Enigma Plays Roles in Survival of Thyroid Carcinoma Cells through PI3K/AKT Signaling and Survivin

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Abstract. *Background/Aim: The aim of the present study was to assess the role of enigma protein in survival of thyroid carcinoma cells. Materials and Methods: BCPAP and 8505C human thyroid carcinoma cells were used. Cell viability using CCK-8 assay, the percentage of dead cells using trypan blue assay, cytotoxic activity using cytotoxicity assay, cell growth rate and cell migration using wound-healing assay were performed. Results: In enigma siRNA-transfected cells, cell viability, and the protein levels of AKT and survivin decreased. The percentage of dead cells, cytotoxic activity and cleaved poly (ADP-ribose) polymerase (PARP) protein levels increased. After transfection of p110α plasmid, the alterations in cell viability, the percentage of dead cells, cytotoxic activity, and protein levels of AKT, survivin and cleaved PARP were abrogated. Cell growth rate and cell migration were reduced with reduction of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) protein levels, as well as increased p53 and p21 protein levels. Conclusion: Enigma affects cell survival through modulation of phosphatidylinositol-3 kinase/AKT signaling and survivin, and regulates cell proliferation and migration via involvement of MMP-2, MMP-9, p53 and p21 in thyroid carcinoma cells.*

Well-differentiated thyroid carcinoma (WDTC), including papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), has an excellent outcome, whereas WDTC

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in approximately 70% of patients with distant metastasis is refractory to radioactive iodine therapy (1, 2). Meanwhile, undifferentiated thyroid carcinoma, predominantly anaplastic thyroid carcinoma (ATC), shows highly aggressive behavior characterized as extrathyroidal invasion and distant metastasis, thereby presenting an unfavorable prognosis (1, 2). Since radioactive iodine therapy-refractory WDTC and undifferentiated thyroid carcinoma can be resistant to conventional treatments, new therapeutic target molecules to improve the outcome of patients are under investigation (1, 2).

The PDZ-LIM family is composed of proteins with multiple functional domains, and the proteins share an *N*-terminal PDZ domain combined with at least one *C*-terminal LIM domain (3). The PDZ domain attaches the protein to the cell membrane, and the LIM domain communicates with protein kinases which are responsible for insulin and mitogenic signaling such as rearranged during transfection (RET)/PTC2 (RET/PTC2) (4-8). Under physiological conditions, the PDZ-LIM family proteins modulate various steps during development and homeostasis, and maintain cytoskeletons and cell adhesion through complementary interaction with integrin (3, 9, 10). In contrast, the PDZ-LIM family protein Epstein-Barr virus latent membrane protein-1 (LMP-1) suppresses cell death by regulating phosphatidylinositol-3 kinase (PI3K)/AKT signaling and the chemoresistance factor survivin in nasal natural killer/T-cell lymphoma *in vitro* and *in vivo* models (11).

Enigma (also named PDLIM7 and LMP-4) belongs to the PDZ-LIM family, and has a PDZ and three LIM domains (6-8). Functionally, enigma serves as a scaffold protein *via* interconnection with cytoskeleton network, and acts as an adaptor protein through stabilization of cell membrane and signaling machinery (12). With respect to the role of enigma in organ development, enigma promotes heart and skeletal muscle organization, and enhances bone morphogenetic factor-mediated osteogenesis, thereby being involved in direct and endochondral bone formation (3, 13). In regard to the role of enigma in tumorigenesis, enigma facilitates cell survival and chemoresistance by inhibiting p53-mediated

