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1α,25(OH)$_2$D$_3$ Analog, MART-10, Inhibits Neuroendocrine Tumor Cell Metastasis After VEGF-A Stimulation

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Abstract. *Aims: Pancreatic neuroendocrine tumors (PanNETs) are usually diagnosed in an advanced stage. Most patients with PanNETs die of metastasis. Vascular endothelial growth factor-A (VEGF-A) is a strong stimulator of angiogenesis and tumor metastasis. We aimed to investigate the effect of MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$], a 1α,25-dihydroxy-vitamin D3 (1α,25(OH)$_2$D$_3$) analog, on PanNET cell metastasis after VEGF-A stimulation. Materials and Methods: Migration and invasion assays, western blot, and immunofluorescent staining were applied in this study. Results: VEGF-A increased PanNET cell migration and invasion, which was attenuated by 1α,25(OH)$_2$D$_3$ and MART-10. VEGF-A treatment stimulated epithelial–mesenchymal transition (EMT) of PanNET cells. During this process, expression of snail family transcriptional repressor 1 and 2, and fibronectin was up-regulated. 1α,25(OH)$_2$D$_3$ and MART-10 counteracted VEGF-A-induced EMT. In addition, expression of neuropilin 1, a key protein in VEGF-A signaling, was down-regulated by 1α,25(OH)$_2$D$_3$ and MART-10. Furthermore, synthesis of F-actin was increased by VEGF-A and reduced by 1α,25(OH)$_2$D$_3$ and MART-10. Conclusion: Our data indicate that MART-10 could be deemed a promising drug for PanNET treatment.*

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Pancreatic neuroendocrine tumors (PanNETs) are rare tumors, comprising 1-2% of all pancreatic tumors. Due to the fact that most PanNETs present with a slow growth without obvious symptoms, the majority of patients with PanNET are thus diagnosed at late stage, excluding the feasibility of radical surgery, which is the cornerstone of treatment, leading to a poor prognosis (1). Despite recent advances in target therapies, which obviously have improved the survival of patients with PanNET metastasis, the outcomes are still not very satisfying (2, 3). Thus, development of new therapeutic agents against PanNETs should be prioritized.

1α,25-Dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$ the active form of vitamin D, is well known for its mineral functions, which include increasing calcium absorption in both small intestine and kidneys, and calcium deposition in the bone. Recent evidence indicates that 1α,25(OH)$_2$D$_3$ acts like a hormone and it has been shown to have pro-apoptosis, pro-differentiation, anti-proliferation, and anti-metastasis effects on a variety of cancer cells (4). However, the clinical application of 1α,25(OH)$_2$D$_3$ in treating cancer is hampered by its side-effect of hypercalcemia. To avoid this, abundant analogs have been created in an effort to produce more potent agents with less likelihood of inducing hypercalcemia (5).

One kind of 1α,25(OH)$_2$D$_3$ analog features 19-nor structure, in which two hydrogen atoms are used to replace C19 methylene group (6). 19-Nor-1α,25(OH)$_2$D$_3$, was produced by Perlman *et al.* and was shown to be less calcemia-inducing and similarly potent as compared to 1α,25(OH)$_2$D$_3$ (7). Further A-ring modification of 19-nor-1α,25(OH)$_2$D$_3$ generates a series of analogs (8), including MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$] (9). MART-10 has been shown to be more active than 1α,25(OH)$_2$D$_3$ in tumor growth

inhibition against cholangiocarcinoma and pancreatic cancer *in vitro* and *in vivo* without inducing hypercalcemia (10, 11). MART-10 was furthermore shown to repress metastatic potential of cancer cells (12-15). For neuroendocrine tumor, MART-10 has been demonstrated to induce G_0/G_1 cell-cycle arrest and apoptosis to repress cancer growth (16).

Cancer growth and metastasis need neo-angiogenesis to provide nutrients. Thus, anti-angiogenesis has widely been applied in cancer treatment and prevention (17). Vascular endothelial growth factor-A (VEGF-A), a strong stimulator of angiogenesis, has become a target for anti-angiogenesis treatment. For NETs, bevacizumab, a monoclonal antibody against VEGF-A, has been shown to increase progression-free-survival of patients with NETs (18).

