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miR-520c and miR-373 target mTOR and SIRT1, activate the Ras/Raf/MEK/Erk pathway and NF- κ B, with up-regulation of MMP9 in human fibrosarcoma cells

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Abstract

MicroRNA 520c and 373 (miR-520c and miR-373) have been characterized as oncogenes and play critical roles in cancer cell metastasis. However, the relationship between these two microRNAs and matrix metalloproteins (MMPs), which are important in cancer cell metastasis, remains unknown. Here we report new evidence in which miR-520c and miR-373 effects in human fibrosarcoma HT1080 cells are associated with MMP9 activity, and this up-regulation of MMP9 is not only at the activity and protein levels, but also at that of its mRNA. Our experimental data demonstrate that these effects occur not by direct binding to the *MMP9* promoter, but by miR-520c and miR-373 directly targeting the 3'UTRs of mRNAs of mTOR and SIRT1 (negative regulators of expression of MMP9 via inactivating the Ras/Raf/MEK/Erk signaling pathway and transcription factor NF- κ B activity); and thus suppressing translation levels of SIRT1 and mTOR. Moreover, inhibition of key kinases of the Ras/Raf/MEK/Erk signaling pathway and western-blots for selected proteins further identified miR-520c and miR-373 as activating this signaling pathway and NF- κ B. In conclusion, miR-520c and miR-373 increased the expression of MMP9 by directly targeting the 3'UTRs of mRNAs of mTOR and SIRT1 and suppressing their translation; resulting in activation of the Ras/Raf/MEK/Erk signaling pathway and NF- κ B; and finally increasing the mRNA, protein, and activity of MMP9 and enhancing cell migration and cell growth in 3-D type I collagen gels.

Introduction

MicroRNAs (miRNAs) are a set of 21- to 24-nucleotide (nt), endogenous non-protein-coding, regulatory RNA molecules that contribute to modulating the expression level of specific proteins based on sequence complementarities with their target mRNA molecules (Meola *et al.*, 2009; Li *et al.*, 2010). Up to now, thousands of miRNAs have been identified in a wide variety of species. The role of miRNAs as key regulatory molecules that control a wide variety of fundamental cellular processes, such as proliferation, death, differentiation, motility, invasiveness, etc., is increasingly recognized in almost all biological and biomedical fields (Ambros, 2004; Erson *et al.*, 2009). miR-520c and miR-373 are two important miRNAs and have been characterized as oncogenes. In breast and prostate cancer

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cells, miR-520c and miR-373 stimulate cancer cell migration and invasion by suppressing the expression of CD44 (Huang *et al.*, 2008; Yang *et al.*, 2009). miR-373 is identified as an oncogene in testicular germ cell tumors through its direct inhibition of the expression of the tumor suppressor LATS2 (Voorhoeve *et al.*, 2006, 2007). In addition, miR-373 induces E-cadherin and DSDC2 expression by binding to the promoters of these two genes in some cancer cells, including LNCaP, PC3 and HCT-116 cells (Place *et al.*, 2008). Furthermore, it has been found that miR-373 is involved in the regulation of DNA repair gene expression in hypoxic stress (Crosby *et al.*, 2009).

MMP9/gelatinase B is a member of the MMP protein family, which plays a key role in cell growth, proliferation and cancer cell migration (Van den Steen *et al.*, 2002). MMP9 degrades type I collagen under certain conditions, but enhances its degradation initiated by collagenases (Heilpern *et al.*, 2009; Sabeh *et al.*, 2009), allowing tumor cells to break from the site of the primary tumor, leading to invasion and metastasis (Egeblad *et al.*, 2002; Van den Steen *et al.*, 2002). MMP9 protein levels in cells are regulated by a variety of means, including both protein and non-protein factors (Zhao *et al.*, 2008; St-Pierre *et al.*, 2004). mTOR and SIRT1, which play critical roles in many cellular processes (Chung *et al.*, 2010; Laplante *et al.*, 2009), have been reported to decrease MMP9 expression via negatively regulating the Ras/Raf/MEK/Erk signaling pathway (Grant, 2008; Carracedo *et al.*, 2008) and the activity of transcription factor NF- κ B (Mendes Sdos *et al.*, 2009; Nakamaru *et al.*, 2009).

Based on the above information, we hypothesized that miR-520c and miR-373 could affect the expression of MMP9 in highly aggressive cancer cells which express MMP9. To explore this hypothesis, we employed the HT1080 cell line as a model to test whether miR-520c and miR-373 affected the activities of MMP9 by using transfection and a zymography assay. Then by combining a series of methods and experiments including predicting binding sites of miRNAs, luciferase assay, and kinase inhibition etc., we determined the molecular mechanism of miR-520c and miR-373 effects on the activities of MMP9. Our data showed that up-regulation of MMP9 by miR-520c and miR-373 was on both the mRNA and protein levels. miR-520c and miR-373 increased MMP9 activity by indirectly targeting the promoter region of the MMP9 gene via binding to the 3'-UTRs and suppressing translation of the mRNAs of mTOR and SIRT1, resulting in activation of the Ras-Raf-MEK-Erk signaling pathway and the transcriptional factor NF- κ B. This resulted in increasing MMP9 mRNA, protein and activity levels. These data led us to propose a model in which miR-520c and miR-373 acting as oncogenes promoted cancer cell migration via enhancing the transcription and activity of MMP9 in human fibrosarcoma cells.

Materials and Methods

Cell culture and Cell transfections

Human fibrosarcoma HT1080 cells, obtained from ATCC (Manassas, VA; CCL-121TM), were cultured in MEM medium with 10% FBS and incubated in 5% CO₂ at 37°C. For transfection and downstream experiments, cells were pre-cultured in 5% FBS MEM medium over night, followed by the transfection and cultured in 5% FBS MEM medium until starting the experiments.