A

BCPAP

8505C

B

BCPAP

8505C

C
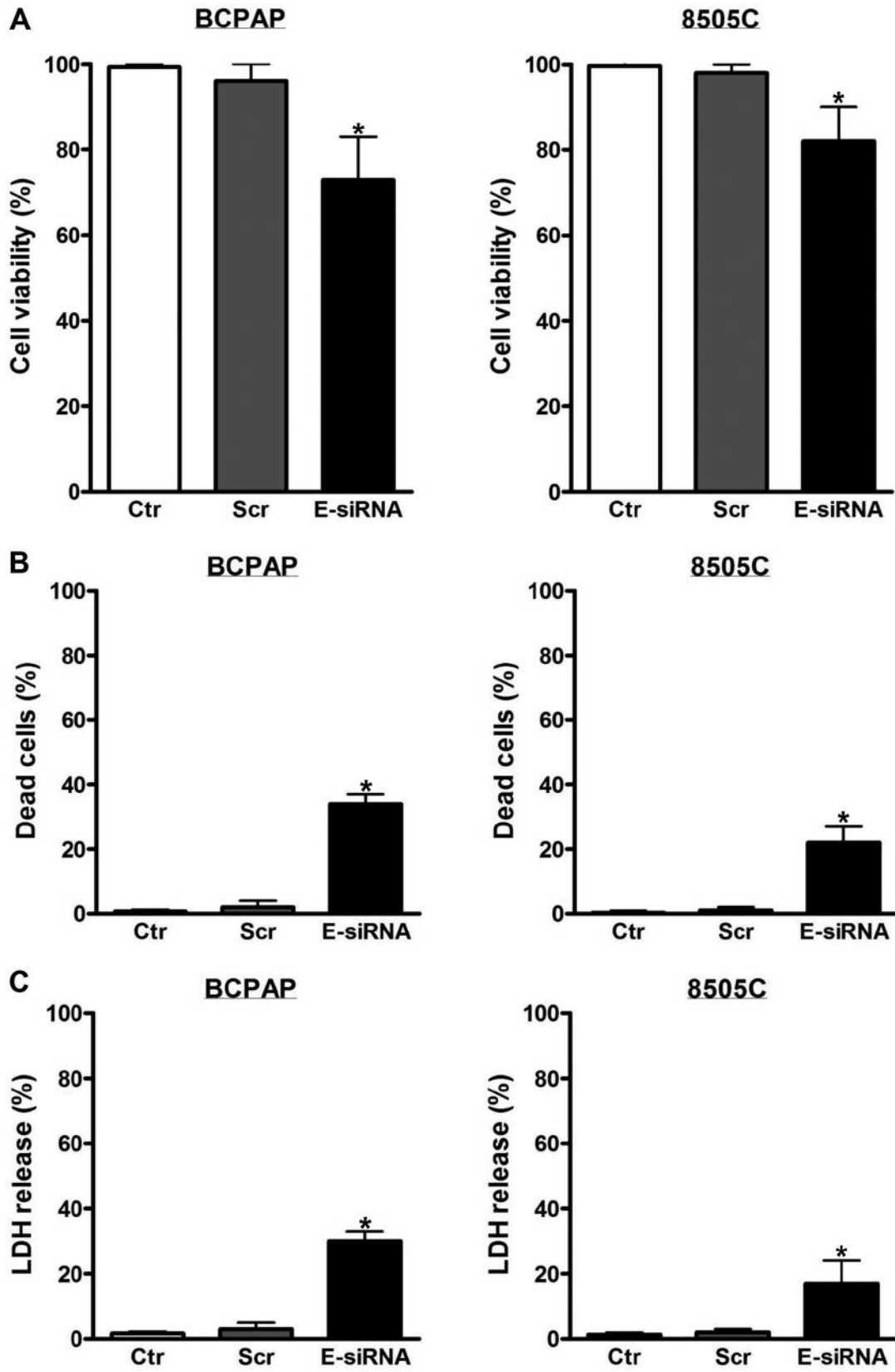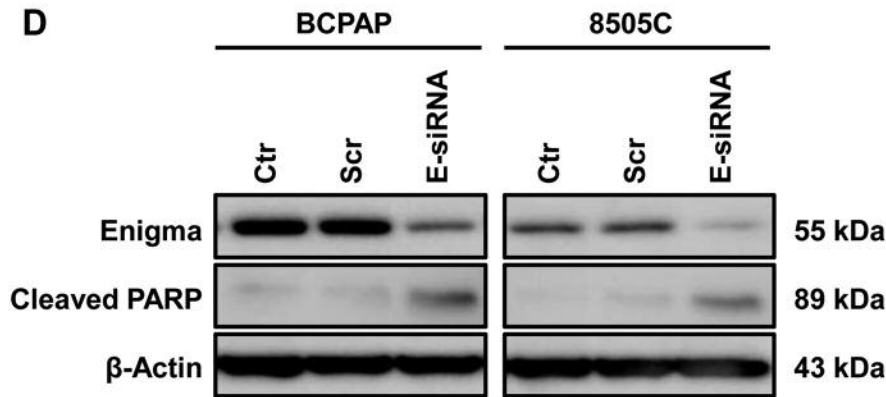
BCPAP

8505C

Figure 1. *Continued*

Figure 1. *The role of enigma in survival of thyroid carcinoma cells. BCPAP and 8505C cells were transfected with enigma siRNA (E-siRNA) for 48 h, and then cell viability using CCK-8 assay (A), the percentage of dead cells using trypan blue assay (B), cytotoxic activity using cytotoxicity assay (C), and the protein levels of enigma and cleaved poly (ADP-ribose) polymerase (PARP) (D), were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05 vs. control (Ctr). LDH: Lactate dehydrogenase; Scr: scramble RNA.*

death of human hepatoma and colon cancer cells (14). Moreover, enigma blocks Casitas B-lineage lymphoma-c (CBL-c)-mediated degradation of oncogenic protein named as RET multiple endocrine neoplasia type 2A (RETMEN2A) (15). In patients with breast cancer with activated RET, overexpression of enigma correlates with a poor survival outcome (15). In thyroid cancer, it was reported that high expression of enigma was exhibited in malignant tissues, and related to advanced pathological staging (16). However, the role of enigma in survival of thyroid carcinoma cells has not been elucidated.

The aim of the present study was to evaluate the role of enigma in survival of thyroid carcinoma cells. We for the first time demonstrate that enigma has a pivotal role in cell survival *via* modulation of PI3K/AKT signaling and survivin in thyroid carcinoma cells. Furthermore, enigma affects cell proliferation and migration in thyroid carcinoma cells.

Materials and Methods

Materials. RPMI1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and streptomycin/penicillin were purchased from Life Technologies (Carlsbad, CA, USA). Primary antibodies against enigma, and total and phospho-AKT (Ser473) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and those against β-actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against cleaved poly (ADP-ribose) polymerase (PARP), survivin, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), p53 and p21 were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). All other reagents were obtained from Sigma-Aldrich unless otherwise stated. *Cell culture*. For experiments, BCPAP human PTC cells and 8505C human ATC cells were used since these are widely popular *in vitro*

models of thyroid carcinoma. Both cells were purchased from Deutsche Sammlung von Mikroorganismenund Zellkulturen (DSMZ GmbH, Braunschweig, Germany). BCPAP cells were grown in RPMI1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS and 1% streptomycin/penicillin. 8505C cells were grown in DMEM 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 70% confluent.

