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ORIGINAL ARTICLE

Non-target Genes Regulate miRNAs-Mediated Migration Steering of Colorectal Carcinoma

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Abstract

MicroRNAs (miRNAs) trigger a two-layer regulatory network directly or through transcription factors and their co-regulators. Unlike *miR-375*, the role of *miR-145* and *miR-224* in inhibiting or driving cancer cell migration is controversial. This study is a step towards addressing the potential of *miR-375*, *miR-145* and *miR-224* expression modulation to inhibit colorectal carcinoma (CRC) cells migration in vitro through regulation of non-target genes *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44*. Transwell migration assay results revealed a significant subdue of migration ability of cells transfected with *miR-375* and *miR-145* mimics and *miR-224* inhibitor. Real time PCR data showed that expression of *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* was downregulated as a consequence of exogenous re-expression of *miR-375* and inhibition of *miR-224*. On the other hand, ectopic expression of *miR-145* did not affect *VEGFA*, *TGFβ1* and *CD44* expression, while it elevated *CD105* and suppressed *IGF1* expression. *MAP4K4,* a predicted target of *miR-145,* was validated as a target that could play a role in *miR-145*-mediated regulation of migration. At mRNA level, no change was observed in expression of *MAP4K4* in cells with restored expression of *miR-145*, while western blotting analysis revealed a 25% reduction of protein level. By applying luciferase reporter assay, a significant decrease in luciferase activity was observed, supporting that *miR-145* directly target 3' UTR of *MAP4K4*. The study highlighted the involvement of non-target genes *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* in mediating anti- and pro-migratory effect of *miR-375* and *miR-224*, respectively, and validated *MAP4K4* as a direct target of anti-migratory *miR-145*.

Keywords MiRNAs, colorectal carcinoma · Non-target genes · *MAP4K4* · Migration

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death among adults. Targeting dysregulated pathways represents the best way to improve cancer treatment and increase the overall survival in cancer patients [43]. MicroRNAs (miRNAs) are small non-coding RNAs that usually bind to recognition sites on target mRNAs leading to either degradation of the transcript, inhibition of translation or rapid deadenylation [20]. Many studies have linked a well-known sub-cellular structure, the cytoplasmic processing bodies (PBs), to miRNA pathway [30]. MiRNAs have been shown to act as tumor suppressors by negatively regulating gene expression of oncogenic factors. Alternatively, oncomirs, miRNAs which down-regulate expression of tumor suppressor genes, have been identified in a variety of cancer types [14]. MiRNAs provide new therapeutic targets for many diseases, including cancer. The progress in selecting specific anti-miRNA inhibitor chemistries and delivery system suggests that miRNA-based therapeutics may soon be applied in the clinic [5, 32]. In the context of CRC, several miRNAs have been reported to regulate different cellular processes that control migration and metastasis by inhibition of multiple target genes [9, 37, 52, 53].

MiR-375 is down-regulated and evinces significant tumor suppressive properties in CRC tissue [12] and could predict

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the metastatic potential of CRC patients [45]. It inhibited the proliferation, invasion and migration by directly targeting Sp1 transcription factor and regulating matrix metalloproteinase 2 (MMP2) and epithelial-mesenchymal transition (EMT)-associated genes [8]. Tumor suppressor *miR-145* inhibited invasion and metastasis in many cancers by targeting several genes [10] and was expressed at reduced levels in colon cancer epithelial cells [27]. However, a study by Yuan et al. [47] demonstrated a significant relation between *miR-145* expression and lymph node metastasis of CRC. The potential function of *miR-224* as oncogene or tumor suppressor remains contradictory. Previous data have founded that *miR-224* was significantly up-regulated in CRC tissue samples and associated with disease relapse and a relative poorer disease-free survival rate, suggesting the oncogenic role of *miR-224* in the tumorigensis of CRC, and its prognostic function as a novel biomarker for patients relapse [50]. In contrast, another study indicated a significantly lower expression level of *miR-224* in CRC tissues than those in non-tumor tissues. The differences in *miR-224* expression levels in the pathogenesis of cancer were suggested to be related to mutation status of the adenomatous polyposis coli (APC) gene and racial and environmental diversity [21].