Transfection experiments utilizing plasmids, siRNAs (mTOR siRNA from Cell Signaling, Danvers, MA; and SIRT1 siRNA from Santa Cruz, Santa Cruz, CA.) and precursor of miRNAs (miR-520c, miR-373 and miR-control; all purchased from Ambion, Foster City, CA.) were carried out using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, Carlsbad, CA).

Western-blot analysis, Antibodies and Other reagents

After culturing transfected cells 60h, cells were harvested and lysed in RIPA buffer (Promega, Madison, WI); and analyzed by Western blotting to assess the level of proteins expressed. Antibodies used here were: anti-MMP9, anti-RECK, anti-CD44 and anti- β -actin antibodies from Santa Cruz; anti-mTOR, anti-SIRT1 and anti-p-Erk1/2 antibodies from Cell Signaling.

Kinase inhibitors utilized included: MEK inhibitor U0126 (10 μ M), p38MAPK inhibitor SB203580 (10 μ M), JNK inhibitor SP600125 (10 μ M), PKC inhibitor RO318220 (5 μ M), Ras inhibitor FTA (10 μ M), IKK inhibitor BMS-345541 (10 μ M), PI3K inhibitor LY294002 (20 μ M) and Raf inhibitor GW5074 (10 μ M) (all from Calbiochem, San Diego, CA). Other chemicals were from Sigma-Aldrich Inc (St. Louis, MO).

Immunostaining and Confocal Microscopy

Cells were grown on glass coverslips and transfected as indicated. After being cultured for 60h in 5% FBS MEM medium, cells were fixed with 4% paraformaldehyde for 20 min and incubated with PBS containing 0.1% Triton X-100 for 5 min. After blocking with 3% goat serum in PBS, 0.2 μ g/ml anti-MMP9 antibody (mouse anti-human) was added to the cells and they were incubated at 4°C for two hours. The secondary antibody used to detect the primary antibody was Alexa Fluor® 488 labeled goat anti-mouse IgG (Invitrogen). Confocal microscopy was carried out in the Biomedical Image Processing laboratories at the University of Minnesota using a Bio-Rad MRC 1024 system attached to an Olympus microscope (Melville, NY) with a 60X oil objective. The images were processed in Photoshop 7.0 (Adobe, San Jose, CA).

Gelatin zymography gel assay

Zymography gel assays were performed as described before (Jiang *et al.*, 2003; Pei, 1999). In brief, cells were cultured in 12-well plates and transfected as indicated in individual figures. The media were harvested at 60h after transfection and cleared by centrifugation at 12,000 rpm for 10 min; and subjected to analysis by SDS-PAGE impregnated with 1 mg/ml gelatin. The gels were incubated at 37°C overnight, stained with Coomassie Blue, destained, and then scanned.

Cell growth and migration assay

Cells were seeded in 12-well plates at the concentration of 1×10^3 , and after 24h incubation, were transfected as indicated by using Lipofectamine 2000. At 12h after transfection, these cells (1×10^3) were mixed with 500 μ l of type I collagen (2.5 mg/ml), placed into 24-well plates, and incubated at 37°C to give rise to a three-dimensional collagen lattice. Fresh medium containing 10% FBS was added to the wells and changed every 2 days. One week later, cells were photographed with a video camera at the University of Minnesota Biomedical Image Processing Laboratories as described previously (Jiang *et al.*, 2003).

Cell motility was tested using a scratch wound assay as described before (Mercure *et al.*, 2008). In brief, equal numbers of HT1080 cells were seeded in a 12-well culture plate. After growth to about 70% confluency, cells were transfected as indicated in individual figures. When the plates were grown to confluency in 5% FBS MEM medium, a scratch was made along the axis of the plate using a pipette tip. Cells were washed three times with PBS buffer, and media were changed. Migration of cells into the scratch area was photographed 48 h later.

miRNA target prediction

By employing PicTar (Krek *et al.*, 2005), MiTarget (Kim *et al.*, 2006) and Method of Multiple Sequence Alignment by CLUSTALW, we predicted targets of miRNAs in the promoter of genes or the 3' un-translational region (UTR) of their mRNAs. Luciferase reporter plasmids were constructed with wild type 3'UTRs and/or with miRNA binding site deletion mutants of the target mRNAs.

Luciferase activity assay

The wild-type promoter and its mutants (described in results and figure 2) of the human MMP9 gene were amplified by PCR using their primers (Table 1); and then the PCR products were cloned into the luciferase assay vector pGL3/Luc (this vector and the basic and control vectors were all from Promega.); and named pGL3-MMP9pro(w)/Luc, pGL3-MMP9pro(mI)/Luc, pGL3-MMP9pro(mII)/Luc and pGL3-MMP9pro(mIII)/Luc, respectively (Table 1 and fig. 3B). The wild-type 3'UTRs of mRNAs of human mTOR and SIRT1 (containing target sites of miR-520c and miR-373) and their target-site deletion mutants were amplified by PCR using their primers (table 1). The PCR products were cloned into the downstream of the luciferase gene in the pMIR-REPORT™ luciferase vector (Ambion). These constructs were named pMIR-SIRT1-3'UTR, pMIR-SIRT1-3'UTRm, pMIR-mTOR-3'UTR and pMIR-mTOR-3'UTRm, respectively (Table 1 and fig. 6B).

