Original Article

MicroRNA miR-182 cluster mediated modulation of RECK without changes in cell surface membrane type-1 matrix metalloproteinase (MT1-MMP)

Milagros Silva¹², María E Hernandez¹², Fausto Rojas¹², Lihua Li³, Subbaya Subramanian³⁴, Michael J Wilson²⁴⁵⁶

¹Centro de Investigaciones Cerebrales, Universidad Veracruzana, Xalapa, Veracruz, MX; ²Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA; ³Department of Surgery, University of Minnesota, Minneapolis, MN, USA; ⁴Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA; ⁵Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA; ⁶Minneapolis VA Medical Center, Minneapolis, MN, USA

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Abstract: Cell surface localized membrane type 1-matrix metalloproteinase (MT1-MMP) plays an important role in physiological and pathological processes and its function can be regulated by proteins such as RECK. We examined the ability of miR-182 (one of the miR-183 cluster miRNAs), which can target RECK, to control cell surface MT1-MMP activity. Expression of RECK mRNA and protein was increased with anti-miRs to miR-182, miR-183 or miR-96 in HT1080 fibrosarcoma cells, but, decreased RECK mRNA and increased its protein in the benign prostatic hyperplasia cell line BPH-1. Treatment of BPH-1 and HT-1080 cells with the anti-miRs did not change the level of cell surface MT1-MMP activity, nor their rate of migration in an in vitro wound-healing assay. Trichostatin A (TSA) did not increase the level of RECK, but blocked cell surface MT1-MMP activity and decreased cell motility. Anti-miRs mediated increased RECK levels did not interfere with cell surface MT1-MMP function, and TSA may block cell surface localization of MT1-MMP by a mechanism independent of RECK.

Keywords: MT1-MMP, EMMPRIN, RECK, microRNA, trichostatin A (TSA), miR-182

Introduction

Cell surface proteolysis regulates cell properties such as adhesion, proliferation, and motility that enable tumor cells to become invasive and metastatic. Evidence for a causal role for matrix metalloproteinases (MMPs) (a family of 23 proteins in humans), and especially membrane type-MMPs (MT-MMPs), in tumor invasion and metastasis is substantial and overwhelming [1-6]. MT1-MMP (MMP-14) has been implicated in the aggressiveness of a variety of cancers [4-6]. Regulation of MT1-MMP occurs at different levels of control of its expression and function, including transcription; translation; activation by proprotein convertases; transport to the cell surface; endocytosis, and inhibition by specific inhibitors (TIMPs and RECK); [4-7]. One of the most well-characterized functions of MT1-MMP is the cell surface activation of proMMP-2 and proMMP-13, but it has also a large array of cellular substrates, including proteins regulating angiogenesis, inflammation, cell adhesion, cell growth, apoptosis, and cell migration [3, 8]. In addition, MT1-MMP may stimulate cellular activities without expression of its proteolytic activity through TIMP-2 binding and activation of the Ras-Raf-ERK signaling cascade [6].

A mechanism of post-transcriptional regulation of gene expression that can control developmental and cellular processes is via microRNAs (miRNAs), molecules of ~18-21 nucleotides that in general function by binding to the 3’UTR of mRNAs and prevent their translation or increase their degradation [9]. There are ~1400 miRNAs in humans that may control the activity of 50% of protein coding genes [9]. miRNAs have been detected and measured in a variety of tumors and their dysregulation is associated with both oncogenic transformation and tumor
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suppressor activity [10, 11] and are often expressed downstream of pro- or anti-metastatic signaling pathways regulated for example by NFkB, EGFR, TWIST1, BRMS1, ZEB1/2, and HIF1a [11, 12]. Because of their small size and ability to target miRNAs with limited complementarity, miRNAs have multiple mRNA targets and hence can potentially block expression of a large number of genes.