Molecularly-targeted inhibitors that blocked migration mediators, therefore subdue cancer cell migration, would be expected to be clinically beneficial to cancer patients [54]. One of the most prominent angiogenic factors is vascular endothelial growth factor (VEGF) that widely studied for its significant role in tumor growth, migration and metastasis [41]. Transforming growth factor β (TGFβ) pathway also plays a pro-tumoral role [28] and promote migration and metastasis of colon cancer cell into the liver and lung [29]. TGFβ was found to cooperate in an autocrine/paracrine fashion with other signaling cascades, including VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietin and Notch, to regulate migration [29, 33]. Insulin-like growth factor (IGF) family molecules act through binding to IGF receptors (IGFRs) which are receptor tyrosine kinases (RTKs) and most frequently signal through phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) dependent mechanisms [19, 40]. It was demonstrated that IGF1 signaling is important in mediating protease activation and cell migration and invasion, and it combined with TGFβ signaling to bring about an EMT [40]. Endoglin/CD105 functions as a co-receptor for TGFβ1, it specifically enhanced TGFβ1-induced phosphorylation of intracellular effectors Smad 1/5/8 and increased a Smad 1/5/8 responsive promoter [22]. Expression of both TGFβ1 and CD105 correlated with increased expression of tumor vascular marker CD44 [3].

MAPK kinase kinase kinase isoform 4 (MAP4K4) is a serine/threonine (S/T) kinase belongs to the mammalian STE20/MAP4K family, which is associated with cell motility, rearrangement of the cytoskeleton, and cell growth [18]. *MAP4K4* was demonstrated to play a central role in cell migration and invasion through the JNK pathway [7, 26], and is regulated by *miR-141* [16] and *miR-194* [43].

The complexity of miRNAs is attributed to the ability of single miRNA to fine-tune the expression of hundreds mRNAs, and the potential of each mRNA to be regulated by hundreds of miRNAs [1] through the two-layer regulatory network. in this network, miRNAs directly regulate the genes by mRNA degradation or translational inhibition at the primary level of the regulatory cascade; whereas the secondary targets of miRNAs are the genes that regulated by miRNA-targeted TFs [38]. This study is a step towards highlighting the involvement of non-target genes *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* in mediating the anti - or pro-migratory effect of *miR375*, *miR-145* and *miR-224,* and validating *MAP4K4* as target of *miR-145*.

Methods

Cell Line Propagation and Transient Transfection

Human CRC cell line HCT116 (ATCC®) was maintained as monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mM L-glutamine at 37 °C under a humidified atmosphere of 5% CO_2. HiPerFect transfection reagent, miScript miRNA mimic of *miR-375* and *miR-145* and miScript miRNA inhibitor of *miR-224* were purchased from Qiagen (Germany). Transfection was carried out according to the manufacturer's instructions. Briefly, cells were seeded, transfected with transfection complex of HiPerFect transfection reagent and 5 nM miRNA mimic or 50 nM miRNA inhibitor diluted in serum free medium, and incubated until monitoring gene or protein expression. For co-transfection of mimic and plasmid, HiPerFect transfection reagent was complexed with 400 ng plasmid DNA and 5 nM *miR-145* mimic diluted in medium without serum or antibiotics.

MiRNAs Target Prediction

Bioinformatics algorithms MIRDB (http://mirdb.org) and Target Scan (http://targetscan.org) were used to predict downstream targets of miRNAs. Mature sequences of miRNAs were downloaded from miRBase and the 3' UTR sequence of human *MAP4K4* was downloaded from Ensembl genome browser.

Reverse Transcription and Real-Time PCR

Total RNA, containing miRNA, was extracted from transfected cells using Direct-zol™ RNA MiniPrep (Zymo Research) according to the manufacturer's instructions. The

extracted mRNA and miRNA were reverse transcribed into single stranded complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and miScript II RT Kit (Qiagen), respectively. For quantitative real-time PCR of *VEGFA*, *TGFβ1*, *IGF1*, *CD105*, *CD44* and *MAP4K4*, amplification mixtures were prepared using KAPA SYBR_FAST q PCR master mix (Kapa Biosystem). Primer of MAP4k4 was designed using free available Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). *GAPDH* was used as an internal reference gene to normalize the expression. Primers of *GAPDH*, *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* were previously reported [2, 6, 35, 39, 48, 49]. Expression analysis of miRNAs was carried out using miScript SYBR Green PCR Kit according to manufacturer using included universal (reverse) primers, while forward primers were designed using online miRNA primer design tool (http://genomics.dote.hu:8080/mirnadesigntool/). Expression was normalized to RNU6B. Results were expressed as ratio of reference to target gene using $2^{-\Delta\Delta Ct}$ method. Primers are listed in Table 1.