Transfection of small interfering RNA (siRNA) and plasmid. An enigma siRNA from Santa Cruz Biotechnology and a p110α plasmid from Addgene (Cambridge, MA, USA) were purchased. Cells were transfected with siRNA and plasmid using Lipofectamine 3000 Reagent (Life Technologies) according to the manufacturer's protocol. Transfection efficiency was tested by western blotting.

CCK-8 assay. After transfection of enigma siRNA and p110α plasmid, viability of BCPAP and 8505C cells was assessed using CCK-8 assay. Cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells ($5\times10^3/100$ μl) in each well on 96-well plates were incubated overnight. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). All experiments were performed in triplicate.

Trypan blue assay. The percentage of dead cells after transfection of enigma siRNA and p110α plasmid in BCPAP and 8505C cells was measured using trypan blue assay. Cells ($5\times10^4/500$ μl) in each well on 12-well plates were incubated, and mixed with trypan blue dye at 37°C. Stained cells were counted using hemocytometer. All experiments were performed in triplicate.

Cytotoxicity assay. In BCPAP and 8505C cells, cytotoxic activity under transfection of enigma siRNA and p110α plasmid was assessed using cytotoxicity assay. Cytotoxic activity was measured by the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (BioVision, Linda, CA, USA). Cells ($5\times10^3/100$ μl) in each well on

96-well plates were incubated, and centrifuged at 250 × *g* for 10 min. Supernatant of 100 μl was transferred in clear 96-well plates. After addition of reaction mixture (2.5 μl Catalyst solution in 112.5 μl Dye solution), cells were incubated for 30 min at room temperature. Absorbance was measured at 495 nm using a spectrophotometer. All experiments were performed in triplicate.

Wound-healing assay. Cell migration after transfection of enigma siRNA in BCPAP and 8505C cells was assessed using 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, and then a wound (0.9 mm) was created in the cell monolayer. The wound closure was monitored by light microscopy, 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.

Western blotting. The total protein was extracted by RIPA buffer (Sigma-Aldrich) containing 1× protease inhibitor cocktail and 1× phophatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, USA). Western blotting was performed using the specific primary antibodies detailed above and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. Bands were detected using ECL Plus Western Blotting Detection System (Thermo Fisher Scientific, Rockford, IL, USA). The protein levels were quantified by densitometry using ImageJ software (NIH, Bethesda, Maryland, USA), and normalized to β-actin levels. All experiments were performed in triplicate.

Statistical analysis. All data are expressed as mean±standard error (S.E). Data were analyzed by unpaired Student's *t*-test or ANOVA as appropriate. A *p*-value less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS program version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Silencing of enigma induces death of thyroid carcinoma cells. To evaluate the role of enigma in survival of thyroid carcinoma cells, BCPAP and 8505C cells were transfected with enigma siRNA for 48 h. After transfection, cell viability was significantly diminished (Figure 1A), and the percentage of dead cells (Figure 1B) and cytotoxic activity (Figure 1C) were significantly enhanced. Moreover, enigma protein levels were significantly diminished, and cleaved PARP protein levels were significantly enhanced (Figure 1D). Taken together, these results demonstrate that repression of enigma causes death of thyroid carcinoma cells.

Enigma modulates PI3K/AKT signaling and survivin in thyroid carcinoma cells. PI3K/AKT signaling plays an essential role in survival, growth and differentiation of thyroid carcinoma cells (17, 18). Survivin, a chemoresistance factor, participates in survival of thyroid carcinoma cells (19). In the present study, to investigate the influence of enigma on PI3K/AKT signaling and survivin, cells were

transfected with enigma siRNA for 48 h, after which the protein levels of total and phospho-AKT, and survivin were measured. As a result of transfection, the protein levels of total and phospho-AKT, and survivin were significantly reduced (Figure 2A and B), indicating that suppression of enigma inhibits PI3K/AKT signaling and survivin in thyroid carcinoma cells.

Stimulation of PI3K/AKT signaling mitigates cell death in conjunction with increment of survivin in enigma-inactivated thyroid carcinoma cells. In the present study, underexpression of enigma impeded PI3K/AKT signaling in thyroid carcinoma cells, and thus the involvement of PI3K/AKT signaling in survival of cells under transfection with enigma siRNA was explored. For augmentation of PI3K/AKT signaling, cells were transfected with p110α plasmid prior to transfection of enigma siRNA for 48 h. Compared with enigma siRNA-transfected cells, in cells transfected with both p110α plasmid- and enigma siRNA, the protein levels of total and phospho-AKT, and survivin, and cell viability significantly increased, while the cleaved PARP protein level significantly decreased (Figure 3A-C). Correspondingly, the percentage of dead cells and cytotoxic activity significantly decreased (Figure 3D and E). In summary, these results reveal that activation of PI3K/AKT signaling curtails cell death with retrieval of survivin in enigma-deprived thyroid carcinoma cells.

Knockdown of enigma ameliorates proliferation and migration of thyroid carcinoma cells. Firstly, in order to evaluate the relevance of enigma to cell proliferation, cells were transfected with enigma siRNA and the cell growth rate was measured after 12, 24, 36 and 48 h. The cell growth rate was significantly diminished at 24, 36 and 48 h after transfection (Figure 4A).

