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Herbimycin A inhibits cell growth with reversal of epithelial–mesenchymal transition in anaplastic thyroid carcinoma cells

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ABSTRACT

We aimed to elucidate the effect of herbimycin A (HMA), a heat shock protein 90 inhibitor, on cell growth and epithelial–mesenchymal transition (EMT) in anaplastic thyroid carcinoma (ATC) cells. HMA inhibited cell growth and migration concomitantly with increase of E-cadherin as well as decrease of N-cadherin and vimentin. Moreover, HMA upregulated p21 and p27, while it downregulated p53 and Akt. In HMA-treated condition, knockdown of E-cadherin and overexpression of p53 increased N-cadherin and vimentin, and mitigated the inhibitory effects of HMA on cell growth and migration. Furthermore, knockdown of p21 and p27 ameliorated inhibition of cell growth and reversal of EMT. In addition, the activation of Akt attenuated growth inhibition, cell death and EMT reversal. Therefore, we propose that HMA suppresses cell growth, and reverses EMT in conjunction with the activation of E-cadherin, p21 and p27 and the inactivation of p53 and PI3K/Akt signaling in ATC cells.

1. Introduction

Anaplastic thyroid carcinoma (ATC) is a highly malignant neoplasm of the thyroid gland characterized by extrathyroidal invasion and distant metastasis [1]. There has been no significant improvement in the survival rate of ATC patients despite the development of multimodal treatment strategies, and thus new therapeutic agents for ATC are currently under exploration [1].

Epithelial–mesenchymal transition (EMT) is a biological process that can occur in embryogenesis, fibrosis and tumorigenesis whereby polarized epithelial cells assume a mesenchymal phenotype [2]. The characteristics of EMT are downregulation of epithelial markers and upregulation of mesenchymal markers, and especially loss of functional expression of E-cadherin is considered as hallmark of EMT [2]. E-cadherin is reduced during EMT progression, and *vice versa* downregulation of E-cadherin induces EMT features, and thereby drives malignant transformation [2–4]. In several cancers, the levels of E-cadherin, which functions as a suppressor of invasion, is reduced, while those of N-cadherin, which acts as a promoter of invasion, is elevated; this effect is referred as cadherin switching [5]. Moreover, decrease of E-cadherin contributes to development and progression of cancer, and increase of N-cadherin renders cancer cells more motile and invasive [4,6,7]. In regard to signaling involved in EMT, inhibition of PI3K/Akt signaling activates expression of E-cadherin, and inactivates expression of N-cadherin and vimentin in human epithelial malignancies [8,9]. As to ATC, E-cadherin is highly expressed in non-neoplastic thyroid and differentiated thyroid carcinoma (DTC) tissues but rarely expressed in ATC tissues, suggesting that ATC has an EMT phenotype [10–13]. Furthermore, PI3K/Akt signaling is deranged in ATC cells, and Akt is stimulated in most ATC patients [14].

Herbimycin A (HMA), a benzoquinone ansamycin antibiotic, is an inhibitor of heat shock protein 90 (hsp90), and suppresses hsp90 client protein expression [15]. HMA has a potent cytotoxic effect on several highly malignant human tumor cells [16]. However, the influence of HMA on growth and EMT of ATC cells has not been investigated.

The aim of the present study was to evaluate the effect of HMA on cell growth and EMT, and to identify the factors related to the impact of HMA in ATC cells. Our results are the first to demonstrate that HMA induces inhibition of cell growth and reversal of EMT, and the activation of E-cadherin, p21 and p27 as well as the inactivation of p53 and PI3K/Akt signaling are involved in the effects of HMA on growth and EMT of ATC cells.

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2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS), L-glutamine and streptomycin/penicillin were obtained from GIBCO (Grand Island, NY, USA). HMA and recombinant human epidermal growth factor (EGF) were purchased from Sigma (St. Louis, MO, USA). HMA was dissolved in dimethylsulfoxide (DMSO), which was provided to the control within permissible concentrations. The final concentration of the vehicle DMSO in the control did not exceed 0.1% in all treatments. The primary antibodies raised against E-cadherin, N-cadherin, vimentin, total and phospho-Akt (Ser473), cyclin-dependent kinase 2 (cdk2) and p27 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies raised against epidermal growth factor receptor (EGFR), total and phospho-Src (Tyr416), p21, p53, cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 were purchased from Cell Signaling Biotechnology (Danvers, MA, USA), and the primary antibody raised against β-actin was obtained from Sigma. All other reagents were purchased from Sigma unless otherwise stated.