In this study, we aimed to investigate the effect of $1\alpha,25(OH)_2D_3$ and MART-10 on the metastatic potential of NETs after VEGF-A stimulation.

Materials and Methods

Vitamin D compounds and VEGF-A. $1\alpha,25(OH)_2D_3$ was purchased from Sigma (St. Louis, MO, USA). MART-10 was synthesized by Kittaka *et al*. (9). VEGF-A was obtained from R&D Systems (Minneapolis, MN, USA).

Cell culture. Rat insulinoma cell line, RIN-m (ATCC® CRL2057™), was purchased from American Type Culture Collection (Manassas, VA, USA). RIN-m cells were grown in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific). Culture medium was changed three times per week.

Transwell filter migration assay. RIN-m cell cells were treated with 20 ng/ml VEGF-A for 24 hours then with either 10^{-7} or 10^{-8} M MART-10 or $1\alpha,25(OH)_2D_3$ for 48 hours. The migration assay was conducted as previously described [15]. Briefly, 2×10^5 cells were seeded on each transwell filter with 8.0-μm pores (Costar, Cambridge, MA, USA). Cells that had migrated through the pores after 24 hours were counted under a microscope (IX71; Olympus, Tokyo, Japan). The experiments were performed in triplicates.

Matrigel invasion assay. RIN-m cell cells were treated with 20 ng/ml VEGF-A for 24 h then with 10^{-7} or 10^{-8} M MART-10 or $1\alpha,25(OH)_2D_3$ for 48 h. The matrigel invasion assay was performed as previously described (15). The cells that had invaded to the opposite side of the matrigel-coated membrane after 48 hours were digitally photographed and counted under a microscope (IX71; Olympus. Experiments were performed in triplicates.

Western blot. After 48 hours of 10^{-7} or 10^{-8} M MART-10 or $1\alpha,25(OH)_2D_3$ M treatment, cells were washed and lyzed in lysis buffers (20 mM HEPES, 1mM EDTA, 1 mM EGTA, 20 mM. NaF, 1 mM Na_3VO_4, 1 mM $Na_2P_2O_7$, 1 mM DTT, 0.5 mM PMSF. 1 μg/ml leupeptin, and 1% Triton X-100). The detailed procedures for western blot were described previously (11). The antibodies used in this experiment were monoclonal antibodies against snail family transcriptional repressor 1 (SNAI1; 1:1,000, #3879; Cell Signaling Technology, Danvers, MA, USA), SNAI2 (1:1000, #9585; Cell Signaling Technology), fibronectin (1:1,000, NBP1-91258; Novus Biological, Littleton, CO, USA), vascular endothelial growth factor receptor 2 (VEGFR2; 1:500, sc-6251, Santa Cruz Biotechnology, Dallas, TX, USA), and neuropilin 1 (1:1,000, #3725; Cell Signaling Technology). The secondary antibodies used in this experiment were rabbit anti-Goat IgG antibody (1:5,000, AP106; Millipore, Billerica, MA, USA) or goat anti-rabbit IgG antibody (1:5,000, ab137914; Abcam, Cambridge, MA, USA).

F-Actin staining. After treatment with 20 ng/ml VEGF-A for 24 h with or without 10^{-7} M MART-10 or $1\alpha,25(OH)_2D_3$ treatment for 48 h, RIN-m cells were seeded onto glass-bottom culture dishes and stained (MatTek, Ashland, MD, USA). The detailed procedures for F-actin staining were as previously described (13). Immunofluorescence of RIN-m cells was examined using a confocal microscope (LSM510 Meta; Zeiss, Oberkochen, Germany).

Statistical methods. The data from each group were compared by Sudent *t*-test with differences with $p<0.05$ being considered significant. The program of Excel 2010 was employed to conduct the statistical analysis.

Results

Effect of $1\alpha,25(OH)_2D_3$ and MART-10 on migration and invasion of VEGF-A-stimulated RIN-m cells. Since migration and invasion are two vital steps in cancer metastasis, we first investigated the effect of VEGF-A on RIN-m cell migration and invasion. As shown in Figure 1, VEGF-A treatment significantly increased RIN-m cell migration and invasion. 10^{-7} M and 10^{-8} M $1\alpha,25(OH)_2D_3$ and MART-10 significantly attenuated the increased migration and invasion of RIN-m cells induced by VEGF-A treatment in a dose-dependent manner. Our data indicate that VEGF-A increases the metastatic potential RIN-m cells, which was repressed by both $1\alpha,25(OH)_2D_3$ or MART-10, with MART-10 being more potent than $1\alpha,25(OH)_2D_3$.