All luciferase assay constructs, control vectors and miRNA expression plasmids (including pMIRH-520c and pMIRH-373, from SBI-System Biosciences, Mountain View, CA) were transfected into HT1080 cells together with pRL/TK vector containing *Renilla* luciferase by using lipofectamine 2000 and incubated in 5% FBS DMEM for 60h; firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay kit (Promega). Each transfection was repeated twice in triplicate.

RT-PCR and Real-time quantitative RT-PCR analysis

Cells were transfected with miRNAs and control miRNA, and then incubated in 5% FBS MEM medium; 60h later, total RNA was extracted from the cells by using TRIzol reagent (Invitrogen). Reverse transcription was performed using 1µg of total RNA as a template and random hexamer as a primer. The cDNA levels of related protein genes were amplified by PCR using related specific primers (table 2). The PCR conditions were performed by denaturing the DNA at 94°C for 3 min, followed by 30 cycles of amplification: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and final extension step at 72°C for 10 min. β-actin was used as an internal control according to the manufacturer's instruction (Ambion). Amplified fragments were assayed on 1% agarose gel.

For expression levels of mature miRNAs in cells, the real-time quantitative PCR method was employed. The same number of cells (1×10^3 /well) was seeded in 96-well plates and transfected and incubated as above in a four parallel experiment set for each transfection. After 60h of transfection, the miRNA levels were detected using TaqMan MicroRNA Cells-to CT Kit (Ambion) according to the the manufacturer's instructions. The miR-520c and miR-373 specific primers, and control miRNA primers were all purchased from Applied Biosystems. The real-time quantitative PCR experiments were carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Data analysis and Statistics

Experiments were carried out in duplicate or triplicate. All quantitative data were presented as mean ± SD as noted in the figure legends. Statistical analysis was done with Student's t test using the Microsoft Excel computer program. Values of P<0.05 were considered significant (i.e. ANOVA test).

Results

miR-520c and miR-373 up-regulate the expression and activity of MMP9

Based on our hypothesis, we undertook an investigation of the roles of these two miRNAs on controlling MMP9 expression in HT1080 cells (human fibrosarcoma cancer cells). After transfection of precursors of miR-520c and miR-373 into HT1080 cells, we found that the miRNAs not only inhibited the translation of CD44 (fig. 1B, 1C), but greatly enhanced the activity of MMP9 as observed in zymographic gels (both in 5% FBS medium and in medium without FBS; fig. 1A(a)); and the effect of these two miRNAs on MMP9 activity was additive when co-transfecting miR-520c with miR-373 (data not shown). In addition, there was a significant increase in MMP9 protein (fig. 1B, 1E) and mRNA levels (fig. 1C). The MMP9 activity was also up-regulated by transfecting with miR-520c and miR-373 expression plasmids pMIRH-520c and pMIRH-373, that expressed mature miRNAs (fig. 1A(b)) [the transfection efficiency was about 90% (fig. 1A(c))]. Quantitative real-time RT-PCR data showed that the levels of the mature miRNAs in cells transfected with precursors of miR-520c and miR-373 (fig. 1D(a), (b), and (c)) or cells transfected with miRNA expression plasmids (data not shown), pMIRH-520c and pMIRH-373, were much higher than control and non-transfected cells.

Since miR-520c and miR-373 expression increased MMP9 activity, we designed experiments to test if these miRNAs could affect cell interaction with their microenvironment in growth in 3-D type I collagen gels and cell migration. The 3-D type I collagen lattices containing HT1080 cells transfected with miR-520c and miR-373 were much smaller than those of mock and control cells (fig. 2A); indicating possible greater type I collagen lattice degradation by cells transfected with the miRNAs and producing more MMP9. This may be due in part to possible direct degradation of type I collagen by MMP9 (Heilpern *et al.*, 2009; Sabeh *et al.*, 2009), but most probably by a cooperative role degrading collagen cleaved by HT-1080 cell surface MT1-MMP. miR-520c and miR-373 also enhanced cell migration in the scratch wound healing assay (fig. 2B). After transfection with the precursors of miR-520c and miR-373, the cellular migration ability was enhanced together with an increased MMP9 protein level; whereas, attempting to block endogenous miR-520c and miR-373 by their inhibitors (Ambion, Foster City, CA), resulted in limited inhibition of the migratory ability of the cells (fig. 2B, indicating that endogenous miR-520c and miR-373 levels were very low in HT1080 cells). These bio-function experiments indicated that cell growth in 3-D type I collagen lattices and cell migration ability were also up-regulated by miR-520c and miR-373.

All of these results demonstrated that miR-520c and miR-373 up-regulated the activity of MMP9 in fibrosarcoma cells by increasing the MMP9 mRNA and protein levels; and also indicated that miR-520c and miR-373 could possibly promote the transcription of the *MMP9* gene directly or indirectly via activating the promoter of the *MMP9* gene or regulating the availability of a transcriptional factor(s).