Next, in order to investigate the relation of enigma to cell migration, cells were transfected with enigma siRNA for 48 h and cell migration was assessed using wound-healing assay. After transfection, cell migration was significantly diminished (Figure 4B and C). The protein levels of MMP-2, MMP-9, p53 and p21 were also measured (Figure 4D) and found to be significantly diminished, while those of p53 and p21 were significantly enhanced (Figure 4E).

Collectively, these results show that repression of enigma curbs cell proliferation and migration with concomitant alteration in MMP-2, MMP-9, p53 and p21 in thyroid carcinoma cells.

Discussion

To our knowledge, this study is the first to demonstrate that inhibition of enigma induces cell death *via* mediation of PI3K/AKT signaling and survivin in thyroid carcinoma cells. Moreover, repression of enigma reduces cell proliferation
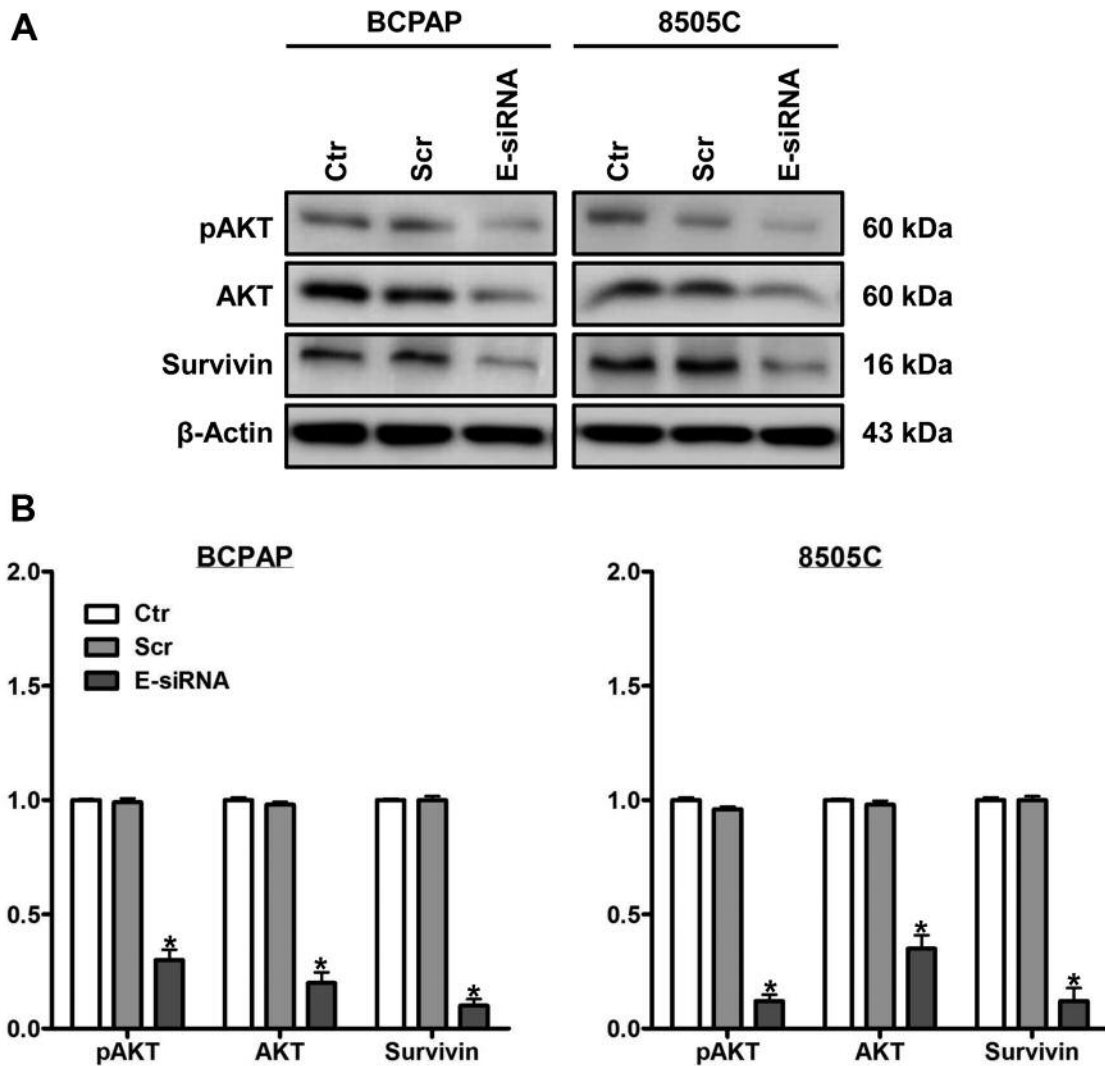
A

B

Figure 2. *The influence of enigma on phosphatidylinositol-3 kinase (PI3K)/AKT signaling and survivin in thyroid carcinoma cells. BCPAP and 8505C cells were transfected with enigma siRNA (E-siRNA) for 48 h, after which the protein levels of total and phospho-AKT, and survivin were measured (A), and quantified by densitometry (B), and normalized to β-actin levels. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05 vs. control (Ctr). Scr: Scramble RNA.*

and migration through modulation of MMP-2, MMP-9, p53 and p21 in thyroid carcinoma cells.

