferation and insufficient cell apoptosis of bone marrow plasma cells in multiple myeloma. Therefore, EEN may represent a potential diagnostic marker or therapeutic target for multiple myeloma.

1. Introduction

Multiple myeloma is a frequent malignancy of the blood. The incidence varies globally from 1 per 100,000 people in China, to about 4 per 100,000 people in most developed countries. In America, about 2000 cases occur every year and this number accounts for about 1% of neoplastic diseases and 13% of hematological malignancies [1]. Multiple myeloma is characterized by an excess of bone marrow plasma cells, monoclonal proteins, osteolytic bone lesions, renal disease, and immunodeficiency [2,3]. To date, the molecular mechanisms of multiple myeloma remain poorly understood. Molecules that play key roles in the proliferation and survival of multiple myeloma cells are thought to be potential promising targets for the diagnosis and treatment of multiple myeloma.

EEN is a SH3 domain-containing protein. It expresses ubiquitously in various tissues. The amino acid sequence of EEN is highly conserved in mammalians. To date, EEN exerted most of its functions by regulating the location and internalization of membrane proteins. During clathrin-mediated endocytosis, EEN is recruited, with other auxiliary proteins (including dynamin, CIN85 and amphiphysin) together, to under cell membrane, where it facilitates to shape endocytic vesicles and endosomes. As a result, the membrane proteins are shipped into lysosomes for degradation. This function underlines the importance of EEN in multiple biological activities such as neuroregulation, tumorigenesis and development [4–6]. Bee Leng Lua and colleagues found that EEN mediates the endocytosis of epidermal growth factor receptor (EGFR) [7]. It was also reported that phosphorylated EEN at tyrosine 315 (Y315) site lost the function to internalize membrane-locating MMP2 enzyme, resulting in degradation of extracellular matrix and migration of malignant cells [8]. Other studies found that EEN and MLL gene fusion resulted from chromosome translocation is an important cause of leukemia [9–11]. However, the roles EEN plays in cell proliferation and survival, as well as in tumor growth, remain to be investigated. In this study, we found that EEN regulated multiple myeloma cells proliferation and survival. IGF-1/Akt pathway was involved in regulating the effect of

* Corresponding authors.
E-mail addresses: mdlijie@sina.com (J. Li), liuchaogaj@21cn.com (C. Liu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.03.127
0006-291X/

EEN on cell proliferation and survival. These data reveal a novel role EEN plays in the pathogenesis of multiple myeloma cells, and suggest that EEN may be a potential diagnostic marker or therapeutic target of multiple myeloma.

2. Materials and methods

2.1. Reagents

Antibodies targeting EEN or IGF-1 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies targeting caspase-3, Bcl-2, Bax, cytochrome C, COX IV, GAPDH, α-tubulin were purchased from Cell Signaling Technology (Beverly, MA). Apoptosis-detecting kit, antibodies targeting Akt, phospho-Akt, mTOR, and phosphor-mTOR were purchased from Beyotime Institute of Biotechnology (Beyotime, China). Antibodies of cyclin D1, p27, Rb and phospho-Rb (S807/811) were purchased from Signalway Antibody (SAB, USA). JC-1 dye, interlukin-6 (IL-6), insulin-like growth factor (IGF-1), and antibody targeting flag were purchased from Sigma (Sigma–Aldrich, USA). RNA interference reagent Hiper Fect and siRNA were purchased from Qiagen Sample & Assay Technologies (Qiagen, Germany). Cell Counting Assay Kit (CCK-8) was purchased from Yiyuan Biotech (Yiyuanbiotech, China). Fetal bovine serum (FBS) and DMEM medium were purchased from Gibco (Gibco, USA). Human IGF-1 ELISA Kit was purchased from Cusabio Tech. Ltd. (CUSABIO, China).

2.2. Cell culture

Human Multiple myeloma cell line ARH-77, U266, SKO-007 were cultured in DMEM medium containing 10% FBS, in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell proliferation examination

Cell proliferation was measured using CCK-8 kit, according to the manufacturer's instructions. Briefly, cells were cultured in 96-well plates (1×10^4/well) for 24 h, the media contain different concentrations of FBS, IL-6, IGF-1 or IGF-1-targeting antibody. 10 μl CCK-8 reagent was added and incubated for another 2 h; absorbance at wavelength of 450 nm was measured using a microplate reader (Bio-Tek, Winooski, VT, USA).

2.4. RNA interference

Stealth siRNA duplex oligoribonucleotides 5′-UCUGUCACCGCC UUGCUGGTG-3′ (S1) and 5′-UGCAGGUACUCGAUGGUCCTG-3′ (S2) (QIAGEN, antisense strand sequences) were used respectively to against the target sequences 5′-CACCAGCAAGGCGGTGACAGA-3′ or 5′-CAGGACCATCGAGTACCTGCA-3′ in human EEN gene (GenBank No. NM_001199943). The siRNAs were transfected transiently with Lipofectamine™ RNAiMAX (Qiagen) according to the manufacturer's instructions, and a negative stealth siRNA sequence was used as a control. Briefly, siRNA and Lipofectamine™ RNAiMAX were diluted in Opti-MEMI, respectively. Then, the diluted siRNA and the diluted LipofectamineTM RNAiMAX were combined for 5 min at room temperature, then added to the cells in quiescent state and swirled gently to ensure uniform distribution. After incubation for 6 h at 37 °C, the transfection mixture was removed and the cells were further incubated in normal growth conditions for 48 h.