Promoter of MMP9 gene was not the direct target of miR-520c and miR-373, but the increase in MMP9 depended on transcription factor NF- κ B

To explore if regulation of MMP9 was direct via miRNAs binding to the promoter of the *MMP9* gene, miRNA target prediction software described in Materials and Methods was employed to check the promoter region of the *MMP9* gene for potential binding sites of miR-520c and miR-373. We found such binding sites (fig. 3A) and constructed luciferase reporter plasmids pGL3-MMP9pro by cloning the wild-type and several mutant forms of the promoter region of the *MMP9* gene into the luciferase reporter vector pGL3-Luc (indicated as in fig. 3A, 3B and 3C). The luciferase activities of the plasmid containing the wild-type

promoter region of the *MMP9* gene (pGL3-MMP9pro(w)/Luc) was up-regulated by transfected miR-520c and miR-373 precursors (fig. 3D), whereas the plasmid cloned promoter mutant III (pGL3-MMP9pro(mIII)/Luc; containing just miRNAs binding sites) did not respond to the miRNAs (fig. 4A). Moreover, the plasmid cloned promoter mutant I (pGL3-MMP9pro(mI)/Luc; containing the NF- κ B, AP1 and other transcription factors binding sites) responded to miR-520c and miR-373 (fig. 4B), but plasmid cloned promoter mutant II (pGL3-MMP9pro(mII)/Luc; without the NF- κ B binding site) had no response to the miRNAs (fig. 4C). These results demonstrated that up-regulation of MMP9 by miR-520c and miR-373 was not by direct targeting the promoter of the *MMP9* gene. However, this up-regulation was dependent on transcription factor NF- κ B, and probably was mediated by factors that activate transcription factor NF- κ B.

Up-regulation of MMP9 expression related to the Ras-Raf-MEK-Erk signal pathway

There are several cellular signaling pathways related to transcription factor NF- κ B, such as the MEK (Hwang *et al.*, 2010), PKC (Moscat *et al.*, 2006), JNK (Bubici *et al.*, 2004) and p38MAPK (Guo *et al.*, 2006) pathways. To determine which pathway was important here, we employed inhibitors of key kinases of these signaling pathways to block them, followed by assays of MMP9 activity as affected by miR-520c and miR-373. The experimental results showed that only the MEK inhibitor U0126 blocked the up-regulation of MMP9 activity by miR-520c and miR-373, whereas, there were no/little effects by the other inhibitors, including PKC inhibitor RO318220, JNK inhibitor SP600125, and p38MAPK inhibitor SB203580 (fig. 4D, upper-panel).

To further determine if the signaling pathway Ras-Raf-MEK-Erk has a key role here, we used the Ras inhibitor FTA and Raf inhibitor GW5074 to block this signaling pathway. Both the Ras and Raf inhibitors blocked up-regulation of MMP9 activity by miR-520c and miR-373 (fig. 4D, down-panel). In addition, the phosphorylated Erk1/2 level was increased by transfecting the precursors of miR-520c and miR-373 (fig. 5A), suggesting that up-regulation of MMP9 by miR-520c and miR-373 was through kinases Ras, Raf, and MEK, with activation of kinase Erk1/2.

Taken together, these results demonstrated that the activation of the Ras-Raf-MEK-Erk signaling pathway and transcription factor NF- κ B in increasing MMP9 expression by miR-520c and miR-373 may be mediated via other factors or signaling mechanisms.

3'UTRs of mRNAs of mTOR and SIRT1 were the targets of miR-520c and miR-373

By surveying several endogenous factors related to the activation of the Ras-Raf-MEK-Erk signaling pathway and of transcription factor NF- κ B, we found and focused on two regulators, mTOR and SIRT1, which negatively regulate the expression and activity of MMP9 via inactivating the Ras-Raf-MEK-Erk signaling pathway and transcription factor NF- κ B (Mendes Sdos *et al.*, 2009; Nakamaru *et al.*, 2009; Steelman *et al.*, 2008). Our data showed that the protein levels of both mTOR and SIRT1 were dramatically decreased by the transfected precursors of miR-520c and miR-373 (fig. 5B); whereas their mRNA levels were not changed (fig. 5C). We also examined the protein and mRNA levels of RECK, another reported negative regulator of MMP9 (Loayza-Puch *et al.*, 2010); and found no detectable expression of endogenous RECK in fibrosarcoma HT1080 cells (fig. 5B, 5C).

Blocking mTOR expression has been reported to activate the Ras-Raf-MEK-Erk pathway through activating kinase PI3K (Grant, 2008; Carracedo *et al.*, 2008). We blocked PI3K and another kinase, IKK, which has been linked to the Ras-Raf-MEK-Erk pathway and NF- κ B (Hou *et al.*, 2009) by using their respective inhibitors, LY294002 and BMS-345541. The data showed that up-regulation of MMP9 activity by miR-520c and miR-373 was blocked

by both of these inhibitors (fig. 4D, down-panel), and demonstrated that PI3K and IKK were involved in the regulation of MMP9 expression. These results indicated that miR-520c and miR-373 decreased the protein level of mTOR and activated the Ras-Raf-MEK-Erk pathway and NF- κ B factor via activating PI3K and IKK, and thus resulting in up-regulation of MMP9.

We next checked the acetylation status of subunit p65 of NF- κ B which is the direct deacetylation target of SIRT1 (Nakamaru *et al.*, 2009). The degree of acetylation of NF- κ B p65 was increased by transfected precursors of miR-520c and miR-373 (fig. 5D), suggesting that blocking SIRT1 translation could increase the acetylation of NF- κ B p65, resulting in activation of NF- κ B factor, and finally up-regulating the protein level and activity of MMP9.

Thus, our data indicated that the signaling pathway Ras-Raf-MEK-Erk and transcription factor NF- κ B were activated by miR-520c and miR-373 via inhibiting the translation of mRNAs of mTOR and SIRT1. We hypothesized it was possible that up-regulation of MMP9 was via direct targeting of the 3'UTR of mRNAs of mTOR and SIRT1 by miR-520c and miR-373, and blocking the translation of these two mRNAs.