Overexpression of enigma is associated with prolonged survival of human hepatoma and colon cancer cells, and with poor prognosis in patients with RET-activated breast cancer (14, 15). In thyroid cancer, it was reported that enigma was highly expressed in malignant compared with benign tissues, and overexpression of enigma was positively correlated with pathological staging (16). However, the direct role of enigma in survival of thyroid carcinoma cells remains enigmatic. In the present study, suppression of enigma reduced cell viability, and increased the percentage of dead cells,

cytotoxic activity and cleaved PARP protein level, reflecting that enigma plays a fundamental role in survival of thyroid carcinoma cells. With respect to the position for enigma as a new therapeutic target in thyroid cancer, further studies are necessary to substantiate whether these phenomena are reproducible in *in vivo* models.

PI3K/AKT signaling takes part in various intracellular processes essential for survival in normal and cancer cells (20). In thyroid cancer, aberrant activation of PI3K/AKT signaling was shown to be involved in triggering of carcinogenesis (17, 18). In this regard, we reported that PI3K/AKT signaling plays critical roles in survival of
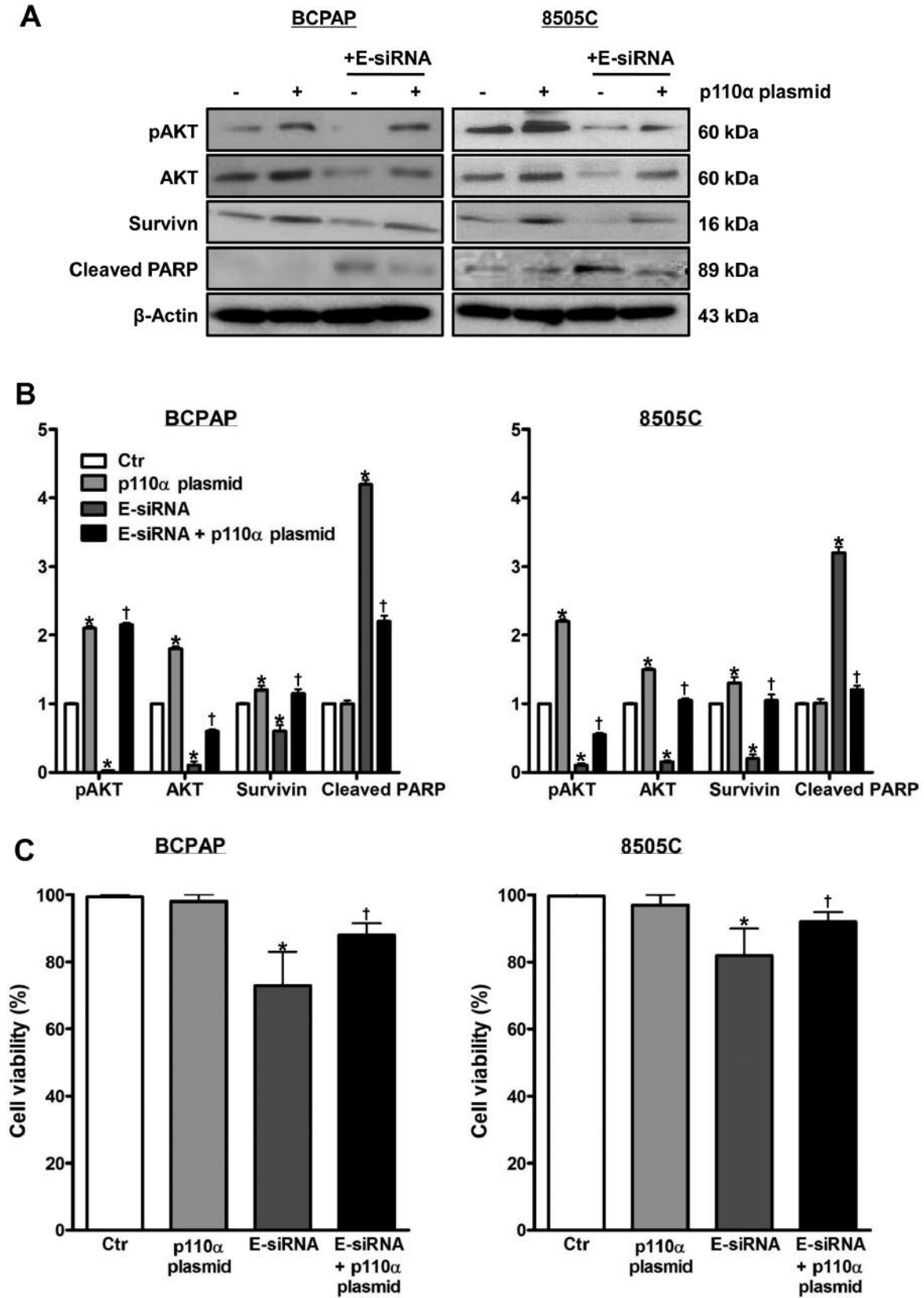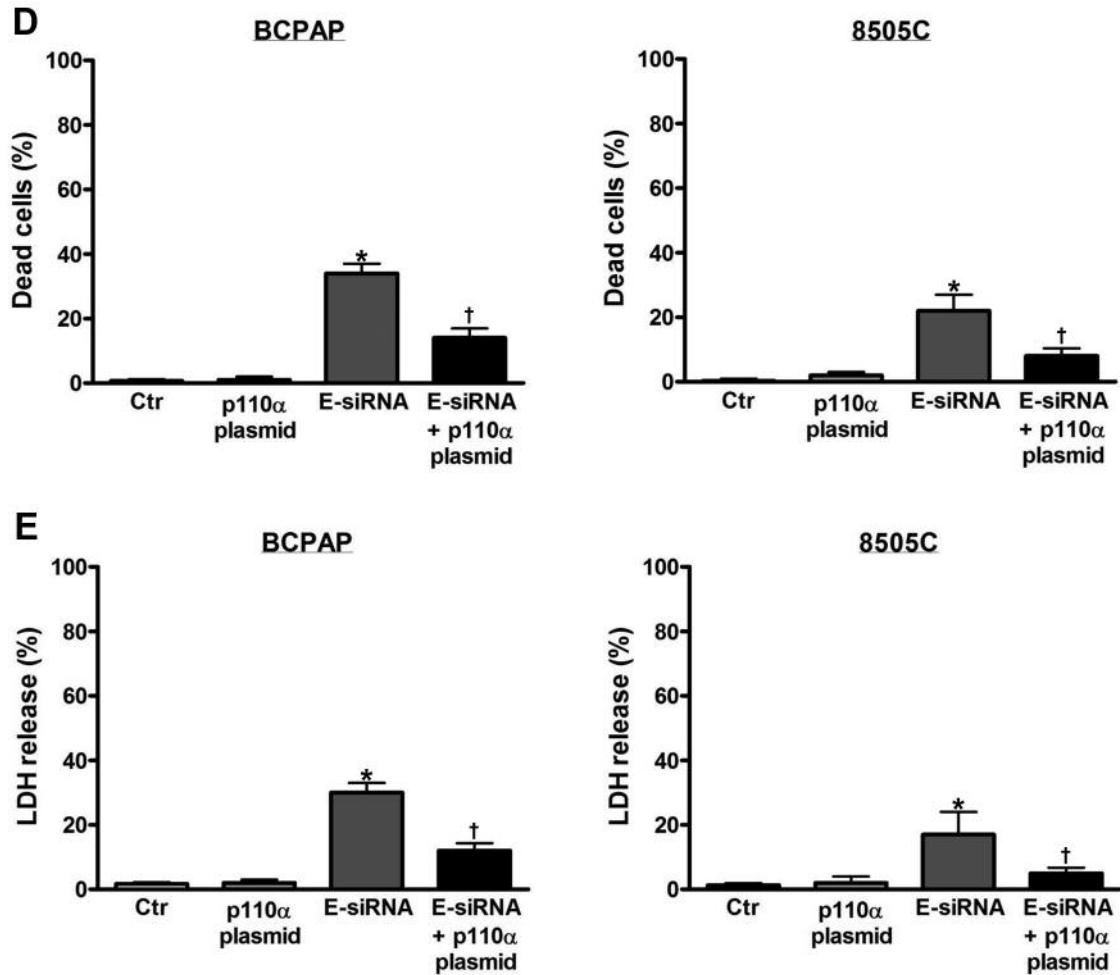
Figure 3. *Continued*