There have been limited studies showing miRNA-induced changes in MMP expression. Decreased expression of MMPs [16-18] have been reported. miRNAs can also regulate proteins that directly modulate MMP activity; e.g., TIMP-3, an endogenous inhibitor to some MMPs, is a validated target for miR-181b [17] and RECK, a membrane-anchored MMP regulator, is the target of 4 groups of miRNA (miR-15b/16, miR-21, miR-372/373, and miR-182/183) [19-23]. miR-183, which is a member of an evolutionarily conserved miRNA cluster including miR-96 and miR-182, inhibits migration and invasion of lung cancer cells [24], but functions as an oncogene in colon cancer and specific types of sarcoma [25]. Since miR-182 can target RECK, decreasing RECK protein and thus contribute to malignancy of tumor cells by releasing the inhibition of pericellular MMP activities, we examined the ability of anti-miRs to miR-182, -96 and -183 as well as the effect of trichostatin A [reported to up-regulate RECK; [26]], to affect cell surface MT1-MMP activities.

Materials and methods

Cell culture, treatment conditions, and transfection

Human cancer cell lines HT1080 (fibrosarcoma), LnCaP (human prostate), U87 (human primary glioblastoma), and RH30 (alveolar rhabdomyosarcoma, ARMS) were from ATCC (Monassas, VA); colon cancer cell line HCT116 and human benign prostate hyperplasia epithelial cell line BPH-1 were kindly provided by Dr. Clifford Steer and Dr. Haojie Huang (University of Minnesota) respectively, HCT116, BPH-1 and LNCaP cells and HT1080, U87 and Rh30 cells were routinely cultured in RPMI-1640 or DMEM media, respectively, supplemented with 10% heat-inactivated FBS and antibiotics [18]. Upon reaching 60-70% confluence, the cultures were changed to serum-free media or media with appropriate treatment agents and were continuously cultured for 48 h at which time culture media, cell protein extracts, or cellular RNA were isolated. Treatments included 0, 10 or 50 µg/ml Concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO); 100 nM trichostatin A (TSA) (Sigma Chemical Co.) or vehicle (0.01% methanol). Media were centrifuged at 3500 rpm for 5 min, and frozen and stored at -80°C until used. The anti-miRNAs Mirzip-182, Mirzip-183 and Mirzip-96 plasmids, which also encoded green fluorescent protein (GFP), (System Biosciences, Mountain View, CA) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a method published previously [25].

Gelatin zymography and western blotting

Zymographic gel assays of culture media (5 µl/ lane) and western blotting (constant amount protein/lane) were performed as described previously [27]. Cells were harvested after 48 hr culture and lysed in 100 µl of SoluLyse-M™ Mammalian Protein Extraction Reagent (proprietary non-ionic detergent in 25 mM Tris pH 7.4 and 250 mM sucrose) (Genlantis, San Diego, CA) containing a mixture of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany); and then analyzed by western blot to assess the level of proteins expressed. Antibodies used were anti-human RECK (D8C7, Cell Signaling Technology, Danvers, MA), anti-human MT1-MMP (sc-30074 Santa Cruz Biotechnology, Dallas, TX), and anti-human GAPDH (sc-25778 Santa Cruz Biotechnology).

RNA extraction and quantitative real time-PCR

Total RNA and microRNAs were isolated from cells using the miRvana miRNA isolation kit following the manufacturer’s protocol (Ambion, Applied Biosystems, Grand Island, NY). cDNA synthesis was performed at 37°C for 60 min and 95°C for 5 min by using a miScript Reverse transcription kit (Qiagen, Valencia, CA). RECK, MT1-MMP, and EMMPRIN mRNAs and miRNAs were examined in a LightCycler 480 (Roche Applied Science, Indianapolis, IN) by using QuantiTect SYBR Green PCR kit according to the manufacturer’s protocol (Qiagen), GAPDH and U6 were used as internal controls to check the efficiency of cDNA synthesis and PCR amplification [25]. The PCR primers were RECK: (forward) 5’ TGGCAAGAGTTTGATCGCTT 3’ and
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Figure 1. The effect of ConA and anti-miRs to miR-183, -182, and -96 on cell surface proMMP-2 activation by MT1-MMP. A. Low levels of proMMP-2 proteolytic processing is observed for all cell lines represented except for LNCaP in which it was not detected. Increased activation of MMP-2 is observed in HT1080 and U87 cancer cells, and in the benign prostatic hyperplasia cell line BPH-1 upon treatment of cells with ConA (10 or 50 μg/ml). HCT116, LNCaP and RH-30 cancer cells do not respond to ConA. B. The expression of miR-182, miR-183, and miR-96 was determined by real time RT-PCR and found to be low in untreated BPH-1 and HT1080 cells. The levels of miR-182 and miR-183 were greater than miR-96 in BPH-1 cells and miR-183 was greater in HT1080 cells. C. The efficiency of transfection of the anti-miRs was demonstrated by the consistent fluorescent expression of the GFP in HT1080 cells. D. Zymography was used to determine proMMP-2 activation upon cellular treatment with the anti-miRs. No change in activation of proMMP-2 was found for HT-1080, BPH-1, and HCT-116 cells.