To evaluate this possibility, we checked for target sites of miR-520c and miR-373 on the 3'UTRs of mRNAs of mTOR and SIRT1 by using prediction software as described in Materials and Methods. Targeting sites of miR-520c and miR-373 were, in fact, found (fig. 6A). We generated the 3'UTR luciferase reporter plasmids as shown in fig. 6B by using the primers shown in table 1. The reporter plasmids included constructs cloned with the wild-type 3'UTRs of mRNAs of mTOR and SIRT1, and constructs cloned with binding site deletion mutations in the 3'UTR of each mRNA. The experimental data demonstrated that the luciferase values of reporter plasmids cloned with the wild-type 3'UTR of mRNAs of mTOR and SIRT1 were blocked by transfected miR-520c and miR-373; whereas no changes were found with the plasmids cloned with target site deletion mutants of 3'UTRs of mRNAs of mTOR and SIRT1 (fig. 6C, 6D).

In addition, siRNAs of mTOR and SIRT1 were employed to transfect into HT1080 cells to test their effect on MMP9 activity. Blocking mTOR and SIRT1 in HT1080 cells resulted in increased MMP9 activity by elevating its transcription and protein levels (fig. 6E, 6F). Furthermore, when we transfected the cDNAs of mTOR and SIRT1 into HT1080 cells, the endogenous MMP9 activities were all decreased (fig. 7A). These results were consistent with inhibition of MMP9 expression by mTOR and SIRT1, as has been reported in previous publications (Mendes Sdos *et al.*, 2009; Nakamaru *et al.*, 2009).

In conclusion, our results indicated that miR-520c and miR-373 blocked the translation of mRNAs of mTOR and SIRT1 by targeting their 3'UTRs, resulting in activation of the Ras-Raf-MEK-Erk signaling pathway and transcription factor NF- κ B, and elevating the transcription and translation levels of MMP9, resulting in enhanced MMP9 activities.

Discussion

miR-520c and miR-373 were first reported as oncogenes in cancer cells in vivo and in vitro (Voorhoeve *et al.*, 2006; Voorhoeve *et al.*, 2007). These two miRNAs have been well-defined in some types of cancer cells to regulate the levels of several important proteins related to cell adhesion and migration (Huang *et al.*, 2008; Yang *et al.*, 2009; Lee *et al.*, 2009; Place *et al.*, 2008). Loayza-Puch *et al.* (2010) reported that MMP9 levels were regulated by miR-373 via targeting the 3'UTR of mRNA of RECK in RECK stable expression fibrosarcoma HT1080 cells. In this study, we found that indeed the MMP9 protein and transcription levels were up-regulated by transfected precursors of miR-520c

and miR-373, but, RECK protein was undetectable in wild-type fibrosarcoma HT1080 cells (fig. 5). This indicated that up-regulation of MMP9 levels by miR-520c and miR-373 was not through blocking translation of mRNA of RECK. We further identified that miR-520c and miR-373 increased MMP9 transcription and protein levels via activating the Ras/Raf/MEK/Erk pathway and transcription factor NF- κ B by binding to the 3'UTRs and blocking the translation of mRNAs of mTOR and SIRT1 (fig. 5, 6 and 7).

mTOR, which exists in two distinct complexes--mTORC1 and mTORC2 (only mTORC1 was sensitive to rapamycin), is involved in many signaling pathways (Gibbons *et al.*, 2009). Inhibition of mTOR/mTORC1 can activate the Ras/Raf/MEK/Erk pathway via RTK/IRS-1/PI3K (Grant, 2008; Carracedo *et al.*, 2008), whereas, inhibition of mTORC2 by blocking Rictor (one component of the complex) can activate the Raf/MEK/Erk pathway via Akt (Das *et al.*, 2011). MMP9 over-expression can be induced by rapamycin, presumably via inhibition of mTOR and the mTORC1 complex (Mendes Sdos *et al.*, 2009; Hwang *et al.*, 2010; Hou *et al.*, 2009), and also by blocking Rictor and blocking the mTORC2 complex (Das *et al.*, 2011). In addition, inhibition of mTOR can result in Akt hyperactivation (Nakamaru *et al.*, 2009) and direct formation of a complex with IKK, activating NF- κ B leading to increased expression of MMP9 (Lu *et al.*, 2005; Romashkova *et al.*, 1999). Inhibition of mTOR by miR-520c and miR-373 implies that both mTORC1 and mTORC2 were blocked. Thus, there are three possible pathways related to MMP9 over-expression: (1) inhibition of mTOR--mTORC1--RTK/IRS-1/PI3K--Ras/Raf/MEK/Erk--IKK/NF- κ B/MMP9 pathway; (2) inhibition of mTOR--mTORC2--Akt--Raf/MEK/Erk--IKK/NF- κ B/MMP9 pathway; and (3) inhibition of mTOR--mTORC2--Akt/IKK/NF- κ B/MMP9 pathway. In this study, our series of kinase inhibition experiments showed that up-regulation of MMP9 was not through Akt directly phosphorylating IKK, but via RTK/IRS-1/PI3K activating the Ras/Raf/MEK/Erk--IKK/NF- κ B pathway. Our data thus showed that decreased translation of mRNA of mTOR by miR-520c and miR-373 is via targeting its 3'UTR, resulting in activation of the Ras/Raf/MEK/Erk pathway and the transcription factor NF- κ B (presumably via activating IKK and degrading I κ B), finally resulting in more active NF- κ B entering the nucleus and increasing expression of the MMP9 gene and thus elevation of MMP9 protein and activity (fig. 1, 7B). However, at this time the details and actual mediator(s) between mTOR and the Ras/Raf/MEK/Erk pathway are not clear, and other mTOR signal pathways may also be involved in this regulation process. These need to be established in future studies.

In addition, we tested the classic activators, SOS1 and Grb2, of the Ras/Raf/MEK/Erk pathway, (Roberts *et al.*, 2007), on MMP9 expression by using siRNAs to inhibit the translation of their mRNAs. The results demonstrated that SOS1 and Grb2 were not involved in the pathway of up-regulating MMP9 by miR-520c and miR-373 (data don't shown).