Western Blotting

After 72 h of transfection, monolayers were washed with ice-cold Dulbecco's phosphate buffer saline (DPBS) and then scrapped in RIPA lysis buffer with 1x protease inhibitor cocktail. Total protein concentration was estimated using Bradford assay [4]. Twenty five μg of protein/lane were separated on 10% SDS-polyacrylamide gel in Tris-Glycine buffer (200 Volts for 1 h). Proteins were then transferred to 0.45 μm nitrocellulose membrane (100 Volts for 90 min). Equal loading was confirmed using reverse staining with Ponceau Red solution (Seva electrophoresis, Germany) Membranes were blocked in 5% non-fat milk for 1 h, and then incubated overnight at 4 °C with monoclonal primary antibodies, anti-MAP4K4 (Abcam, UK). Blots were then washed using Tris buffered saline Tween 20, TBST (3 × 10 min washes) and incubated with appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. After 3 × 10 min washes in TBST, membranes were developed using Enzyme chemiluminescence (ECL Optiblot, Abcam, UK). Protein bands were visualized on developed membranes by exposure to x-ray film. Protein band intensities were determined using Image Studio Lite software v 5.2 (Li-COR® Biosciences, USA).

Vector Construction and Luciferase Reporter Assay

Oligonucleotides corresponding to the surrounding predicted target sequence of *miR-145* in 3' UTR of *MAP4K4* was

Table 1 Primers and oligonucleotides used in the study

Name	Sequence (5` to 3`)
miR-375-S	TGGTTTTTGTTCGTTCGGCT
miR-145-S	GGTTTTTTTGTCCAGTTTTCCCAG
miR-224-S	TGGTGCAAGTCACTAGTGGT
MAP4K4-S	CAAATCAGCAGGCACAAGAA
MAP4K4-A	GTTGCCCCATCAACCTCTAA
GAPDH-S	ACCCACTCCTCCACCTTTGAC
GAPDH-A	TGTTGCTGTAGCCAAATTCGTT
VEGFA-S	ATGAGGACACCGGCTCTGACCA
VEGFA-A	AGGCTCCTGAATCTTCCAGGCA
TGFβ1-S	AAGGACCTCGGCTGGAAGTGC
TGFβ1-A	CCGGGTTATGCTGGTTGTA
IGF1-S	GCAATGGGAAAAATCAGCAG
IGF1-A	GAGGAGGACATGGTGTGCA
CD 105-S	CTCTGCTGCTGAGCTGAATG
CD105-A	GATCTGCATGTTGTGGTTGG
CD 44-S	AGAAGGTGTGGGCAGAAGAA
CD 44-A	AAATGCACCATTTCCTGAGA
PmirGLO vector-S	ATTAAGGCCAAGAAGGGCGG
PmirGLO vector-A	CCTGCAGGTCGACTCTAGACTCG
MAP4K4 UTR-S	AAAGAACTTGGAATTCCTTGT***AACTGGA***GCTC
MAP4K4 UTR-A	TCGAGAGCTCCAGTTACAAGGAATTCCAAGTTCTTT

S sense, *A* antisense, *UTR* 3` untranslated region

MiR-145 recognition site is in bold and italic; restriction sites are underlined

designed and cloned into the Dra1 and Xho1 sites of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). Oligonucleotides were annealed and ligated to the pmirGLO Vector which was linearized with the same restriction enzymes to generate overhangs that are complementary to the annealed oligonucleotide overhangs. Insertion was verified by PCR (primers and oligonucleotides sequences are listed in Table 1). In 6 well plates, HCT116 cells were co-transfected with 400 ng of pmirGLO vector and 5 nM of *miR-145* mimic. 72 h. after co-transfection, the relative luciferase activity was measured using Luciferase Assay System (Promega, USA).

Trans-well Migration Assay

After 48 h of transfection, migration assay was performed using Thincerts™ (Greiner Bio-One) in 24 well plates using polycarbonate membranes with 8-μm pores. HCT116 cells were serum-starved by incubating cells in serum-free media and kept in a 37 °C and 5% CO_2 incubator for 24 h. At a density of 2×10^5 cells/ml in 100 μl of serum free medium, cells were placed in the upper chamber of the transwell assembly while the lower chamber contained 650 μl of RPMI. After incubation at 37 °C and 5% CO_2 for 24 h, the upper surface of the membrane was scraped gently to remove non-migrating cells and washed with phosphate-buffered saline) PBS). The membrane was then fixed in 4% paraformaldehyde for 15 min and stained with crystal violet. The cells were then imaged in five fields for each membrane and counted using image J.