Effect of VEGF-A, $1\alpha,25(OH)_2D_3$ and MART-10 on epithelial–mesenchymal transition (EMT) of RIN-m cells. Since EMT is a vital process to render cancer cells more invasive, we evaluated the effect of VEGF-A, $1\alpha,25(OH)_2D_3$ and MART-10 on EMT of RIN-m cells. Figure 2A shows that VEGF-A induced expression of SNAI1 and SNAI2 in RIN-m cells, which was significantly repressed by both $1\alpha,25(OH)_2D_3$ and MART-10, with the latter being more potent. Fibronectin, a mesenchymal cell marker, was significantly up-regulated by VEGF-A and down-regulated by $1\alpha,25(OH)_2D_3$ and MART-10 (Figure 2B). Our data indicate that VEGF-A induced EMT in RIN-m cells, which was attenuated by both $1\alpha,25(OH)_2D_3$ and MART-10.

Effect of $1\alpha,25(OH)_2D_3$ and MART-10 on neuropilin 1 and VEGFR2 expression in VEGF-A-stimulated RIN-m cells. Neutropilin 1 and VEGFR2 are two important proteins in VEGF-A-induced cell migration and invasion. As shown in

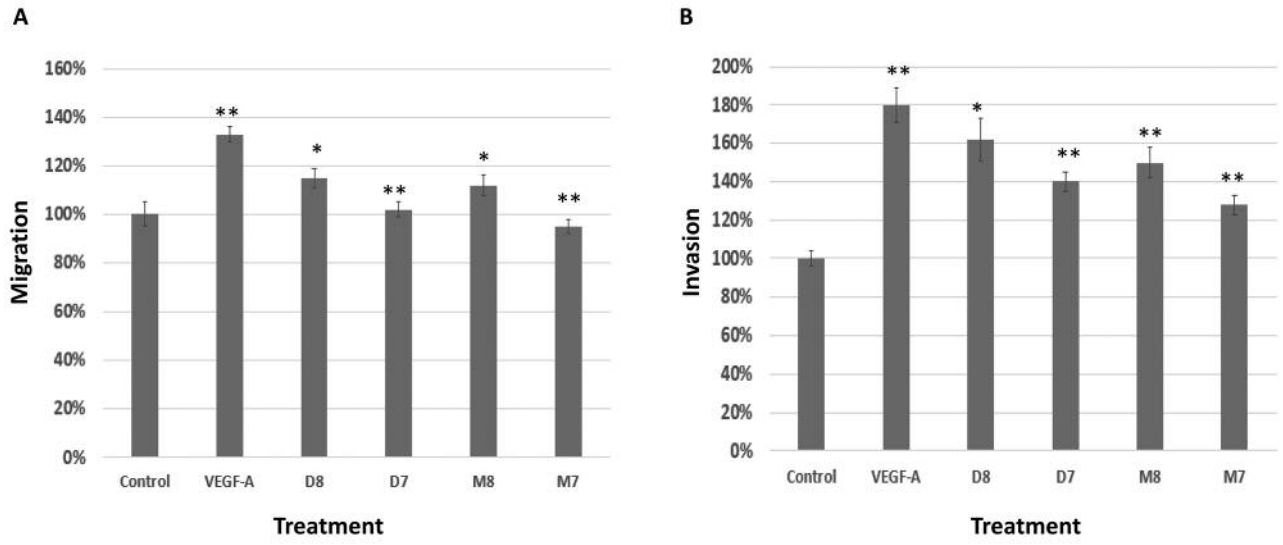

Figure 1. *The effect of is a strong stimulator of angiogenesis and tumor metastasis. We aimed to investigate the effect of MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$], and 1α,25-dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$) on vascular endothelial growth factor-A (VEGF-A)-stimulated migration (A) and invasion (B) of RIN-m cells. Migration assay: RIN-m cells were treated with 20 ng/ml VEGF-A for 24 hours then with either 10^{-7}μM (D7)/10^{-8} μM (D8) 1α,25(OH)$_2$D$_3$ or 10^{-7} μM (M7)/10^{-8} μM (M8) MART-10 for 48 hours. Migrating (A) and invading (B) cells were photographed and counted using a microscope (IX71; Olympus, Tokyo, Japan) 24 and 48 hours later, respectively. Data are presented as mean percentage (relative to the control) ±SD. Experiments were performed in triplicate and repeated at least three times. Significantly different at *p<0.05 and **p<0.01, as compared to the VEGF-A treatment group.*