Wound healing/scratch test

In brief, cells were seeded in 12 well plates. After growth to about 70-80% confluency, cells were treated with TSA or Con A, or transfected with indicated plasmids, and then grown to confluency. Cell motility was then tested using a wound assay in which a scratch was made along the axis of the plate using a pipette tip. Cells were washed two times with PBS buffer to remove free cells, complete media with 5% FBS was added, and cells were grown under normal conditions. Migration of cells into the scratch was photographed every 4 h.

Immunostaining and confocal microscopy

Confocal microscopy was carried out as described previously [18]. Cells were grown on glass cover slips and treated as indicated. After culture for 48 hr in 10% FBS media, cells were fixed with 4% paraformaldehyde, incubated with 0.1% Triton X-100 in PBS, blocked with 3% goat serum in PBS, and then incubated with mouse anti-human MT1-MMP antibody (Santa Cruz Biotechnology) overnight at 4 C. The secondary antibody was Alexa Fluor®488-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Confocal microscopy was carried out in the University of Minnesota Imaging Center using an Olympus FluorView 1000 BX2 Upright
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Confocal Microscope with a 60X oil objective. The images were processed in Power Point 14.3.1 software for Mac.

Data analysis and statistics

Experiments were carried out with three replicates. All quantitative data were presented as mean ± SD. Statistical analysis was done with ordinary one-way ANOVA for Quantitative Real Time-PCR using the Prism 6 for Mac OS X computer program. Values of P<0.05 were considered significant.

Results

It is well known that the expression and cell surface localization of MT1-MMP in HT-1080 cells are increased by ConA [28, 30]. We examined the cell surface activities of MT1-MMP in a number of cell lines as indicated by their ability to process proMMP-2 in the serum of the culture media. Low levels of intermediate and active forms of MMP-2 were detected in media from HT-1080, U87, HCT116 and RH30 (not LNCaP) cancer cells, and also the benign prostate BPH-1 cell line (Figure 1A). ConA stimulated MMP-2 activation by HT-1080 and U87, but not HCT116 and RH30 cells, indicating a difference in the ability of tumor cells to respond to ConA. It was interesting that there was a strong response to ConA by the benign prostate BPH-1, but not by the prostate carcinoma LNCaP cell lines. These data show that tumor cells may differ not only in the level of expression of MT1-MMP, but also in their ability to assemble MT1-MMP on the plasma membrane.

Since miR-182 and miR-96 target RECK, we examined the effect of knocking down their expression, as well as that of miR-183 from the same cluster, on the level of RECK mRNA expression by transfecting their anti-miRs in HT-1080 and BPH-1 cells. The endogenous levels of these miRs were low in both BPH-1 and HT-1080 cells, but with concentrations being higher in the former (Figure 1B) and only significant concentrations of miR-183 in HT-1080 cells. The effectiveness of transfection of the anti-miRs is shown by the cellular expression of GFP encoded in the transfected plasmids (Figure 1C). The transfection of the anti-miRs into HT-1080 cells resulted in a
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small increase in RECK mRNA (Figure 2A) and protein (Figure 2B), but no change in cell surface activation of proMMP-2 (Figure 1D). However, for similarly treated BPH-1 cells, there was a decrease in RECK mRNA (Figure 2A), but increased RECK protein (Figure 2B) and a small/no effect on proMMP-2 activation. Thus, HT-1080 cells respond to the anti-miRs with small increase in RECK, but no change in proMMP-2 activation; whereas in BPH-1 cells there
Figure 4. Confocal localization of MT1-MMP in HT1080 cells and the effects of ConA and TSA treatment on its subcellular localization. TSA treatment reduced levels of MT1-MMP observed on the cell surface in each cell line, either alone or in conjunction with ConA treatment.
Figure 5. The effect of TSA treatment on cells migrating into the cell denuded space created in an *in vitro* wound-healing assay. Migration of both BPH-1 and HT1080 cells into the void created by the scratch in the cell cultures was blocked in response to TSA treatment.
was a minimal change in cell surface MT1-MMP activity even with a greater increase in RECK protein.