Statistical Analysis

Data were analyzed using SPSS 16.0 software and presented as mean ± standard error. Statistical significance of the data was analyzed by One-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Exogenous Regulation of miRNAs Expression Subdue Migration of CRC Cells

Real time PCR analysis revealed that transfection of *miR-375* and *miR-145* mimics and *miR-224* inhibitor resulted in restoring *miR-375* and *miR-145* expression 2.5 fold, and inhibition of *miR-224* expression 2.7 fold. This miRNAs expression modulation resulted in a significant reduction of migrated cells comparing to control. Relative to control, migrated cells reduced to 14, 27 and 24% in cells transfected with *miR-375* and *miR-145* mimics and *miR-224* inhibitor, respectively (Fig. 1a–e).

Regulation of Non-target Genes Involved in Migration

VEGFA, *TGFβ1*, *IGF1*, *CD105* and *CD44* expression analysis was carried out to test whether they are regulated by the candidate miRNAs as a part of their mechanism of influencing

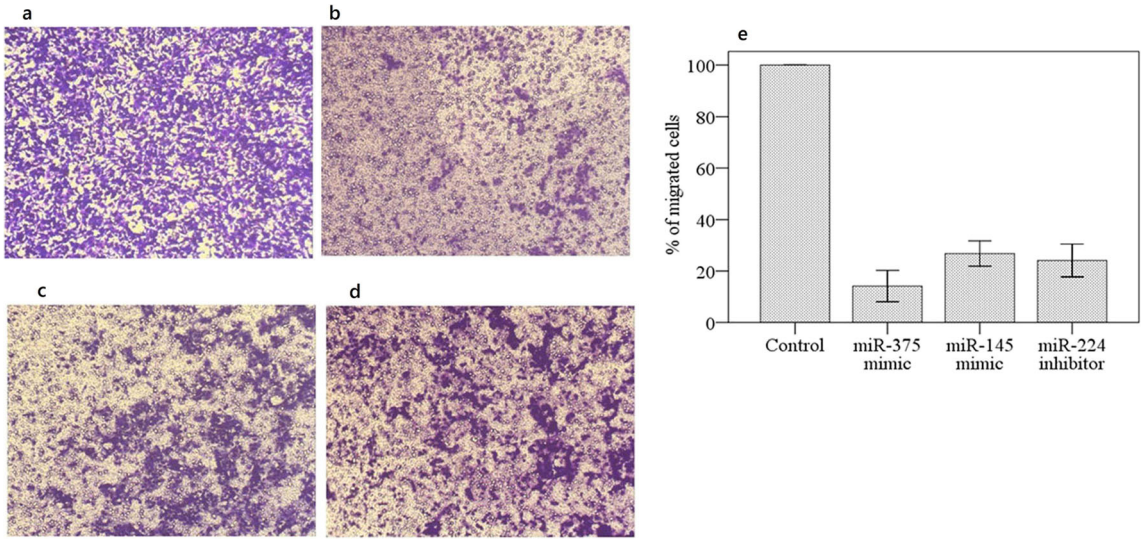

Fig. 1 Migration inhibition of CRC cells in response to exogenous manipulation of miRNAs expression. **a**, **b**, c and **d**: Representative images of migrated cells using transwell migration assay after transfection with miR-375 mimic (**b**), miR-145 mimic (**c**) and miR-224 inhibitor (**d**) comparing to control (**a**). **e**: Diagram represents percent of migrated cells relative to control ($P < 0.01$)