Figure 3. *The involvement of phosphatidylinositol-3 kinase (PI3K)/AKT signaling in survival of enigma-deprived thyroid carcinoma cells. BCPAP and 8505C cells were transfected with p110α plasmid prior to transfection of enigma siRNA (E-siRNA) for 48 h, and then the protein levels of total and phospho-AKT, survivin and cleaved poly (ADP-ribose) polymerase (PARP) were measured (A), and quantified by densitometry (B), and normalized to β-actin levels. Cell viability (C), the percentage of dead cells (D) and cytotoxic activity (E) were also measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05 vs. control (Ctr); †p<0.05 vs. cells transfected with E-siRNA alone. LDH: Lactate dehydrogenase; Scr: scramble RNA.*

thyroid carcinoma cells (21-26). Although the PDZ-LIM family protein LMP-1 was shown to inhibit cell death by regulating PI3K/AKT signaling in nasal natural killer/T-cell lymphoma cells (11), the participation of PI3K/AKT signaling in pro-survival effect of enigma in thyroid carcinoma cells has not been evaluated before. In the present study, repression of enigma reduced total and phospho-AKT protein levels. Furthermore, activation of PI3K/AKT signaling with transfection of p110α plasmid in enigma siRNA-transfected cells increased cell viability, and decreased the percentage of dead cells, cytotoxic activity and cleaved PARP protein levels. Collectively, our findings show that suppression of enigma inactivates PI3K/AKT signaling

in thyroid carcinoma cells, and activation of PI3K/AKT signaling mitigates cell death in enigma-deprived thyroid carcinoma cells. In addition, these results denote that enigma is involved in cell survival *via* PI3K/AKT signaling in thyroid carcinoma cells. Further to this suggestion, our results may illuminate another pathway for PI3K/AKT signaling to enhance survival of thyroid carcinoma cells.