2.2. Cell culture

For experiments, FRO cells, authenticated as shown previously, were provided by Professor Young Joo Park (Division of Endocrinology and Metabolism, Seoul National University, Republic of Korea) [17]. FRO cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. Cells received fresh medium at regular intervals. Treatments and experiments were performed using cells that were 50% confluent.

2.3. CCK-8 assay

Cell growth was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells (5×10^3/100 μl) in each well on 96-well plates were incubated overnight, and treated for an additional 4 h at 37 °C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). All experiments were performed in triplicate.

2.4. Cell number count

Cells (1×10^5/500 μl) in each well on 12-well plates were incubated, and mixed with trypan blue dye at 37 °C. Stained cells were counted using a hemocytometer. All experiments were performed in triplicate.

2.5. FACS analysis

The dead cells were analyzed by the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Pharminogen, San Diego, CA, USA). Cells (1×10^5/ml) in each well on 6-well plates were incubated, harvested, washed, and fixed according to manufacturer's protocol. FITC annexin V and propidium iodide in 1× binding buffer was added for 15 min at room temperature, and analysis was made using a FACSsort flow cytometry (Becton Dickinson, San Jose, CA, USA) and CellQuest software program (Becton Dickinson, San Jose, CA, USA). All experiments were performed in triplicate.

2.6. Wound healing assay

Cell migration was measured by the CytoSelect™ 24-Well Wound Healing Assay Kit (Cell Biolabs, San Diego, CA, USA). Cells (1×10^5/500 μl) in plates were incubated overnight to generate wound field (0.9 mm), washed, and treated at 37 °C. The wound closure was monitored by light microscope, and the cell migration rate was calculated according to the following equation: cell migration = [length of cell migration (nm)/migration time (h)]. All experiments were performed in triplicate.

2.7. Immunofluorescence (IF)

Cells were fixed, permeabilized with acetone for 10 min at room temperature, and blocked. Cells were incubated with primary antibodies against E-cadherin, N-cadherin and vimentin overnight at 4 °C and then with FITC-conjugated IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Cells were mounted, and identified by flexible confocal microscope LSM 700 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

2.8. Transfection of small interfering RNA (siRNA) and plasmid

The human siRNAs against E-cadherin, p21 and p27, and the control siRNA were obtained from Cell Signaling Biotechnology and Bioneer (Daejeon, Republic of Korea). Two different single sequence siRNAs per each gene were used for experiments. The human-specific p53 and PIK3CA (P110α) plasmids, and the control plasmid were purchased from Addgene (Cambridge, MA, USA). Cells were transfected with siRNAs and plasmids using Lipofectamine 3000 Reagent (Life Technologies, Grand Island, NY 14072, USA) according to manufacturer's protocol. Transfection efficiency was tested by Western blotting.

2.9. Western blotting, reverse transcription-polymerase chain reaction and statistical analysis

Western blotting and reverse transcription-polymerase chain reaction were performed, and quantitative data were statistically analyzed. More details are included in Supplemental Materials and Methods.

3. Results

3.1. HMA inhibits cell growth with concomitant reversal of expression of EMT markers in ATC cells

In ATC cells, to evaluate the effect of HMA on cell growth, cells were treated with HMA at various doses and times, and cell growth was measured. After treatment, cell growth was reduced in a dose- and time-dependent manner (Fig. 1A).

To investigate the influence of HMA on EMT markers, cells were treated with HMA, and the protein and mRNA levels of E-cadherin, N-cadherin and vimentin were measured. As a result of treatment, the protein and mRNA levels of E-cadherin were elevated, while those of N-cadherin and vimentin were reduced (Fig. 1B). When cells were immunostained with antibodies against E-cadherin, N-cadherin and vimentin after treatment with HMA, E-cadherin-stained cells were elevated, whereas N-cadherin-stained cells and vimentin-stained cells were reduced (Fig. 1C).