Figure 3, VEGF-A treatment affected neither neuropilin 1 nor VEGFR2 expression in RIN-m cells. 1α,25(OH)$_2$D$_3$, and MART-10 treatment significantly inhibited neutropilin expression as compared to the VEGF-A-stimulated control RIN-m cells. VEGFR2 expression was not influenced by either agent.

Effect of VEGF-A, 1α,25(OH)$_2$D$_3$ and MART-10 on F-actin synthesis in RIN-m cells. Since F-actin plays a vital role during cell migration and invasion, we evaluated F-actin synthesis of RIN-m cells after different treatments using immunofluorescent stain. Figure 4 shows that F-actin synthesis in RIN-m cells was increased by VEGF-A, which was repressed by both 1α,25(OH)$_2$D$_3$ and MART-10.

Discussion

Our results indicate that VEGF-A induced RIN-m cell migration and invasion, thus increasing metastatic potential. Both 1α,25(OH)$_2$D$_3$ and MART-10 repressed VEGF-A-stimulated RIN-m cell migration and invasion, with MART-10 being more potent than 1α,25(OH)$_2$D$_3$. VEGF-A induced EMT and F-actin synthesis in RIN-m cells, which were attenuated by 1α,25(OH)$_2$D$_3$ and MART-10. Collectively, our data suggest that MART-10 could be a promising agent for PanNET treatment.

Most patients with cancer die due to metastasis, which is initiated by cancer cell dissemination, characterized by attenuated cell–cell adhesion and strengthened cell motility and invasiveness (19). EMT is a vital process during normal tissue development, but EMT can also be reactivated by cancer cells to render them more invasive and resistant to chemotherapies (20). VEGF was originally identified as an endothelial cell-specific mitogen and was found to induce angiogenesis both physiologically and pathologically (21, 22). VEGFs represent a growth factor family consisting of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor, with VEGF-A being the most prominently studied. Besides angiogenesis, VEGF-A has been found to have other functions, such as affecting host responses to cancer and fibroblasts in the tumor stroma (3, 24). Furthermore, it has been shown that VEGF-A induces EMT of cancer cells, thus promoting cancer metastasis (25, 26).

Three families of transcription factors have been implicated inactivaing EMT, including SNAI1/SNAI2, zinc finger E-box binding homeobox 1/2, and TWIST families (27). After EMT, cancer cells present more mesenchymal cell markers, such as vimentin, fibroblast-specific protein-1, fibronectin, and N-cadherin (28). Figure 2 shows that VEGF-A increased SNAI1 and SNAI2 expression in RIN-m cells, thereby triggering EMT, supported by increased fibronectin expression, which was attenuated by both 1α,25(OH)$_2$D$_3$ and MART-10. Our