Survivin is a member of the inhibitor of apoptosis protein family, and is expressed ubiquitously in fetal tissues (27). Additionally, survivin is related to chemoresistance and unfavorable prognosis in patients with malignant tumors, and overexpression of survivin is relevant to dedifferentiation and progression in thyroid cancer (19, 28). In this respect, we
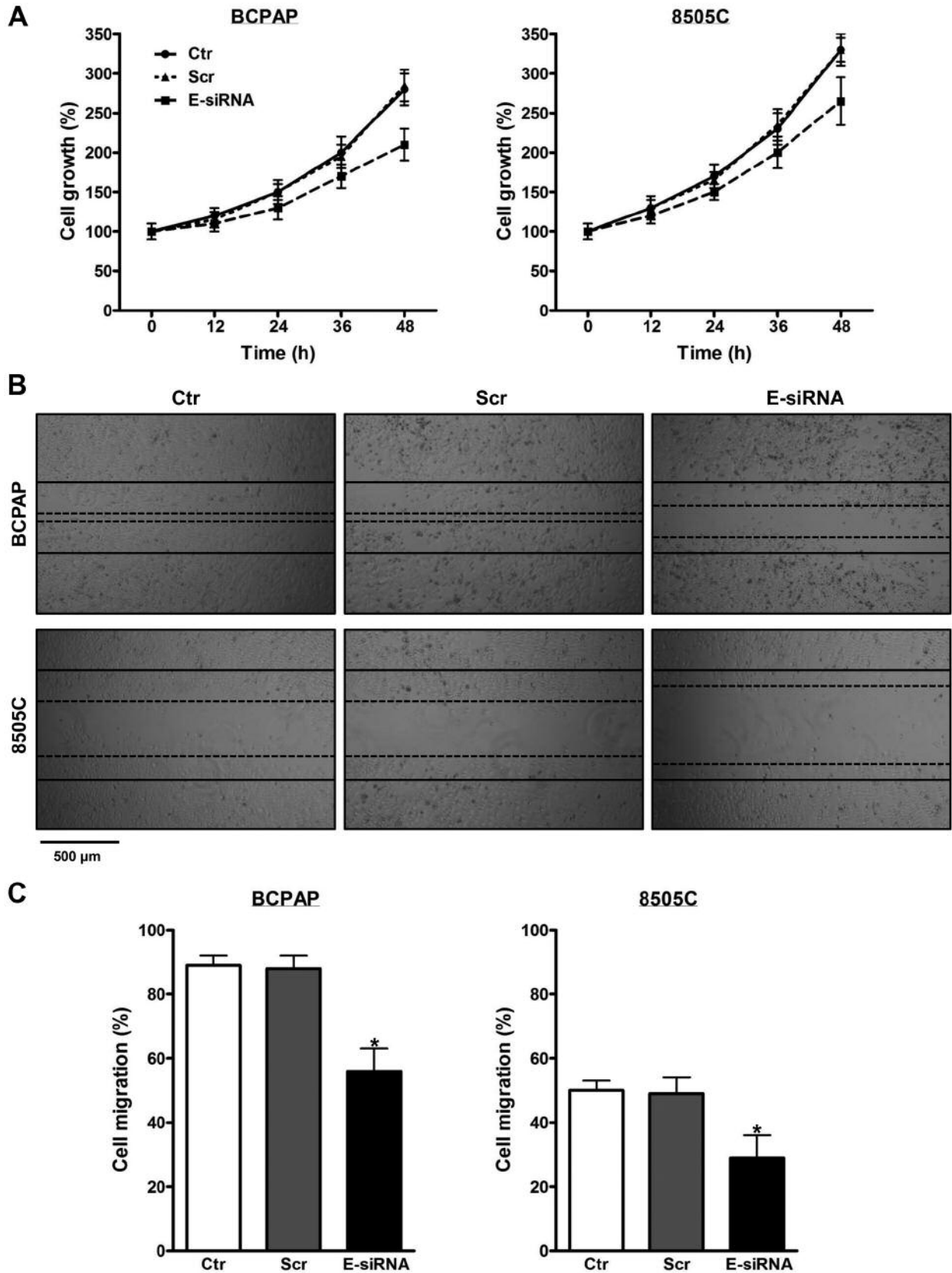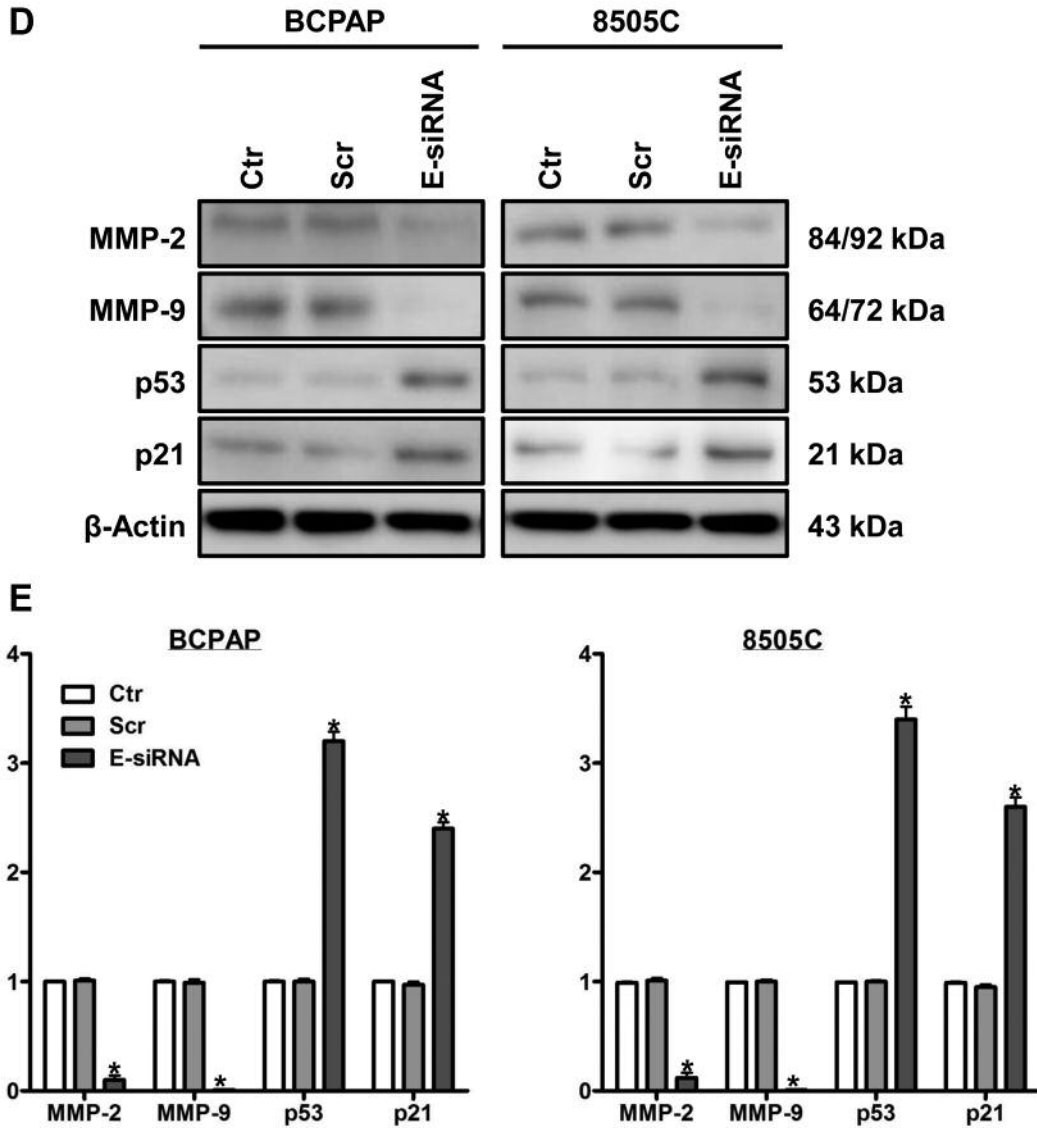
A