2.5. Adenovirus infection

Full-length cDNA of human EEN was cloned into plasmid pCMV-Tag2 between BamH1 and Xho1 restriction sites, and this recombinant plasmid was packaged into adenovirus. For expression of exogenous EEN, cells were infected with the adenovirus vector. Briefly, full media were removed from cells. After washing with PBS, the cells were cultured in media without serum and antibiotics but with adenovirus vectors (MOI = 20, 40, 80) for 6 h. Then the cells were transferred into fresh full medium and cultured for another 24 h.

2.6. Cell cycle analysis

Cells were harvested and processed for cell cycle analysis using propidium iodide-staining method as described previously [12]. Then the cells were analyzed for DNA content using flow cytometry. The percentages of cells containing different DNA multiple were quantified.

2.7. Apoptosis detection

Cellular apoptosis was detected with Annexin V-FITC/PI double staining using flow cytometry as described previously [13]. Briefly, cells were harvested, suspended at a density of 1×10^6/ml and centrifuged, cell pellets were washed twice with ice cold PBS and then suspended in binding buffer, then the cells were incubated with Annexin V-FITC and PI for 15 min in the dark at room temperature. Finally, the cells were detected using flow cytometry. Apoptotic ratio was analyzed.

2.8. Mitochondrial membrane permeability determination

Mitochondrial membrane permeability was determined with JC-1 fluorescence method. In normal cells, JC-1 fluorescent dye accumulates as aggregates form in mitochondria, emitting red fluorescence. When the cells are induced to apoptosis, the mitochondrial membrane permeability was increased, the dye outflows from mitochondria into cytoplasm and becomes as monomeric form, emitting green fluorescence. The fluorescence was measured using confocal microscopy.

2.9. Western blot

All cell protein preparation and concentration examination were performed as described previously [14]. Isolation of mitochondrial and cytoplasmic proteins was carried out as previously described [15]. Cellular protein expression or phosphorylation levels were determined with Western blot analysis as described elsewhere [16].

2.10. ELISA assay

After cells were harvested, the media were centrifuged and supernatants were collected. IGF-1 levels in the media were assessed using a human IGF-1 ELISA kit according to the manufacturer's instructions. Data were obtained using a microplate reader (Perkin Elmer; Boston, MA).

2.11. Statistical analyses

All data were expressed as means ± S.D.; for each result, *n* value represents the number of independent experiments. ANOVA followed by the Bonferroni multiple comparison *post hoc* test was employed in SPSS system. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. EEN expression parallels with rate of ARH-77 proliferation, and EEN accelerates proliferation of multiple myeloma cells

First of all, we investigated whether EEN expression correlates with the rate of cell proliferation. We cultured multiple myeloma cell line ARH-77 using various concentrations (0.5%, 2.5%, 5%, 10%, 20%) of fetal bovine serum (FBS) for 24 h, then detected viable cell number and EEN protein expression levels. As shown in Fig. S1A, cells proliferate faster with higher FBS concentration. Cell number in 5% FBS was more than 1.5-fold compared to that in 0.5% FBS, and cell number in 10% FBS reached over 2-fold. Interestingly, we found that EEN protein expression paralleled to FBS-induced cell proliferation. Compared to 0.5% FBS, 5% FBS elevated EEN expression level to 1.94 ± 0.35-fold, and 10% FBS further elevated it to 2.89 ± 0.28-fold (Fig. S1B).

IL-6 is one of the most important cell growth factors, multiple studies reported that uncontrolled up-regulation of IL-6 associates with various cancers, including multiple myeloma [17,18]. We thus explored the effect of IL-6 on EEN expression in ARH-77 cell. As shown in Fig. S1C and D, IL-6 accelerated cell proliferation, consistent with previous data, meanwhile, IL-6 at the concentrations of 25, 50, and 100 ng/ml markedly enhanced EEN expression.

Given that FBS and IL-6 affect EEN expression in ARH-77, we speculated that EEN may be involved in the proliferation of multiple myeloma cells. To verify this notion, we determined the effects of EEN knockdown and over-expression on ARH-77 proliferation. Efficiencies of RNA interference and exogenous expression were firstly tested. Compared to control, two EEN-targeting siRNAs (S1, S2) respectively reduced EEN protein expression to 26.0 ± 10.1% and 22.0 ± 13%, while negative siRNA (NS) had no effect (Fig. 1A). Infection of adenovirus that package EEN-flag expression vectors enhanced EEN expression markedly (Fig. 1B). Expectedly, S1 and S2 suppressed ARH-77 cell proliferation to 44 ± 14.0% and 38 ± 15.7%, respectively (Fig. 1C), while EEN over-expression promoted cell proliferation significantly (Fig. 1D). Moreover, we tested these phenomena in two other multiple myeloma cell lines, U266 and SKO-007. As a result, similar results were observed in the both cell lines (Fig. 1 middle and lower).