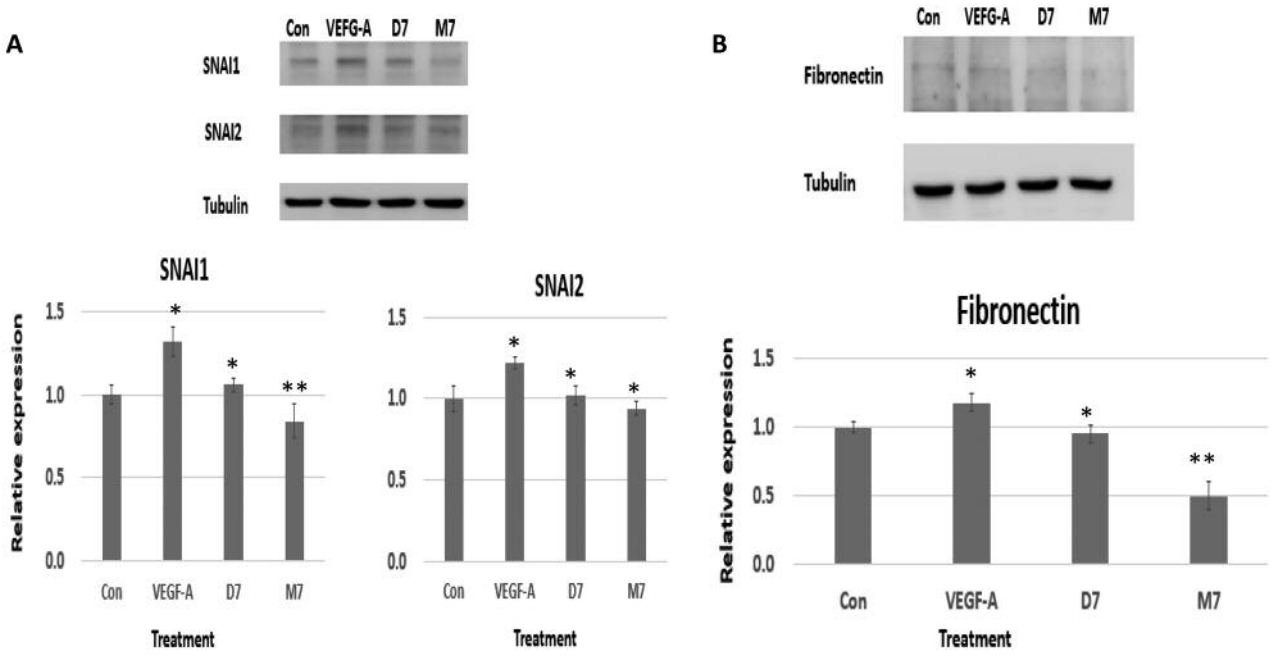

Figure 2. *The effect of 1α,25-dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$) and MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$] on vascular endothelial growth factor-A (VEGF-A)-induced expression of snail family transcriptional repressor 1 (SNAI1) and SNAI2 (A), and fibronectin (B) in RIN-m cells. RIN-m cells were stimulated with 20 ng/ml VEGF-A for 24 h with or without 48 h of F-A for 24 h then with either 10^{-7} μM (D7)/10^{-8} μM (D8) 1α,25(OH)$_2$D$_3$ or 10^{-7} μM (M7)/10^{-8} μM (M8) MART-10 treatment. Western blot analysis of SNAI1, SNAI2 and fibronectin (upper panels) and quantitation of the western blot (lower panels) were then carried out. Data are presented as mean fold expression relative to the control ±SD. Experiments were performed in triplicate. Significantly different at *p<0.05 and **p<0.01, as compared to the VEGF-A treatment group. Con: Control.*