EGF stimulates migration and invasiveness by activation of EGFR in ATC cells [18–20]. HMA accelerates degradation of EGFR, and thereby inhibits EGF-induced EMT in human epidermoid carcinoma cells [21]. Thus, we examined whether HMA affects cell growth and EMT markers under condition of EGF exposure in ATC cells. When cells were treated with EGF in absence of serum, the protein levels of EGFR, Akt, total and phospho-Src, N-cadherin and vimentin were enhanced, while those of E-cadherin were

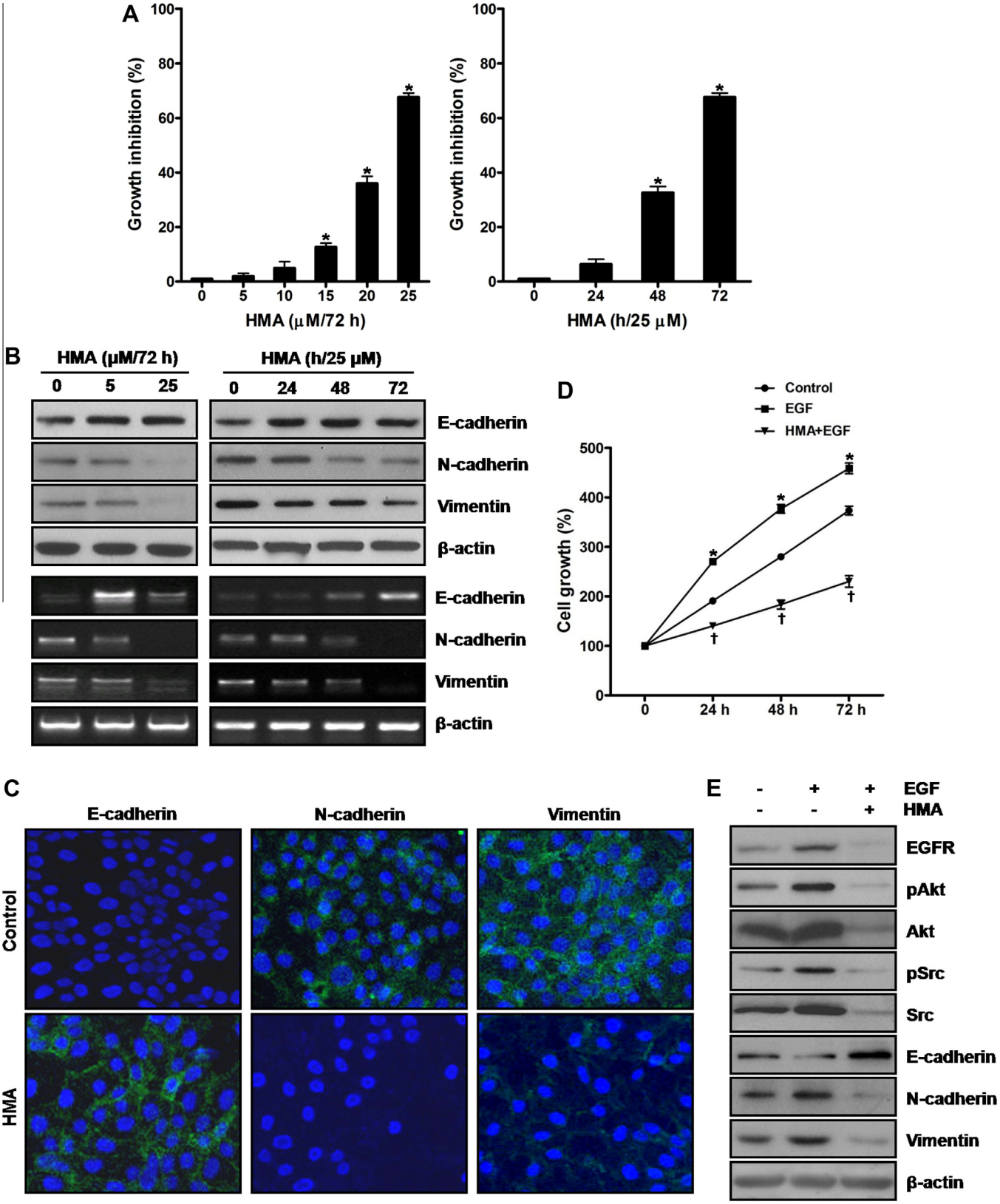

Fig. 1. The effects of HMA on cell growth and EMT markers in ATC cells. (A) Cells were treated with HMA at 5, 10, 15, 20 and 25 μM for 72 h, and at 25 μM for 24, 48 and 72 h, and cell growth was measured using CCK-8 assay. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. each matched control. (B) Cells were treated with HMA at 5 and 25 μM for 72 h, and at 25 μM for 24, 48 and 72 h, and the protein and mRNA levels of E-cadherin, N-cadherin and vimentin were measured. (C) Cells stained with antibodies against E-cadherin, N-cadherin and vimentin following treatment with HMA at 25 μM for 72 h were measured using IF. DAPI was used to label nucleus. (D) Cells were treated with EGF at 50 nM with or without simultaneous treatment with HMA at 25 μM in presence of serum for 24, 48 and 72 h. Cell growth was measured. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. EGF-treated cells. (E) Cells were simultaneously treated with EGF at 50 nM and HMA at 25 μM in presence of serum for 72 h. The protein levels of EGFR, Akt, Src, E-cadherin, N-cadherin and vimentin were measured.