3.2. EEN facilitates G1/S cell cycle transition

We found that EEN knockdown by S1 or S2 in ARH-77 resulted in a significantly increased percentage of cell population in G1 phase and decreased percentages in S and G2/M phases (Fig. 2A). On the contrary, EEN over-expression decreased cell percentage in G1 phase. Although the increase of cell percentages in S and

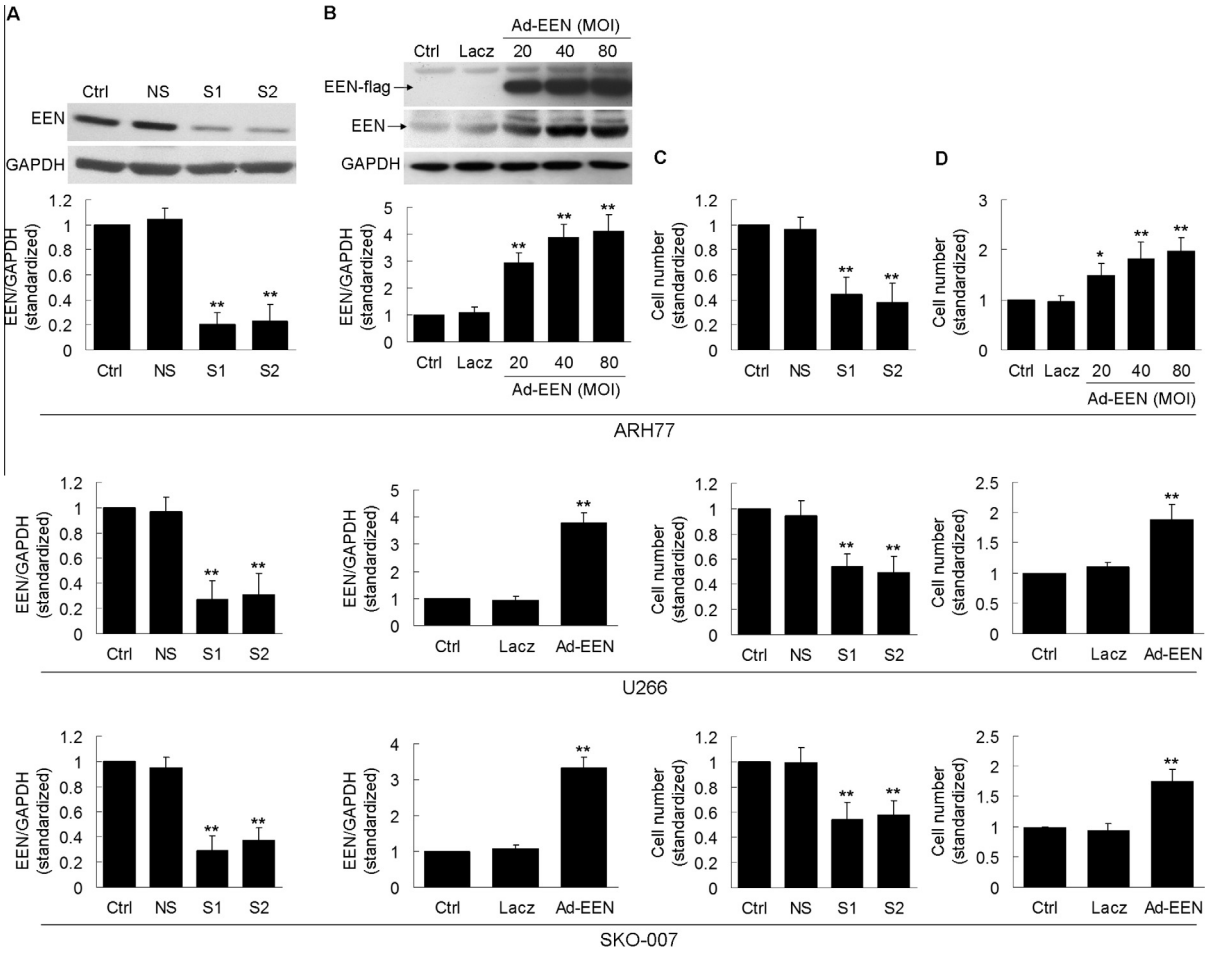

Fig. 1. EEN regulates the proliferation of multiple myeloma cells. Upper panel, ARH-77: (A) silencing efficiencies of siRNA oligos were tested. Cells were transfected with 20 nM EEN-targeting siRNA (S1 or S2) or negative control siRNA (NS), 48 h later, the cells were harvested, and EEN expression was determined by Western blot. (B) Cells were infected with adenovirus packaging expression vectors of flag-tagged EEN (Ad-EEN, MOI = 20, 40, 80) or of flag-tagged Lacz (Lacz, MOI = 80), and cultured for 48 h, then the cells were harvested. With antibody targeting flag or EEN, expression of flag-tagged EEN or total EEN was examined respectively by Western blot. (C) EEN expression was knockdown by RNAi as described in (A), then cell proliferation was tested with CCK-8 kit. (D) EEN was exogenously expressed by the method mentioned in (B), then cell proliferation was tested with CCK-8 kit. Middle and lower panels, the experiments carried out in ARH-77 were all performed in U266 and SKO-007. ($n = 4$ for each experiment, bars represent mean ± SD, $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, ANOVA-test was employed).