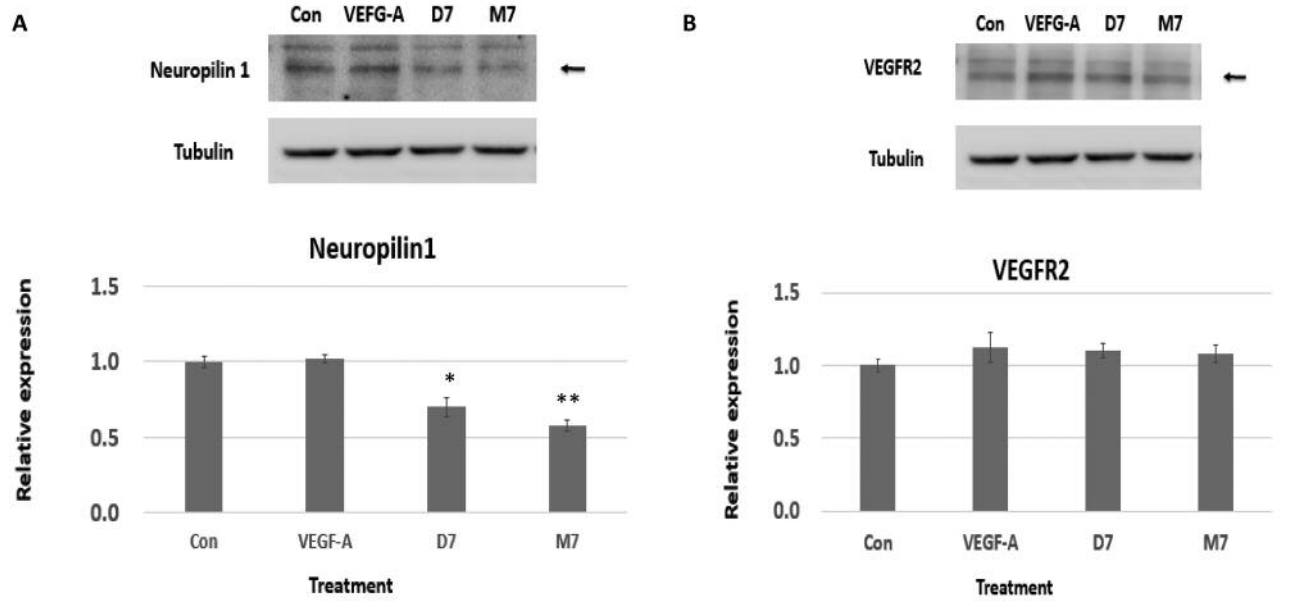

Figure 3. *The effect of 1α,25-dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$) and MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$] on vascular endothelial growth factor-A (VEGF-A)-induced expression of VEGF receptor 2 (VEGFR2) (A) and neuropilin 1 (B) in RIN-m cells.RIN-m cells were stimulated with 20 ng/ml VEGF-A for 24 h with or without 48 h of 10^{-7} μM (D7)/10^{-8} μM (D8) 1α,25(OH)$_2$D$_3$ or 10^{-7} μM (M7)/10^{-8} μM (M8) MART-10 treatment. Western blot analysis of VEGFR2 and neuropilin 1 (upper panels) and quantitation of the western blot (lower panels) were then carried out. Data are presented as mean fold expression relative to the control ±SD. Experiments were performed in triplicate. Significantly different at *p<0.05 and **p<0.01, as compared to the VEGF-A treatment group. Con: Control.*