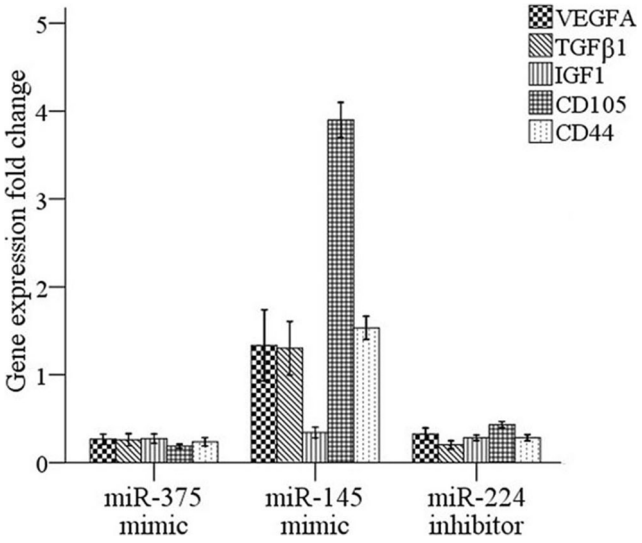

Fig. 2 Expression regulation of *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* after ectopic expression of *miR-375* and *miR-145* and depletion of *miR-224*

migration ability. Data from real time PCR demonstrated that the expression of all selected genes were significantly inhibited in cells transfected with *miR-375* mimic and *miR-224* inhibitor ($P < 0.01$), whereas *miR-145* exhibited diverse effects on the expression of different genes. Considering 2 fold change as a cut off value, restoring expression of *miR-145* resulted in increased *CD105* (4 fold), suppression of *IGF1* (3 fold) without change in *VEGFA*, *TGFβ1* and *CD44* expression (Fig. 2).

MAP4K4 is Directly Regulated by *miR-145*

MIRDB (http://www.mirdb.org/miRDB/) and TARGETSCAN (http://www.targetscan.org) bioinformatics tools indicated that none of *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* is targeted by *miR-375, miR-145* or *miR-145*. On the other hand, *MAP4K4* was predicted as target of *miR-145* (Fig. 3). To validate this prediction, real time PCR, western blotting, and luciferase reporter assay were carried out. No change of mRNA level was observed while protein level was reduced by 25%. Insertion of *miR-145* binding site into reporter vector and co-transfection with *miR-145* mimic resulted in a significant ($P < 0.05$) decrease of luciferase activity comparing to control (Fig. 4a–c).

Discussion

MiRNAs play important roles in tumorgenesis and metastasis of human cancers [10]. In colon cancer, tumor suppressor *miR-375* [42] and *miR-145* [31] and oncomiR *miR-224* [50] were found to be dysregulated. Tumor suppressive function of *miR-375* was suggested by directly acting upon frizzled-8 *(FZD8)*, which may serve as a therapeutic target to inhibit tumor metastasis in CRC [45]. Wang et al. [44] reported that *miR-375* can regulated the invasion and migration of laryngeal squamous cell carcinoma via AKT-mediated EMT synergistically. *MiR-145* inhibited migration and invasion of SW620 and LoVo metastatic cell lines and resulted in a decrease of lung metastases in nude mice by targeting Fascin-1 [13]. In addition, *miR-145* was reported to suppress the invasiveness and metastasis of neuroblastoma cells by targeting hypoxia-inducible factor *2α* (*HIF-2α)* [51] and inhibiting *N-cadherin* protein translation, and in turn indirectly downregulate its downstream effector MMP9 [15]. In breast cancer [17] and glioblastoma [36], targeting junctional adhesion molecule A (*JAM-A*) and *NEDD9*, respectively, could mediate the inhibitory impact of *miR-145* on migration, invasion and metastasis. Conversely, Yuang et al. [47] reported that increased *miR-145* could improve migration and invasion of HCT-8 cells and lead to lymph node metastasis of CRC. Oncomir *miR-224* expression was reported to increase consistently with tumor burden and enhances CRC metastasis [24]. In HCC, *miR-224* upregulation was found to promote migration through targeting Homeobox D 10 (*HOXD10*) gene [23]. Conversely, data by Liu et al. [25] indicated that *miR-224* inhibited migration of breast cancer cells by targeting *frizzled 5*. For evaluating the potential of miRNAs expression modulation to inhibit tumor cells migration in vitro, we transfected HCT116 cells with *miR-275* and *miR-145* mimics and *miR-224* inhibitor and subjected them to transwell migration assay. A significant reduction of migration ability was observed after transfection, supporting the anti-migratory role of *miR-375* and *miR-145* and the pro-migratory activity of *miR-224*.