G2/M phases were mild compared to blank control, the difference is significant ($p < 0.05$) compared to Lacz control group (Fig. 2B). The transition checkpoint of G1 to S phase is regulated by CDK4 and CDK6, the activities of which are promoted by cyclin D1 but attenuated by p27. Activated CDK4 and CDK6 phosphorylate Rb and consequently promote transition of cell phase [19,20]. So, we further studied the expression and phosphorylation levels of cyclin D1, p27 and Rb. Fig. 2C shows that in EEN knockdown groups, cyclin D1 expression and Rb phosphorylation were markedly attenuated, while p27 expression was elevated, and inverse results were observed in group of EEN over-expression.

3.3. EEN is indispensable for cell survival

To explore the effect of EEN on cell survival, we explored its effect on cell apoptosis. In ARH-77 cell line, Annexin V-FITC/PI staining followed by flow cytometric analysis revealed that EEN knockdown increased the percentage of apoptotic cell population significantly, and EEN over-expression decreased apoptotic cell population (Fig. 3A). Similar results were observed in U266 (Fig. 3B) and SKO-007 (Fig. 3C) cells, although EEN over-expression just mildly inhibited U266 apoptosis, which may be owing to very low background apoptotic level in control groups.

To understand the mechanism by which EEN regulates cell apoptosis, we examined the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax. As Fig. 3D shows, EEN RNAi markedly up-regulated Bax expression but down-regulated Bcl-2 expression in ARH-77, while EEN over-expression has the opposite effects. Because the stability of mitochondrial membrane potential is essential for cellular homeostasis [13], we tested the effect of EEN on mitochondrial membrane potential using JC-1 staining. As shown in Fig. 3E, in groups of EEN RNAi, the intensity of green fluorescence emitted by JC-1 dye in cells was remarkably enhanced, paralleled with an increased ratio of green/red fluorescence intensity. However, there is no significant difference between blank control and negative siRNA control. This result suggested that EEN knockdown increased mitochondrial membrane permeability and decreased mitochondrial membrane potential. Increase of mitochondrial membrane permeability will trigger the release of cytochrome C from mitochondrial to cytoplasm, and consequently activate downstream apoptotic signal [21]. So we further examined the release of cytochrome C. As Fig. 3F shows, in groups of EEN knockdown, mitochondrial cytochrome C was reduced while cytoplasmic cytochrome C was increased, compared to control groups. Western blot results also show that EEN knockdown activated caspase-3 (Fig. 3G). These results indicated that EEN regulates cell apoptosis via the mitochondrial-dependent pathway.

3.4. IGF-1 secretion is involved in regulating the effect of EEN on cell proliferation and survival

To study the signal transduction pathway via which EEN regulates cell proliferation and survival, we explored IGF-1 pathway, which is involved in progression of many malignancies including multiple myeloma [22]. We found that diverse levels of EEN expression had no effect on intracellular IGF-1 levels (Fig. 4A upper), but altered its secretion, EEN knockdown decreased the concentration of IGF-1 in media and EEN over-expression had the opposite effect (Fig. 4A lower). Akt-mTOR is an important downstream pathway of IGF-1 [23]. Our results showed that EEN knockdown significantly attenuated phosphorylation levels at both Thr308 and Ser473 sites in Akt, as well as mTOR phosphorylation, and that addition of IGF-1 into media partially reversed the effects of EEN knockdown. On the other hand, exogenous expression of EEN markedly elevated phosphorylation levels of Akt and mTOR,