The activity of NF- κ B is also known to be regulated by acetylation both *in vitro* and *in vivo* (Yang *et al.*, 2007). SIRT1 has been shown to be specifically related to deacetylation of the p65 subunit of NF- κ B at lysine 310, and this deacetylation attenuates NF- κ B transcriptional activation (Chen *et al.*, 2005). Thus, reduction of SIRT1 protein could result in hyperacetylation of active NF- κ B located in the nucleus, leading to added enhancement of its activity with the activation resulting from decreased mTOR. Furthermore, SIRT1 inhibition has also been reported to increase histone-4 acetylation of NF- κ B binding sites (-594 to -604) of the MMP9 promoter; and thus increasing the binding of NF- κ B to the MMP9 promoter. In short, miR-520c and miR-373 decreased the protein level of SIRT1 by targeting 3'UTR of its mRNA (fig. 5, 6); and this could lead to enhanced activity of NF- κ B by inhibiting deacetylation of subunit p65 of NF- κ B and inhibiting the deacetylation of

histone-4 at the NF- κ B binding site of the MMP9 promoter; finally resulting in increased transcription level and activity of MMP9 (fig. 7B).

NF- κ B is a critical transcription factor involved in a broad range of biological processes, including the immune system, cell survival, stress responses and maturation of various cell types (Shih *et al.*, 2011). Several regulation modes of NF- κ B have been found, including phosphorylation and acetylation of subunits of NF- κ B (Lu *et al.*, 2005; Romashkova *et al.*, 1999; Yang *et al.*, 2007; Chen *et al.*, 2005). In this study, we found that both phosphorylation and acetylation of NF- κ B were involved in the regulation of MMP9 expression by miR-520c and miR-373. From our data, when the Ras/Raf/MEK/Erk pathway was blocked by kinase inhibitors, the MMP9 activity was also blocked, although the protein level of SIRT1 was still decreased by transfected miR-520c and miR-373. It indicated that phosphorylation of NF- κ B by the Ras/Raf/MEK/Erk pathway was required for activation of NF- κ B by acetylation; and also indicated that mTOR has the key role in regulation of MMP9 by miR-520c and miR-373, and SIRT1 was a cooperative factor with mTOR in the activation of NF- κ B.

In summary, we found that miR-520c and miR-373 up-regulated transcription, protein expression and activity of MMP9 in HT1080 cells. This up-regulation of MMP9 was via miR-520c and miR-373 targeting the 3'UTRs of mRNAs of mTOR and SIRT1 and suppressing translation of their mRNAs, thus decreasing the protein levels of mTOR and SIRT1. This was followed by activation of the Ras/Raf/MEK/Erk pathway and transcription factor NF- κ B by phosphorylation and enhanced acetylation of NF- κ B. In this process, phosphorylation of NF- κ B by PI3K--Ras/Raf/MEK/Erk--IKK pathway is a crucial step for the activation of NF- κ B by enhancing acetylation; and indicates that mTOR plays a key role and SIRT1 is a cooperative factor for the activation of NF- κ B here. All of these mean that we have characterized a molecular mechanism of cooperative up-regulation of the expression of the MMP9 gene simultaneously by different signaling pathways via miR-520c and miR-373 targeting the 3'UTRs of mRNAs of SIRT1 and mTOR (fig. 7B).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

MMP	matrix metalloprotein
mTOR	mammalian target of rapamycin

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Figure 1.

Up-regulation of expression and activity of MMP9 by miR-520c and miR-373 in HT1080 cells. Cells were seeded in 12-well plates, transfected with precursors or plasmids of miRNAs as indicated; and incubated in DMEM media with or without 5% FBS. (A) At 60h after transfection, zymography of media showed increased MMP9 activity as affected by miR-520c and miR-373 (a), whether cells were grown with or without 5% FBS. Cells transfected with the expression plasmids of miR-520c and miR-373 (pMIRH-520c and pMIRH-373) and grown in 5% FBS DMEM demonstrated elevated MMP9 activities (b) and GFP expression (c) when co-transfected with pMIRH-plasmids containing GFP marker gene using Confocal microscope. The transfection efficiency of plasmids is indicated. (B) Cells demonstrated decreased CD44 and increased MMP9 (β -actin as control) by Western blot assay 60h after transfection with precursors of miR-520c and miR-373. (C) Cells examined by RT-PCR 60h after transfection with precursors of miR-520c and miR373 demonstrated

no change in CD44 mRNA, but a strong increase in MMP9 mRNA. (D) The expression levels of mature miR-520c and miR-373 was confirmed by quantitative real-time RT-PCR of HT-1080 cells 60h after transfection with precursors of miR-520c and miR373: (a) Changes of Ct values; (b) Changes of Rn values; (c) Levels of DNA products of quantitative real-time RT-PCR separated in 1.8% agarose gels. (E) Increased cellular MMP9 was demonstrated by confocal microscopy in HT-1080 cells 60h after transfection with precursors of miR-520c and miR-373.

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Figure 2.

Bio-functional effects of miR-520c and miR-373 on growth and migration of fibrosarcoma HT1080 cells. (A) Cells were seeded in 12-well plates and transfected as described; 12 h after transfection the same number of cells (1×10^3) were mixed with 0.5 mL type I collagen, the collagen allowed to gel at 37°C. The collagen matrices were photographed 7 days later, and the diameters of the collagen gels were measured. The decreased diameters of the collagen gels indicate increased MMP9 secretion and subsequent collagen degradation. (B) The effect of miR-520c and miR-373 on cell migration. The same number of cells was seeded in 12-well plates, transfected 24 h later as indicated, and at near full confluency, a wound scratch was made along the axis of each well using a pipette tip. Migration of cells into the scratch area was photographed at 48 h (the border areas of the scratch wound are marked by two white lines as noted in the figures).