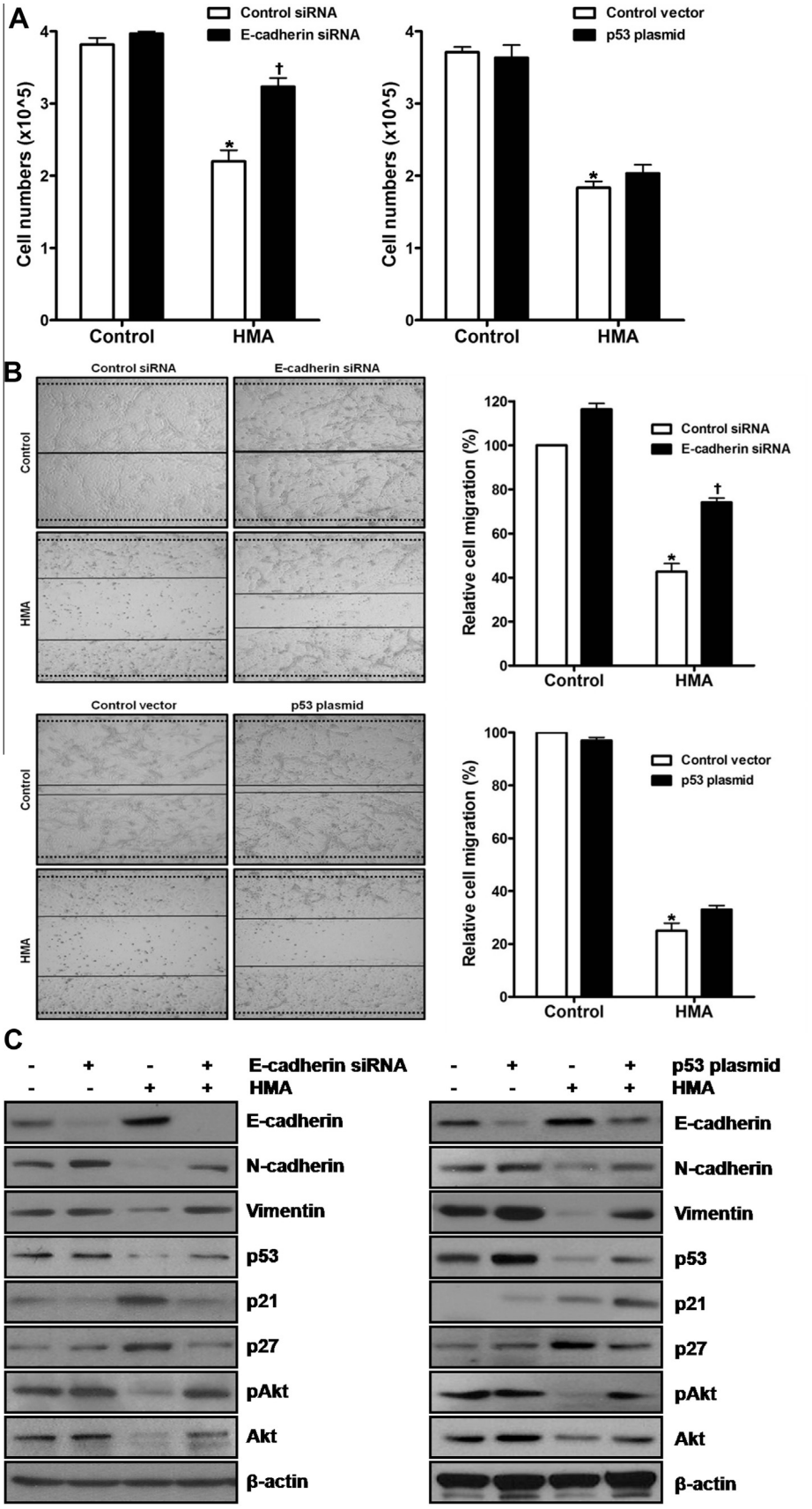

Fig. 2. The relation of E-cadherin and p53 to HMA-induced effects on cell growth and EMT markers in ATC cells. (A–C) Cells were transfected with E-cadherin siRNA and p53 plasmid before treatment with HMA at 25 μM for 72 h. Cell numbers (A) and migration (B) were measured using a hemocytometer and wound healing assay, respectively, and the protein levels of E-cadherin, N-cadherin, vimentin, p53, p21, p27 and Akt were measured (C). Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. HMA-treated cells.

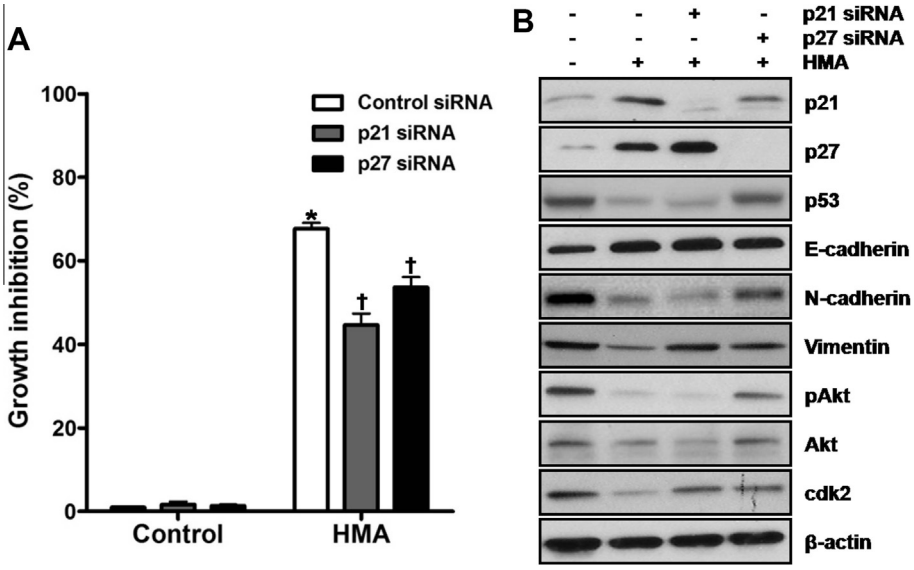

Fig. 3. The relevance of p21 and p27 to the effects of HMA on cell growth and EMT markers in ATC cells. (A and B) Cells were transfected with siRNAs against p21 and p27 before treatment with HMA at 25 μM for 72 h. Cell growth (A), and the protein levels of p21, p27, p53, E-cadherin, N-cadherin, vimentin, Akt and CDK2 (B) were measured. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. HMA-treated cells.

diminished (Fig. S1). Cells were simultaneously treated with EGF and HMA in presence of serum, after which cell growth, and the protein levels of Akt, E-cadherin, N-cadherin and vimentin were measured. In EGF-treated cells, cell growth was enhanced (Fig. 1D). The protein levels of Akt, N-cadherin and vimentin were enhanced, whereas E-cadherin protein levels were diminished (Fig. 1E). The increase of cell growth and the change in these protein levels caused by EGF were rescued by treatment with HMA.

3.2. Knockdown of E-cadherin and overexpression of p53 mitigate the effects of HMA on cell growth and EMT in ATC cells

While E-cadherin suppresses invasion and EMT phenotype of cancer cells, p53 has a role in EMT process linked with initiation and progression of cancer cells [5,22,23]. Thus, we documented whether E-cadherin and p53 are related to HMA-induced effects on cell growth and EMT in ATC cells. When cells were transfected with E-cadherin siRNA and p53 plasmid prior to treatment with HMA, knockdown of E-cadherin attenuated the HMA-induced decrease of cell numbers (Fig. 2A) and migration (Fig. 2B). Furthermore, overexpression of p53 also tended to ameliorate the HMA-induced decrease of cell numbers and migration.