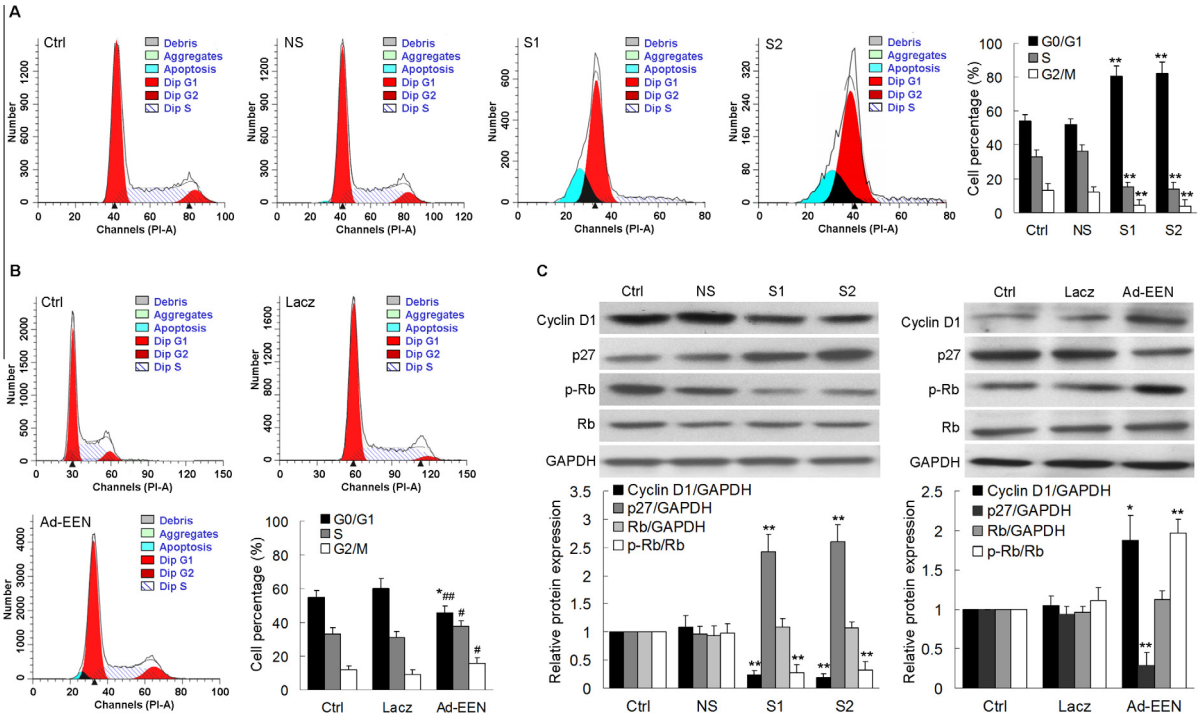

Fig. 2. EEN facilitates G1/S cell cycle transition. ARH-77 cells: (A) EEN expression was knockdown by RNAi, 48 h later cells were harvested and cell cycle phases were determined by flow cytometry. Percentage of cells in G0/G1, S, G2/M phases was statistically analyzed. (B) Cells were infected with EEN- (Ad-EEN) or Lacz-packaging (Lacz) adenovirus (MOI = 40), 48 h later, the cells were harvested, and cell cycle phases were determined and analyzed as described in Fig. 2A. (C) EEN was knockdown or exogenous expressed, then changes of G1/S phase transition-regulating proteins were tested by Western blot ($n \geqslant 4$ for each experiment, bars represent mean ± SD, $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs Lacz, $^{\#\#}p < 0.01$ vs Lacz, ANOVA-test was employed).