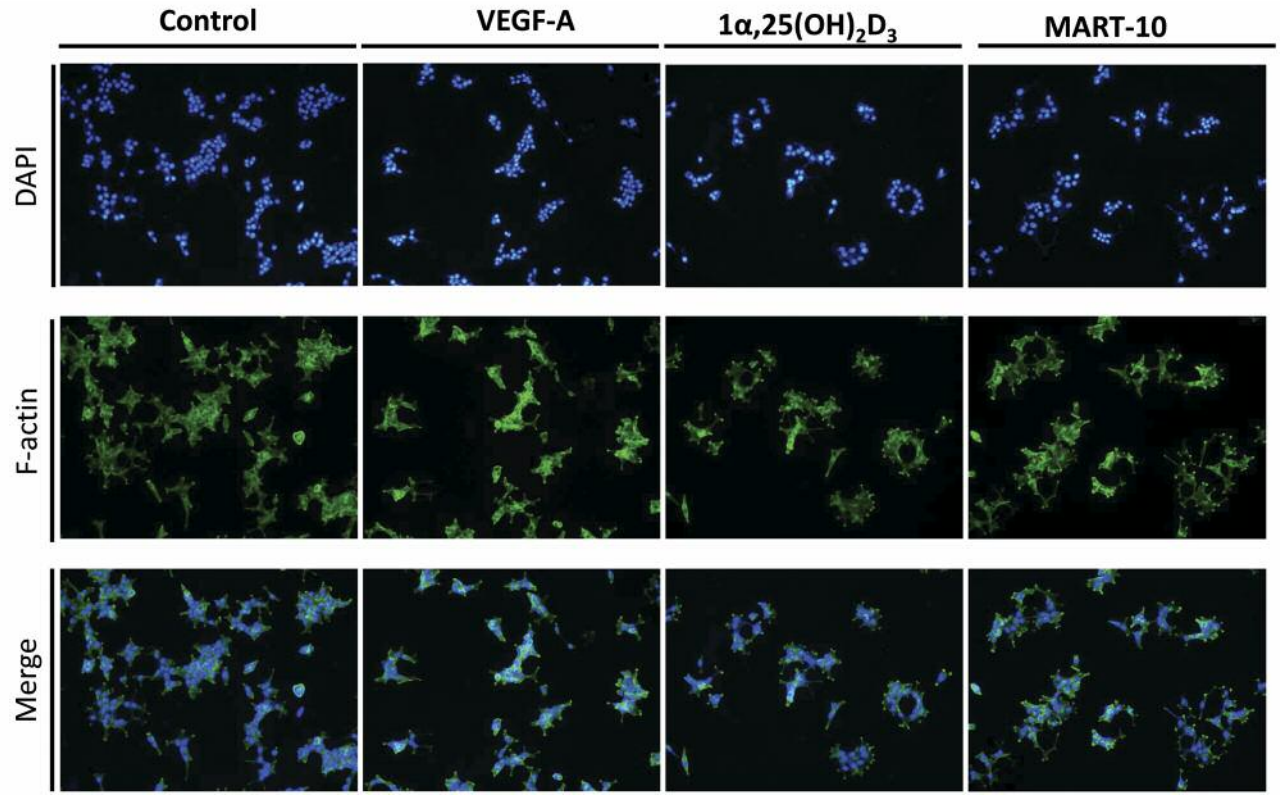

Figure 4. *The effect of 1α,25-dihydroxyvitamin D3 (1α,25(OH)$_2$D$_3$) and MART-10 [19-nor-2α-(3-hydroxypropyl)-1α,25(OH)$_2$D$_3$] on F-actin synthesis in RIN-m cells. Cells were treated with 20 ng/ml vascular endothelial growth factor-A (VEGF-A) for 24 hours with and without 10^{-7} μM 1α,25(OH)$_2$D$_3$ or 10^{-7} μM MART-10 treatment for 48 hours. Immunofluorescence staining of F-actin (green) in RIN-m cells was then performed. 4',6-Diamidino-2-phenylindole (DAPI) (blue) was applied as a nuclear stain.*

results thus clearly indicate that VEGF-A appears to activate EMT in RIN-m cell cells. 1α,25(OH)$_2$D$_3$ and MART-10 seem to be able to block this process, thus reducing RIN-m cell metastatic potential after VEGF-A treatment.

VEGF-A needs to bind with VEGFRs to achieve its functions. Three main kinds of VEGFRs have been identified, which are VEGFR1, VEGFR2, and VEGFR3 (29), with VEGFR2 being the predominant in mediating VEGF-A signaling (30). A variety of cancer types have been found to express VEGFRs, correlating with some clinical parameters (31). Pancreatic cancer has previously been shown to express VEGFR2 (32). Figure 3A shows that RIN-m cells exhibited expression of VEGFR2, which was not influenced by VEGF-A or 1α,25(OH)$_2$D$_3$, or MART-10 treatment. Neuropilin 1, originally known as a neuronal receptor (33), is able to bind with VEGFR2 to increase VEGF-A and VEGFR2 binding affinity (34). As shown in Figure 3B, RIN-m cells expressed neuropilin 1, which was not affected by VEGF-A but was down-regulated by 1α,25(OH)$_2$D$_3$ and MART-10. Our results suggest that 1α,25(OH)$_2$D$_3$ and MART-10 inhibited the effeCt of VEGF-A on RIN-m cell partly through attenuation of neuropilin 1 expression.

Since F-actin synthesis provides force to drive cancer cell migration (35), we evaluated F-actin synthesis in RIN-m cells after different treatments using immunofluorescence stain. Figure 4 shows that VEGF-A increased F-actin synthesis. Both 1α,25(OH)$_2$D$_3$ and MART-10 repressed VEGF-A-upregulated F-actin synthesis in RIN-m cells, thus inhibiting cell migration.

Conclusion

Our results indicate that both MART-10 and 1α,25(OH)$_2$D$_3$ effectively inhibit VEGF-A-induced metastatic potential of RIN-m cells through reducing EMT and F-actin synthesis by RIN-m cells. 1α,25(OH)$_2$D$_3$ and MART-10 attenuated the effects of VEGF-A partly by repressing neuropilin 1 expression. Since MART-10 is more potent than 1α,25(OH)$_2$D$_3$ and is non-calcemic, MART-10 could be deemed as a promising agent to treat PanNETs.

Conflicts of Interests

All Authors declare no conflict of interest in regard to this study.

Acknowledgements

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