Next, cells were transfected with E-cadherin siRNA and p53 plasmid before treatment with HMA, and the protein levels of E-cadherin, N-cadherin, vimentin, p53, p21, p27 and Akt were measured. In HMA-treated cells, the protein levels of p21 and p27 increased, whereas those of p53 and Akt decreased (Fig. 2C). Transfection of E-cadherin siRNA increased the protein levels of N-cadherin, vimentin, p53 and Akt, while it decreased those of p21 and p27. Transfection of p53 plasmid increased the protein levels of N-cadherin, vimentin, p21 and Akt, whereas it decreased those of E-cadherin and p27.

3.3. Silencing of p21 and p27 partially rescues HMA-induced effects on cell growth and EMT in ATC cells

p21 represses Ras- and c-Myc-dependent EMT and cancer stem cell-like gene expression in an *in vivo* breast tumor model [24]. p27 mediates inhibition of E-cadherin-dependent growth in mouse breast cancer cells [25]. In this study, HMA elevated the protein levels of p21 and p27, and thus we determined whether p21 and p27 are relevant to the effects of HMA on cell growth and EMT in ATC cells. When cells were transfected with siRNAs against p21 and p27 prior to treatment with HMA, growth inhibition resulted from HMA was relieved by transfection of both siRNAs (Fig. 3A).

Next, cells were transfected with siRNAs against p21 and p27 before treatment with HMA, and the protein levels of p21, p27, p53, E-cadherin, N-cadherin, vimentin and Akt were measured. To identify knockdown of p21 and p27, the protein levels of cdk2, which is inactivated by p21 and p27, were also measured. In HMA-treated cells, transfection of both siRNAs did not alter E-cadherin protein levels (Fig. 3B). Transfection of p21 siRNA elevated the protein levels of vimentin and cdk2, while transfection of p27 siRNA elevated those of p53, N-cadherin and Akt as well as vimentin and cdk2.

3.4. Activation of PI3K/Akt signaling countervails the effects of HMA on survival as well as growth and EMT of ATC cells

The activation of PI3K/Akt signaling diminishes expression of E-cadherin, and the inactivation of the signaling reverses EMT phenotype [9,26]. In this study, HMA enhanced E-cadherin protein levels, while it diminished Akt protein levels. In addition, knockdown of E-cadherin abrogated HMA-induced suppression of Akt. Thus, we explored the role of PI3K/Akt signaling in HMA-induced effects on cell growth and EMT in ATC cells. When cells were transfected with PIK3CA plasmid prior to treatment with HMA, cell growth was enhanced (Fig. 4A). In PIK3CA plasmid-transfected and HMA-treated cells, compared with HMA-treated cells, the protein levels of N-cadherin, vimentin and p53 were enhanced, whereas those of E-cadherin, p21 and p27 were diminished (Fig. 4B).

As cells were treated with HMA at various doses and times, the percentage of dead cells was diminished in a dose- and time-dependent manner (Fig. 4C). The percentage of dead cells (Fig. 4D), and the protein levels of cleaved PARP and cleaved caspase-3 (Fig. 4E) were diminished in PIK3CA plasmid-transfected and HMA-treated cells, compared with HMA-treated cells.

4. Discussion

The hsp90 inhibitor HMA has a potent cytotoxic effect on human malignant tumor cells [16]. However, the influence of HMA on