The indirect regulatory mechanism of miRNAs, in which genes are targeted by miRNA-regulated TFs, explains how non-target mRNAs can also be influenced by miRNAs [38]. Using quantitative real time PCR we

Fig. 3 Schematic representation of bioinformatics analysis of predicted consequential pairing of *MAP4K4* 3` UTR targeting sequences and *miR-145* seed region

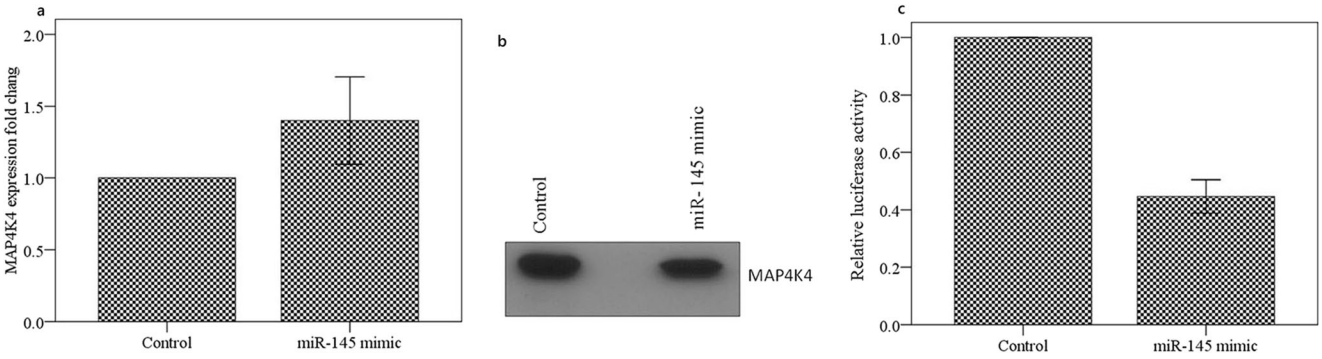

Fig. 4 *MAP4K4* is directly regulated by *miR-145* through translational inhibition rather than mRNA degradation. **a** Considering 2 fold change as cut off value, *MAP4K4* transcript wasn't changed. **b** Reduction of MAP4K4 protein level. **c** Significant decrease of luciferase activity ($P < 0.05$) after restoring expression of *miR-145*

analyzed the expression of non-target pro-migratory factors *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* at mRNA level to test whether they play a role in miRNAs-mediated migration regulation. We found that all genes were downregulated as a consequence of ectopic expression of *miR-375* and inhibition of *miR-224*, so we suggested that *miR-375*- and *miR-224*- mediated downregulation of *VEGFA*, *TGFβ1*, *IGF1*, *CD105*, and *CD44* could interfere with CRC cells migration. Previous findings of Benetti et al. concluded that TGFβ1 and CD105 are mediators of tumor cell migration, and their expression correlated with increased expression of CD44 in HCC [3]. In the same context, increased IGF1 [40] and CD44 [46] together with their dependent activation and association with MMPs can induced TGFβ1 and resulted in promotion of cell migration and EMT.

Unlike *miR-375* and *miR-224*, *miR-145* expression modulation did not exhibit an impact on the expression of *VEGFA*, *TGFβ1* and *CD44*. On the other hand, it elevated *CD105* and suppressed *IGF1* expression. *MiR-145* could mediate its anti-migratory effect via regulation of other target genes. In our previous work [34] we reported that *miR-145* directly target *MTDH*, an oncogene playing a pivotal role in the pathogenesis, progression, apoptosis regulation, angiogenesis, invasion and metastasis [11]. Herein, we focused on *MAP4K4,* a tumor-promoting oncogen, that bioinformatically predicted as *miR-145* target. Cells ectopically expressed *miR-145* have subjected to mRNA and protein level analysis, in addition to luciferase reporter assay. No change was observed in expression of *MAP4K4* transcript, this may be attributed to that *miR-145* regulate *MAP4K4* via translation inhibition rather than mRNA degradation. This suggestion was confirmed by western blotting analysis which indicated that protein level was reduced in cells with restored *miR-145* expression. By applying luciferase assay, we observed a significant decrease in luciferase activity, supporting that *MAP4K4* is directly targeted by *miR-145* and could, at least partially, mediates its anti-migratory effect.

Conclusion

The study is a step towards highlighting the involvement of non-target genes *VEGFA*, *TGFβ1*, *IGF1*, *CD105* and *CD44* in mediating anti- and pro-migratory effect of *miR-375* and *miR-224*, respectively, and validated the oncogene *MAP4K4* as a direct target of anti-migratory *miR-145*.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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