The histone deacetylase inhibitor trichostatin A (TSA) can upregulate RECK expression in lung cancer cells [26]. There was no change in RECK mRNA in TSA treated HT-1080 cells either with or without conA stimulation (Figure 2A). TSA also did not alter the transcript level of RECK in BPH-1 cells but decreased RECK mRNA level in BPH-1 cells co-treated with conA. There was a small decrease in RECK protein with TSA treatment in BPH-1, but not in HT1080 cells (Figure 3A). ProMMP-2 activation was diminished in HT-1080 and BPH-1 cells treated with TSA, with or without conA treatment (Figure 3B), but no change in proMMP-2 processing by HCT-116 cells occurred in response to TSA. The TSA effect focuses on cell surface localization of MT1-MMP as there was no change in MT1-MMP message levels in HT-1080 cells with TSA treatment, although there was a small decrease in MT1-MMP message with combined TSA and ConA treatment (Figure 3C). The message levels of EM-MPRIN, which stimulates MMP expression, did not change with TSA treatment, but was slightly decreased in HT-1080 cells exposed to both TSA and ConA. There was a small, but significant, increase in EM-MPRIN in BPH-1 cells with the combined treatment (Figure 3C), which coincides with increased MT1-MMP message. These data may indicate there to be 2 populations of cell surface MT1-MMP; one of low MT1-MMP quantity not responsive to TSA or to ConA, and a second population in some cells readily mobilized to the cell surface by ConA.

The distribution of MT1-MMP in HT-1080 cells as affected by TSA was examined by confocal microscopy. Treatment with TSA decreased cell surface localization of MT1-MMP compared to untreated or vehicle-treated cells or those treated with ConA (Figure 4). These confocal data support the decrease in cell surface MT1-MMP as determined through functional changes in proMMP-2 activation, but not total cellular levels of MT1-MMP resulting from TSA treatment.

The effect of anti-miRs-182, -183 and -96 were also tested on HT-1080 and BPH-1 cells in an in vitro wound healing assay and there was no effect on the rate of cell migration (Data not shown). Treatment with TSA, however, decreased the rate of migration of both HT-1080 and BPH-1 cells when compared with untreated cells or cells treated with the vehicle alone (Figure 5). The selective inhibition of cell migration by TSA over the anti-miRs suggests that the cell surface localization and activity of MT1-MMP is the important factor in the cell migration response.

Discussion

The results of our study of regulation of RECK levels and MT1-MMP cell surface localization and activity indicated that blocking miR-182, miR-183, and miR-96 through transfecting their respective anti-miRs did increase RECK mRNA expression in HT1080 and protein in BPH-1 cells, but did not decrease cell surface MT1-MMP activities. In contrast, TSA reduced RECK protein expression in BPH-1 but not HT-1080 cells, and reduced cell surface MT1-MMP in both cell lines as evidenced in diminished proMMP2 activating capability. Functionally, the anti-miR treated HT1080 and BPH-1 cells did not demonstrate any change in cell migration in an in vitro wound healing assay, whereas those treated with TSA clearly were restrained in migration capability. These data indicate that increased RECK in anti-miR treated cells did not affect cell surface MT1-MMP and either the changes in RECK levels were inconsequential to affect MMP activities or it is sequestered in a separate subcellular location and not in contact with MT1-MMP. The decrease in cell surface MT1-MMP activation of pro-MMP-2 in response to TSA could be attributed to RECK, however, there was no change or a decrease in RECK mRNA or protein. The cell surface localization of MT1-MMP was clearly associated with cell migratory behavior.