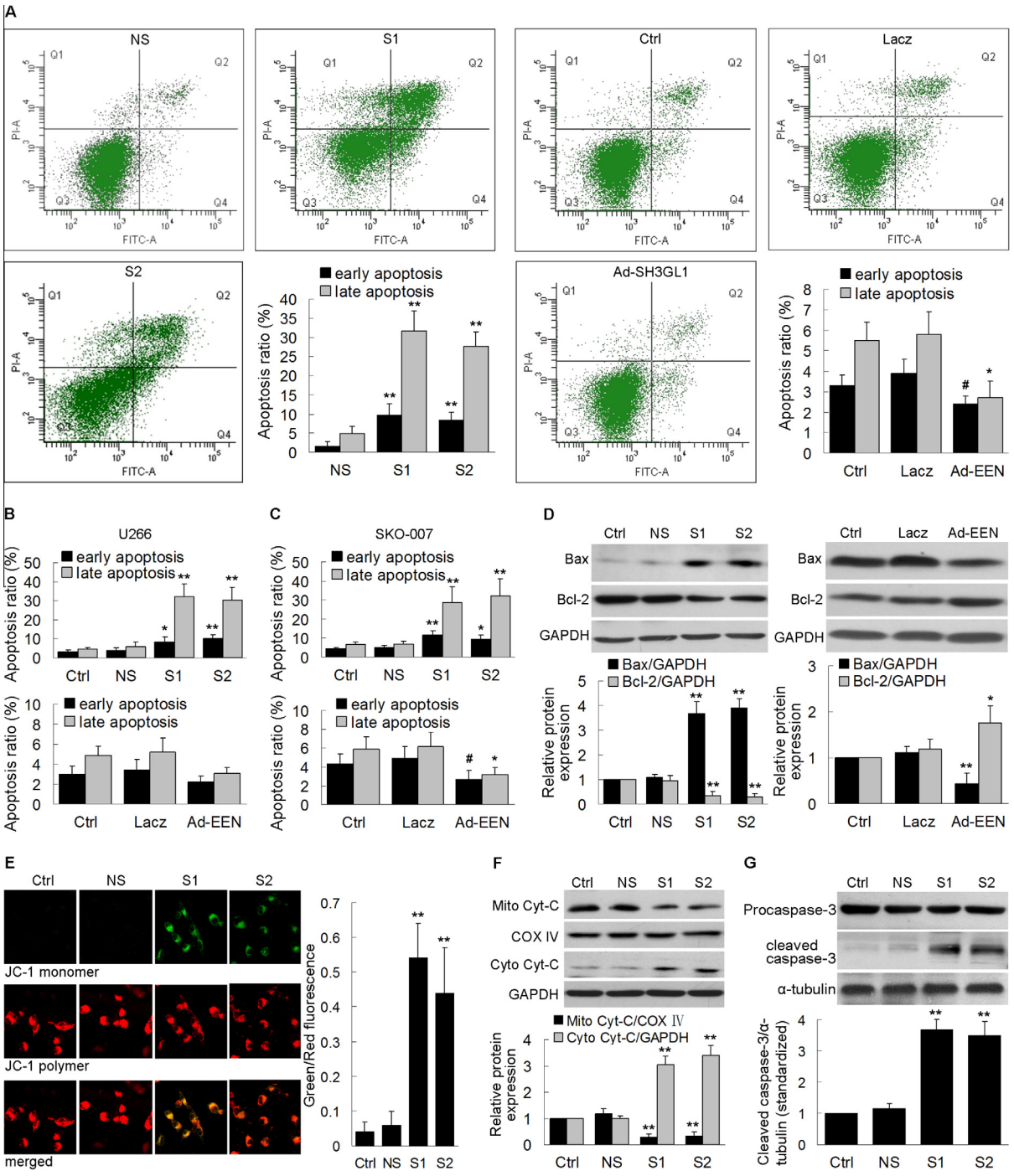

Fig. 3. EEN is essential for cell survival. (A) In ARH-77, EEN was knockdown or exogenously expressed as in Fig. 2, and cell apoptosis was tested with Annexin V/PI double staining method by flow cytometry. Percentage of cell populations undergoing early or late apoptosis were statistically analyzed. (B and C) The experiments carried out in ARH-77 were all replicated in U266 and SKO-007 respectively. (D) EEN was knockdown or exogenously expressed in ARH-77, then Bax and Bcl-2 protein expression were examined by Western blot. (E) Following EEN-knockdown, ARH-77 cells were stained with JC-1, and fluorescence was examined using confocal microscopy. (F and G) After EEN-knockdown, cytochrome C levels in mitochondria and cytoplasm, expression levels of procaspase-3 and cleaved caspase-3 in ARH-77 were examined by Western blot, Cox IV is a loading control of mitochondrial protein, Mito Cyt-C represents cytochrome C in mitochondria and Cyto Cyt-C represents cytochrome C in cytoplasm. ($n = 5$ for each experiment, bars represent mean ± SD, $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs Lacz, ANOVA-test was employed).

and antibody targeting IGF-1 in media eliminated this elevation (Fig. 4B). We further verified whether IGF-1 is indeed involved in regulation of cell proliferation and survival by EEN. As a result, IGF-1 addition eliminated the EEN knockdown-caused suppression of cell proliferation, and IGF-1-targeting antibody inhibited the proliferation-accelerating effect of EEN over-expression (Fig. 4C); moreover, similar results were observed in apoptosis assays (Fig. 4D) and cell cycle tests (Fig. S2). These results suggest that potentiating IGF-1 secretion is involved in the regulation of cell proliferation and apoptosis by EEN.

4. Discussion

Our study is the first to show that EEN is involved in proliferation and survival of multiple myeloma cells. The salient findings of