Figure 3. Schematic representation of the MMP9 promoter and predicted binding sites of miR-520c and miR-373, constructs of luciferase assay plasmids containing wild-type/mutant fragments of the MMP9 promoter; and luciferase assay results of the plasmid containing wild-type MMP9 promoter. (A) Schematic representation of the MMP9 promoter and binding sites of miR-520c and miR-373, the sequence of the miR-520c and miR-373 sites, and the complementary situation of the bases, including G:U/T wobble base-pairing. (B) Representation of wild-type/mutant fragments of the MMP9 promoter designed for the luciferase assay constructs (described in Methods and Materials). (C) Different luciferase assay plasmids constructed by cloning the designed wild-type/mutant fragments of the MMP9 promoter into the pGL3-basic vector (described in Methods and Materials). (D) Dual-luciferase assay results of the constructed plasmid with the wild-type MMP9 promoter (pGL3-MMP9pro(w)/Luc; containing miR-520c and miR-373 binding sites, and the NF-κB, AP1, Ets/Elk and other transcription factor binding sites). HT1080 cells were transfected with plasmids as indicated and harvested at 60h after transfection and assayed using a dual-luciferase assay kit (Promega). Cells co-transfected with precursor of miR-520c or miR-373 demonstrated increased luciferase activity. * $P < 0.05$ compared with pGL3-control/Luc; # $P < 0.05$ compared with pGL3-mmp9pro(w)/Luc. Error bars indicate s.d. among three individual experiments.

Figure 4.

Dual-luciferase assay results of the constructed plasmids containing mutant fragments of the MMP9 promoter, and the effect of kinase inhibitors on up-regulation of MMP9 activity by miR-520c and miR-373. HT1080 cells were seeded in 12-well plates, transfected with different plasmids as indicated; and cells were harvested for dual-luciferase assays 60h after transfection. (A) There was no change in luciferase activity with the plasmid containing mutant III of the MMP9 promoter (pGL3-MMP9pro(III)/Luc; containing only the miR-520c and miR-373 binding sites) with miR-520c or miR-373 as compared to control miRNA or positive control (only mutant III). (B) There was increased luciferase activity with miR-520c or miR-373 and the constructed plasmid containing mutant I of the MMP9 promoter (pGL3-MMP9pro(mi)/Luc; containing the NF- κ B, AP1, Ets/Elk and other transcription factors binding sites, but no miRNAs binding sites). (C) There was no change in luciferase activity with miR-520c or miR-373 and the constructed plasmid containing mutant II of the MMP9 promoter (pGL3-MMP9pro(miII)/Luc; containing AP1, Ets/Elk and other transcription factors binding sites, but lacking the NF-KB binding site and miRNAs binding sites). (D) Effects of inhibitors of MEK (U0126), p38 MAPK (SB203580), JNK (SP600125), and PKC (RO318220) (upper-panel); and of inhibitors of Ras (FTA), Raf (GW5074), PI3K (LY294002), and IKK (BMS-345541) (lower-panel) on the up-regulation of MMP9 by miR-520c and miR-373. Cells were transfected with miR-520c and miR-373 as indicated, cultured for 12h in the 5% FBS medium, and then treated with kinases inhibitors (final concentrations described in Materials and Methods) for 48h, at which time media were harvested for assay. Inhibition of MEK, and inhibition of Ras, Raf, PI3K, and IKK blocked increased MMP9 expression by the miRNAs. *P < 0.05 compared with pGL3-basic/Luc; #P < 0.05 compared with pGL3-control/Luc; Δ P < 0.05 compared with pGL3-mmp9pro(miII)

or mI or mII)/Luc. (A, B, and C) Error bars indicate s.d. among three individual experiments.

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Figure 5.

Effects of miR-520c and miR-373 on phosphorylation level of Erk1/2, expression levels of mTOR and SIRT1, acetylation status of subunit p65 of NF- κ B, and expression of the *RECK* gene in HT1080 cells. Cells were seeded in 12-well plates and cells were harvested for western-blot and RT-PCR assays 60h after transfection with miRNAs as indicated. (A) miR-520c and miR-373 increased the degree of phosphorylation of Erk1/2 (relative quantification was made in figure). (B) miR-520c and miR-373 decreased the protein levels of SIRT1, mTOR, and RECK (β -Actin was used as the control). (C) There was no change in the mRNA levels of SIRT1, mTOR, and RECK genes by using RT-PCR after stimulation by miR-520c and miR-373 (β -Actin was used as control). (D) miR-520c and miR-373 expression resulted in increased acetylation status of subunit of p65 of NF- κ B (Western-blot. β -Actin was used as the control).