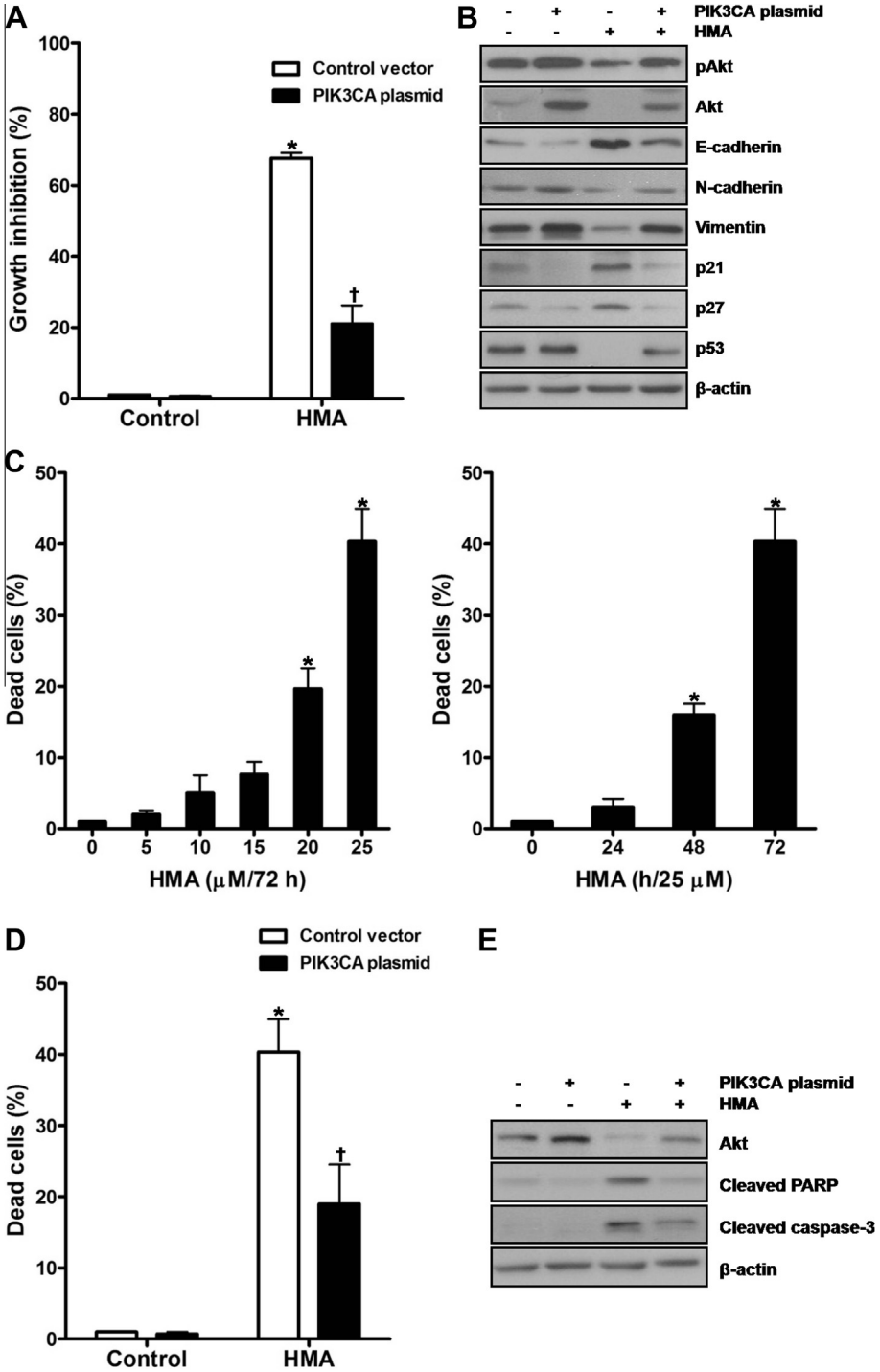

Fig. 4. The role of PI3K/Akt signaling in HMA-induced effects on survival as well as growth and EMT markers of ATC cells. (A and B) Cells were transfected with PIK3CA plasmid before treatment with HMA at 25 μM for 72 h. Cell growth (A), and the protein levels of Akt, E-cadherin, N-cadherin, vimentin, p21, p27 and p53 (B) were measured. (C) Cells were treated with HMA at 5, 10, 15, 20 and 25 μM for 72 h, and at 25 μM for 24, 48 and 72 h, and the percentage of dead cells was measured using FACS analysis. (D and E) Cells were transfected with PIK3CA plasmid, after which were treated with HMA at 25 μM for 72 h. The percentage of dead cells (D), and the protein levels of Akt, cleaved PARP and cleaved caspase-3 (E) were measured. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. HMA-treated cells.

growth and EMT of ATC cells has not been investigated. In this study, we showed for the first time that HMA suppresses cell growth with concomitant reversal of EMT by involvement of E-cadherin, p21, p27, p53 and PI3K/Akt signaling in ATC cells. E-cadherin is a differentiation marker of thyroid cancer, and shows little expression in ATC [3–6,27]. E-cadherin is highly expressed in non-neoplastic thyroid and DTC tissues but rarely expressed in ATC tissues, representing that ATC has an EMT phenotype [10–13]. Meanwhile, EGF stimulates migration and invasiveness by activation of EGFR in ATC cells [18–20]. HMA accelerates degradation of EGFR, and thereby represses EGF-mediated EMT in human epidermoid carcinoma cells [21]. In this study, HMA increased E-cadherin, and decreased N-cadherin and vimentin in experiments of Western blotting and IF. Moreover, HMA suppressed cell growth and migration in conjunction with increase of E-cadherin and decrease of N-cadherin and vimentin. Meanwhile, EGF stimulated cell growth,

and increased N-cadherin, vimentin and EGFR-related signal proteins such as Akt and Src, and decreased E-cadherin. EGF-induced change in cell growth and EMT markers was attenuated by treatment with HMA. Taken together, these results suggest that HMA inhibits cell growth, and reverses EMT in ATC cells with or without stimulation by EGF.