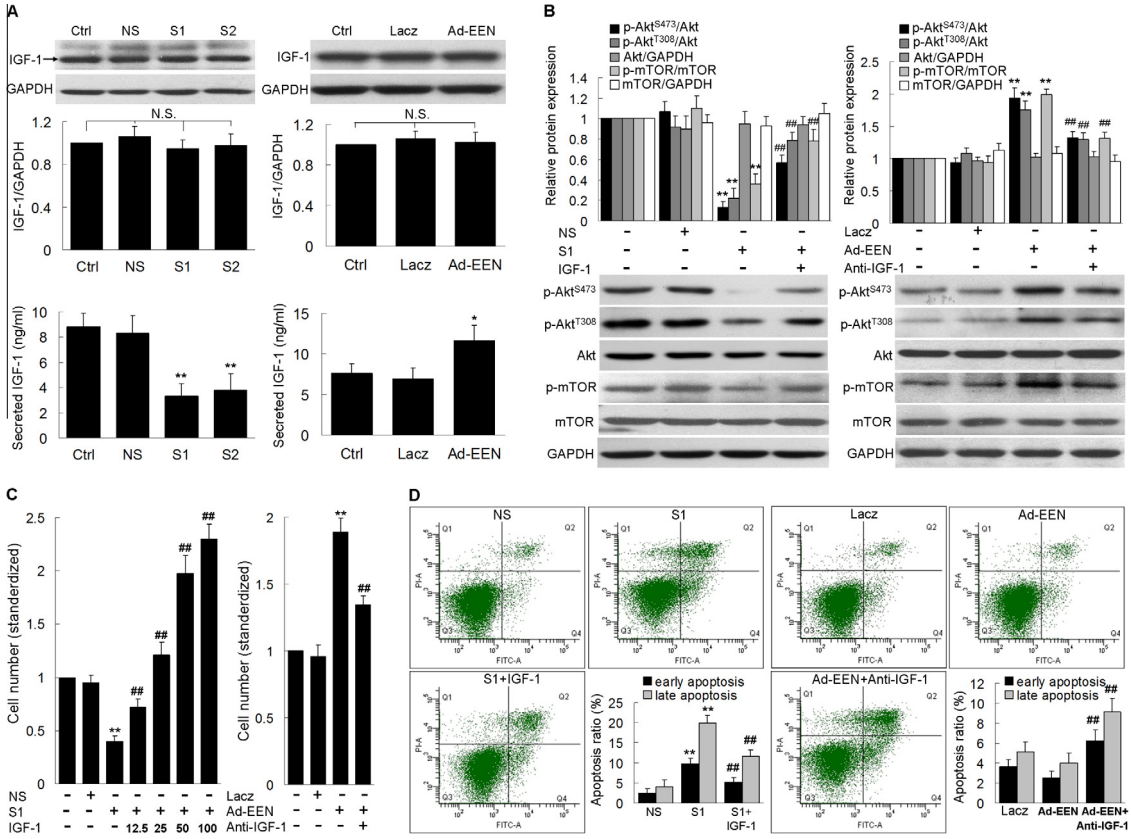

Fig. 4. EEN mediates IGF-1/Akt signal, IGF-1 secretion is involved in the regulation of ARH-77 proliferation and apoptosis by EEN. (A) EEN was knockdown or exogenously expressed, (upper panel) cells were harvested and levels of intracellular IGF-1 were examined by Western blot, (lower panel) media were collected and centrifuged, IGF-1 concentration in supernatant was determined by ELISA assay. (B) Cells were incubated with human IGF-1 (50 ng/ml) for 24 h following EEN knockdown, or were incubated with monoclonal antibody targeting human IGF-1 for 24 h following EEN over-expression, then the phosphorylation levels of Akt kinase at Ser 473 and Thr 308 sites, as well as of mTOR kinase, were examined by Western blot. (C) Effects of IGF-1 (ng/ml) or IGF-1-targeting antibody (Anti-IGF-1) on the regulation of cell proliferation by EEN. (D) Effects of IGF-1 (25 ng/ml) or IGF-1-targeting antibody on the regulation of cell apoptosis by EEN. ($n \geqslant 4$ for each experiment, bars represent mean ± SD, $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs S1 or Ad-EEN, ANOVA-test was employed).

this study were summarized below: (1) expression of EEN paralleled with the rate of ARH-77 proliferation. (2) We observed that serum and IL-6 promoted EEN expression. (3) EEN facilitated cell cycle transition from G1 to S and G2/M phase, by regulating expression of cyclin D1 and p27, as well as phosphorylation of Rb. (4) EEN was involved in keeping the stability of mitochondrial membrane permeability, and consequently in regulating cell survival.

Paracrine and autocrine of cell factors play important roles in cell proliferation and survival. Abnormal secretion of IGF-1 has been reported to be a tumorigenic factor [22]. In this study we observed that EEN is essential for ARH-77 to maintain IGF-1 secretion. Meanwhile, IGF-1 is indispensable for EEN to modify the activities of Akt and mTOR. Mechanistically, the regulation of cell proliferation and survival by EEN is mediated, at least partially, if not all, by IGF-1 signal.

However, further studies are needed to verify the roles of EEN in the pathogenesis and progression of multiple myeloma in vivo, and to identify whether EEN can be exploited as a potential diagnostic marker or therapeutic target of multiple myeloma.

Acknowledgments

This work was supported by the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (No. 2012BAK02B02); Science and Technology Planning Project of Guangdong Province, China (No. 2010A030100011-06); The Fundamental Research Funds for the Central Universities in China (No. 12ykpy26); Guangdong Natural Science Foundation (No. S2012040008068; No.S2013010014514); Fund of Guangdong Province-Ministry of Education Producing, Study and Research Incorporation (No. 2012B091100454).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.127.

References

[1] K. Bauer, M. Rancea, B. Schmidtke, S. Kluge, I. Monsef, K. Hubel, A. Engert, N. Skoetz, Thirteenth biannual report of the Cochrane Haematological Malignancies Group – focus on multiple myeloma, J. Natl Cancer Inst. 103 (2011) E1–E19.
[2] O. Sezer, Myeloma bone disease, Hematology 10 (Suppl. 1) (2005) 19–24.
[3] O. Sezer, Myeloma bone disease: recent advances in biology, diagnosis, and treatment, Oncologist 14 (2009) 276–283.
[4] K.D. Micheva, A.R. Ramjaun, B.K. Kay, P.S. McPherson, SH3 domain-dependent interactions of endophilin with amphiphysin, FEBS Lett. 414 (1997) 308–312.
[5] G.C. Ochoa, V.I. Slepnev, L. Neff, N. Ringstad, K. Takei, L. Daniell, W. Kim, H. Cao, M. McNiven, R. Baron, P. De Camilli, A functional link between dynamin and the actin cytoskeleton at podosomes, J. Cell Biol. 150 (2000) 377–389.
[6] P. Verstreken, O. Kjaerulff, T.E. Lloyd, R. Atkinson, Y. Zhou, I.A. Meinertzhagen, H.J. Bellen, Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release, Cell 109 (2002) 101–112.
[7] B.L. Lua, B.C. Low, Activation of EGF receptor endocytosis and ERK1/2 signaling by BPGAP1 requires direct interaction with EEN/endophilin II and a functional RhoGAP domain, J. Cell Sci. 118 (2005) 2707–2721.
[8] X. Wu, B. Gan, Y. Yoo, J.L. Guan, FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation, Dev. Cell 9 (2005) 185–196.