Figure 6.

mTOR and SIRT1 as targets of miR-520c and miR-373: miR-520c and miR-373 suppressed the translation of mRNAs of mTOR and SIRT1 via targeting 3'UTRs of their mRNAs; and siRNAs of mTOR and SIRT1 increased transcription and activity levels of MMP9. HT1080 cells were seeded in 12-well plates and transfected with precursors of miRNAs and constructed plasmids or siRNAs as indicated. 60h after transfection, media were harvested for zymography assay and cells were harvested for dual-luciferase, western-blots, and RT-PCR assays. (A) Representation of the sequences of miR-520c and miR-373 and their binding sites located in the 3'UTRs of the mRNAs of SIRT1 and mTOR; and the complementary situation of bases, including G:U/T wobble base-pairing. (B) Schematic representation of luciferase assay plasmids constructed by cloning fragments of wild-type/ mutant 3'UTRs of mRNAs of SIRT1 and mTOR into the pMIR-REPORTTM luciferase assay vector. (C) Dual-luciferase assay results of constructed plasmids containing wild-type and mutant 3'UTRs of mRNA of mTOR, pMIR-mTOR-3'UTR and pMIR-mTOR-3'UTRm, respectively. * $P < 0.05$ compared with pMIR-REPOT; # $P < 0.05$ compared with pMIR-mTOR-3'UTR; $\Delta P < 0.05$ compared with pMIR-mTOR-3'UTRm. (D) Dual-luciferase assay results of constructed plasmids containing wild-type and mutant 3'UTRs of mRNA of SIRT1, pMIR-SIRT1-3'UTR and pMIR-SIRT1-3'UTRm, respectively. * $P < 0.05$ compared with pMIR-REPOT; # $P < 0.05$ compared with pMIR-SIRT1-3'UTR; $\Delta P < 0.05$ compared with pMIR-SIRT1-3'UTRm. (E) mTOR siRNA increased the protein level, activity and

mRNA of MMP9 (zymography, western blot and RT-PCR). (F) SIRT1 siRNA increased the protein level, activity and mRNA of MMP9 (zymography, western blot and RT-PCR). (C and D) Error bars indicate s.d. among three individual experiments.

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Figure 7.

(A) Down-regulation of MMP9 activity by over-expression of mTOR and SIRT1. HT1080 cells were seeded in 6-well plates, transfected as described in figures and cultured in 5% FBS MEM. 48 h later, media were harvested for gelatin zymography of MMP9, and cells were harvested for western blot assay of mTOR and SIRT1. Increased mTOR or SIRT1 protein expression was associated with decreased MMP9. (B) Schematic representation of the regulation mechanism pathway described in the study. Briefly, inhibition of translation of mTOR mRNA induced the activation of PI3K (not Akt), and activation of the Ras/Raf/MEK/Erk pathway, resulting in activating IKK and activation of NF- κ B, and finally increased transcription of the MMP9 gene. On the other hand, inhibition of SIRT1 could block deacetylation of the NF- κ B p65 subunit and promoter of the MMP9 gene, and increase active NF- κ B binding to the promoter of the MMP9 gene, and finally up-regulate the transcription level of the MMP9 gene.

Table 1

Primers of constructing luciferase reporter plasmids of mmp9 promoter, SIRT1 3'-UTR and mTOR 3'-UTR

MMP9 promoter luciferase reporter plasmids construction		
	Primer pairs	Constructed plasmid name
Wild-type	Forward 5' acgagctccagatttcagtgtctctccacatcctgcc 3' Reward 5' acgctagctgctgtgtgtggggccttaaggagg 3'	pGL3-mmp9pro(w)/Luc
Mutant I	Forward 5' acgagctctgcccaggtcctgaagg 3' Reward 5' aacgctagcgactgcagctgctgttg 3'	pGL3-mmp9pro(mI)/Luc
Mutant II	Forward 5' acgagctccagagccattccttcc 3' Reward 5' aacgctagcgactgcagctgctgttg 3'	pGL3-mmp9pro(mII)/Luc
Mutant III	Forward 5' acgagctccagatttcagtgtctctccacatcctgcc 3' Reward 5' acgctagcggatctgagagttcagcatggggag 3'	pGL3-mmp9pro(mIII)/Luc
SIRT1 3'-UTR luciferase reporter plasmids construction		
	Primer pairs	Constructed plasmid name
Wild-type 3'UTR	Forward 5' acgagctctgtaataattgtgcaggtagcagg 3' Reward 5' acgccggccagaaaaagtcaatgacaa 3'	pMIR-SIRT1-3'UTR
3'-UTR mutant (miRs target-site deletion)	Forward 5' catcactcctaaattaataaattacaataaaaagggttg 3' Reward 5' caaacctttttattgtatttattatttaggagtgatg 3'	pMIR-SIRT1-3'UTRm
mTOR 3'-UTR luciferase reporter plasmids construction		
	Primer pairs	Constructed plasmid name
Wild-type 3'UTR	Forward 5' acgagctcagatgtgcccatcagtt 3' Reward 5' acgccggctgatgtcatttattggcaca 3'	pMIR-mTOR-3'UTR
3'-UTR mutant (miRs target-site deletion)	Forward 5' ggttttgacttaactcacaagctgatggaagaatgacc 3' Reward 5' ggtcattcttccatcagctgtgtgagtttaagtcaaaacc 3'	pMIR-mTOR-3'UTRm

Table 2

Primers of RT-PCR of some genes

Gene name	Primer pairs	Fragment size (bp)
MMP9	Forward 5' gacgcagacatcgtcatccagttt 3' Reward 5' gccgcgccatctgcgttt 3'	200
CD44	Forward 5' attccagaatggctgatcatc 3' Reward 5' tccttgtctcatcagctgtc 3'	256
SIRT1	Forward 5' gaacaggttcggaatc 3' Reward 5' aacatgaagaggtgtgggtg 3'	553
mTOR	Forward 5' tggaggagagcgtctgaga 3' Reward 5' ctggaaggccgtgtacg 3'	477
RECK	Forward 5' cctcagtgcacagttcaga 3' Reward 5' gcagcacacactgtgta 3'	198
β -Actin	Forward 5' gatgagattggcatggcttt 3' Reward 5' ctcaagttggggacaaaaa 3'	431