B

C

Figure 4. *Continued*

Figure 4. *The role of enigma in proliferation and migration of thyroid carcinoma cells. A: BCPAP and 8505C cells were transfected with enigma siRNA (E-siRNA) for 12, 24, 36 and 48 h, after which cell growth rate was measured. B and C: BCPAP and 8505C cells were transfected with E-siRNA for 48 h, and then cell migration using wound-healing assay was measured. D and E: BCPAP and 8505C cells were transfected with E-siRNA for 48 h. The protein levels of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), p53 and p21 were measured, and quantified by densitometry, and normalized to β-actin levels. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05 vs. control (Ctr). Scr: Scramble RNA.*

reported that expression of survivin was associated with survival of thyroid carcinoma cells (21-26). Survivin is stimulated through PI3K/AKT signaling in response to cytokines, growth factors and chemotherapeutic agents in malignant cells (29). Regarding the interactions among the PDZ-LIM family protein, PI3K/AKT signaling and survivin, LMP-1 was implicated in augmentation of expression of survivin by activating PI3K/AKT signaling in nasal natural

killer/T-cell lymphoma animal model (11). However, the relationship among enigma, PI3K/AKT signaling and survivin in thyroid carcinoma cells has not been investigated. In the present study, inhibition of enigma decreased survivin protein levels, and activation of PI3K/AKT signaling in enigma siRNA-transfected cells diminished decrement of survivin protein levels. Intriguingly, these alterations were accompanied by increment of cell viability and decrement of

the percentage of dead cells, cytotoxic activity and cleaved PARP protein levels. Taken together, our findings reveal that repression of enigma abrogates expression of survivin in thyroid carcinoma cells, and activation of PI3K/AKT signaling attenuates cell death with reexpression of survivin in enigma-inactivated thyroid carcinoma cells. Moreover, these results imply that enigma counteracts cell death *via* intensification of PI3K/AKT signaling and survivin in thyroid carcinoma cells. However, the detailed mechanism for the involvement of enigma in communicating with PI3K/AKT signaling and survivin should be further explored.

MMP-2, MMP-9, p53 and p21 are involved in proliferation, migration, invasion and metastasis of tumor cells (30-32). In thyroid cancer, our report shows that MMP-2, MMP-9, p53 and p21 were responsible for proliferation and migration of thyroid carcinoma cells (33). In terms of the influence of enigma on proliferation and migration, enigma was found to mediate mitogenic signaling of RET/PTC2 and to attenuate antiproliferative activity of p53 through MDM2 (7, 8, 14). In the present study, suppression of enigma reduced cell growth and migration with concomitant decrease of MMP-2 and MMP-9 protein levels and increase of p53 and p21 protein levels, indicating that inhibition of enigma ameliorates cell proliferation and migration through manipulation of MMP-2, MMP-9, p53 and p21 in thyroid carcinoma cells. These results show that enigma modulates proliferation and migration as well as survival of thyroid carcinoma cells. In clinical practice, these findings may highlight enigma protein as a potential drug target in thyroid cancer refractory to conventional therapy.

Acknowledgements

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