[9] H. Liu, B. Chen, H. Xiong, Q.H. Huang, Q.H. Zhang, Z.G. Wang, B.L. Li, Z. Chen, S.J. Chen, Functional contribution of EEN to leukemogenic transformation by MLL-EEN fusion protein, Oncogene 23 (2004) 3385–3394.
[10] J.W. Yam, D.Y. Jin, C.W. So, L.C. Chan, Identification and characterization of EBP, a novel EEN binding protein that inhibits Ras signaling and is recruited into the nucleus by the MLL-EEN fusion protein, Blood 103 (2004) 1445–1453.
[11] L.H. Ma, H. Liu, H. Xiong, B. Chen, X.W. Zhang, Y.Y. Wang, H.Y. Le, Q.H. Huang, Q.H. Zhang, B.L. Li, Z. Chen, S.J. Chen, Aberrant transcriptional regulation of the MLL fusion partner EEN by AML1-ETO and its implication in leukemogenesis, Blood 109 (2007) 769–777.
[12] Y.B. Tang, Y.J. Liu, J.G. Zhou, G.L. Wang, Q.Y. Qiu, Y.Y. Guan, Silence of ClC-3 chloride channel inhibits cell proliferation and the cell cycle via G/S phase arrest in rat basilar arterial smooth muscle cells, Cell Prolif. 41 (2008) 775–785.
[13] S.Y. Li, X.G. Wang, M.M. Ma, Y. Liu, Y.H. Du, X.F. Lv, J.G. Zhou, Y.B. Tang, Y.Y. Guan, Ginsenoside-Rd potentiates apoptosis induced by hydrogen peroxide in basilar artery smooth muscle cells through the mitochondrial pathway, Apoptosis 17 (2012) 113–120.
[14] X.L. Shi, G.L. Wang, Z. Zhang, Y.J. Liu, J.H. Chen, J.G. Zhou, Q.Y. Qiu, Y.Y. Guan, Alteration of volume-regulated chloride movement in rat cerebrovascular smooth muscle cells during hypertension, Hypertension 49 (2007) 1371–1377.
[15] O. Terradillos, S. Montessuit, D.C. Huang, J.C. Martinou, Direct addition of BimL to mitochondria does not lead to cytochrome C release, FEBS Lett. 522 (2002) 29–34.
[16] G.L. Wang, X.R. Wang, M.J. Lin, H. He, X.J. Lan, Y.Y. Guan, Deficiency in ClC-3 chloride channels prevents rat aortic smooth muscle cell proliferation, Circ. Res. 91 (2002) E28–E32.
[17] C.E. Zielinski, F. Mele, D. Aschenbrenner, D. Jarrossay, F. Ronchi, M. Gattorno, S. Monticelli, A. Lanzavecchia, F. Sallusto, Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta, Nature 484 (2012) 514–518.
[18] N.M. Kaskas, T. Moore-Medlin, G.B. McClure, O. Ekshyyan, J.A. Vanchiere, C.A. Nathan, Serum biomarkers in head and neck squamous cell cancer, JAMA Otolaryngol. Head Neck Surg. 140 (2014) 5–11.
[19] K. Nakayama, N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D.Y. Loh, K. Nakayama, Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors, Cell 85 (1996) 707–720.
[20] G. Dimco, R.A. Knight, D.S. Latchman, A. Stephanou, STAT1 interacts directly with cyclin D1/Cdk4 and mediates cell cycle arrest, Cell Cycle 9 (2010) 4638–4649.
[21] I. Kuwabara, Y. Kuwabara, R.Y. Yang, M. Schuler, D.R. Green, B.L. Zuraw, D.K. Hsu, F.T. Liu, Galectin-7 (PIG1) exhibits pro-apoptotic function through JNK activation and mitochondrial cytochrome C release, J. Biol. Chem. 277 (2002) 3487–3497.
[22] M. Cea, A. Cagnetta, M. Fulciniti, Y.T. Tai, T. Hideshima, D. Chauhan, A. Roccaro, A. Sacco, T. Calimeri, F. Cottini, J. Jakubikova, S.Y. Kong, F. Patrone, A. Nencioni, M. Gobbi, P. Richardson, N. Munshi, K.C. Anderson, Targeting NAD+ salvage pathway induces autophagy in multiple myeloma cells via mTORC1 and extracellular signal-regulated kinase (ERK1/2) inhibition, Blood 120 (2012) 3519–3529.
[23] I. Palazzolo, C. Stack, L. Kong, A. Musaro, H. Adachi, M. Katsuno, G. Sobue, J.P. Taylor, C.J. Sumner, K.H. Fischbeck, M. Pennuto, Overexpression of IGF-1 in muscle attenuates disease in a mouse model of spinal and bulbar muscular atrophy, Neuron 63 (2009) 316–328.