ConA stimulation of MMP-2 activation is generated by mobilization of MT1-MMP from intracellular pools to produce a rapid response in cell surface activity, followed by increases in MT1-MMP mRNA production [28, 30]. We found a varied response to ConA among cancer cell lines. This differential response of cell lines to ConA has been attributed to the epithelial mesenchymal transition status of the cell line, i.e., breast cancer cell lines expressing vimentin and thus demonstrating epithelial mesenchymal transition were found to show ConA stimulated proMMP2 activation, whereas those breast cancer cells negative for vimentin did not [29]. This criterion applies to HCT116 cells.
which have no/low vimentin expression and other associated characteristics of epithelial cells [31]. However, it does not apply to BPH-1 cells which demonstrate strong ConA induced proMMP-2 activation but do not express vimentin [32]. However, BPH-1 cells can be transformed, become tumorigenic, and express vimentin by growth with human prostatic carcinoma-associated fibroblasts or by exposure to carcinogen doses of testosterone and estradiol after recombination with rat urogenital mesenchyme and growth in vivo [32].

There appears to be at least 2 populations of MT1-MMP on the cell surface based on MT1-MMP sensitivity to TSA. The effect of TSA on decreasing the level of cell surface MT1-MMP in HT1080 and BPH-1 cells could have affected the cell surface half-life of the enzyme and/or its own proteolytic processing. The regulation of short-term MT1-MMP levels on the cell surface is via intracellular trafficking [33]. MT1-MMP localization, and its proteolytic processing associated with its cell surface function, is associated with the caveolar fraction [34], and both caveolae and clathrin-coated pit internalization of MT1-MMP may be associated with different subpopulations of MT1-MMP present at the cell surface [35]. MT1-MMP in prostate tumor cells is associated with both caveolar and non-caveolar membrane fractions, also indicating more than one plasma membrane population of this proteinase [27]. However, MT1-MMP associated with RECK is internalized preferentially with an endocytic marker that is neither clathrin- or caveolae-dependent and is correlated with degradation and not recycling of MT1-MMP [36]. The presence of proMMP2 activating activity in tumor cells not responding to ConA in the present study further emphasizes the complex regulation of distribution of cell surface subgroups of MT1-MMP.

The effect of TSA on cell migration was dependent on displacing cell surface MT1-MMP or inhibiting its activity. Control of MT1-MMP in cell migration has been attributed to the cytoplasmic tail and not the proteolytic activity of MT1-MMP since mutant MT1-MMP with an inactive catalytic site can stimulate migration and one lacking the cytoplasmic domain, while catalytically functional, cannot stimulate migration in breast cancer cells [6]. LNCaP cells which demonstrated no proMMP2 activation in zymograms, express little/no MT1-MMP. However, if they are transfected with wild type MT1-MMP, they demonstrate increased oxidative stress with associated generation of reactive oxygen species (ROS), and an aggressive phenotype with increased migration, invasion and anchorage-independent growth. This effect required the proteolytic activity of MT1-MMP [37]. Histone deacetylase inhibitors such as TSA have been proposed to be used in a variety of therapeutic roles because they are protective against oxidative stress [38], and there may be other mechanisms outside of affecting RECK expression that may account for TSA effects on cell surface MT1-MMP levels/activity.

RECK expression is regulated by multiple pathways, including diminished RECK expression linked to s-phase kinase-associated protein (SKP2) [39] and TIMP-2 stimulated RECK expression [40]. RECK expression is silenced by promoter hyper-methylation [41-43], but not in hypoxia suppressed RECK expression [44]. Histone deacetylase (HDAC) silencing leads to RECK down-regulation in hypoxia and can be off-set by treatment with TSA [43]. We demonstrate here that knocking down miR-182 or miR-96 increases RECK expression, but does not change the level of cell surface MT1-MMP, which does not conform to a direct relationship of RECK to the level of cell surface MT1-MMP. However, divergent aspects of miRNAs in cell responses have been noted; e.g., miR-182 inhibits migration and invasion of lung cancer cells [24], but functions as an oncogene in colon cancer and specific types of sarcoma [25]. The implications of the miRNA data are that in our experimental conditions RECK remained sequestered and did not interact with MT1-MMP. In contrast to miR-182, TSA did decrease cell surface MT1-MMP but did not alter or lowered the levels of RECK; the former is consistent with published TSA effects, but not the latter [26, 43]. This apparent inconsistency may be due to distribution of MT1-MMP in different cellular and cell surface sub group populations in our study.

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Disclosure of conflict of interest
None.

Address correspondence to: Dr. Michael J Wilson, Research Service, VA Medical Center, One Veterans Drive, Minneapolis, MN 55417, USA. Tel: 612-467-2810; Fax: 612-725-2093; E-mail: Wilso042@umn.edu

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