While E-cadherin represses invasion and EMT phenotype of cancer cells, p53 has a role in EMT process associated with initiation and progression of cancer cells [5,22,23]. In this regards, overexpressed p53 suppresses expression of E-cadherin in colon and breast cancer cells [28,29]. In this study, HMA inhibited cell growth concomitantly with increase of E-cadherin and decrease of p53. In HMA-treated condition, knockdown of E-cadherin and overexpression of p53 increased N-cadherin and vimentin. Knockdown of E-cadherin increased p53, whereas overexpression of p53 decreased E-cadherin, implying that E-cadherin and p53 reciprocally affects each other in HMA-treated ATC cells. Knockdown of E-cadherin and overexpression of p53 mitigated the inhibitory effects of HMA on cell growth and migration. These results suggest that HMA represses cell growth with concomitant reversal of EMT by regulating E-cadherin and p53 in ATC cells. Intriguingly, in HMA-treated condition, knockdown of E-cadherin caused decrement of p21 and p27, while overexpression of p53 caused increment of p21 and decrement of p27, connoting that E-cadherin and p53 modulate p21 and p27 in HMA-treated ATC cells. In addition, different effects of E-cadherin knockdown and p53 overexpression on cell growth and migration may be explained by different expression of p21 and p27 under these conditions.p21 represses Ras- and c-Myc-dependent EMT and cancer stem cell-like gene expression in an *in vivo* breast tumor model, and p27 suppresses E-cadherin-dependent growth in mouse breast cancer cells [24,25]. In this study, HMA-induced growth inhibition was accompanied by increase of p21 and p27 and decrease of cdk2. In HMA-treated condition, knockdown of p21 and p27 ameliorated HMA-induced growth inhibition. As to EMT markers, knockdown of p21 increased vimentin and cdk2, while knockdown of p27 increased N-cadherin and p53 as well as vimentin and cdk2. These results suggest that silencing of p21 and p27 rescues the inhibitory effects of HMA on cell growth and EMT in ATC cells.

PI3K/Akt signaling is deregulated in ATC cells, and Akt is activated in most ATC patients [14]. In regard to EMT, the activation of PI3K/Akt signaling represses expression of E-cadherin, and the inactivation of the signaling reverses EMT phenotype [9,26]. In this study, HMA increased E-cadherin, and decreased Akt. In HMA-treated condition, knockdown of E-cadherin attenuated decrease of Akt. As to EMT markers, the activation of Akt increased N-cadherin and vimentin, and decreased E-cadherin. These findings demonstrate that HMA suppresses EMT and PI3K/Akt signaling, and the activation of PI3K/Akt signaling mitigates HMA-induced inhibition of EMT in ATC cells. Meanwhile, in regard to cell survival, HMA induced cell death with concomitant increase of cleaved caspase-3 protein levels, which was relieved by transfection of PIK3CA plasmid. Our results indicate that HMA represses cell survival as well as growth by suppression of PI3K/Akt signaling in ATC cells.

In this study, HMA decreased p53 and Akt. In HMA-treated condition, overexpression of p53 ameliorated decrease of Akt, and the activation of Akt increased p53, revealing that p53 may be positively related to PI3K/Akt signaling in HMA-treated ATC cells. Meanwhile, in HMA-treated condition, knockdown of E-cadherin attenuated increase of p21 and p27. Furthermore, knockdown of p27 increased Akt, and the activation of Akt decreased p21 and p27. These findings manifest that p21 and p27 may be negatively relevant to PI3K/Akt signaling in HMA-treated ATC cells.

In conclusion, our results demonstrate that HMA inhibits cell growth, and reverses EMT in conjunction with the activation of E-cadherin, p21 and p27 and the inactivation of p53 and PI3K/Akt signaling in ATC cells. The present study will provide new clinical implications of HMA in human ATC, which is refractory to conventional therapies.

Acknowledgments

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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.2014.11.018.

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