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MiR-138 and MiR-135 Directly Target Focal Adhesion Kinase, Inhibit Cell Invasion, and Increase Sensitivity to Chemotherapy in Cancer Cells

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Abstract

Focal Adhesion Kinase is a 125 kDa non-receptor kinase and overexpressed in many types of tumors. Recently, short noncoding RNAs, called microRNAs have been discovered as regulators of gene expression mainly through binding to the untranslated region (UTR) of mRNA. In this report we show that MiR-138 and MiR-135 down-regulated FAK expression in cancer cells. MiR-138 and MiR-135 inhibited FAK protein expression in different cancer cell lines. The computer analysis of 3'FAK-untranslated region (FAK-UTR) identified one conserved MiR-138 binding site (CACCAGCA) at positions 3514-3521 and one conserved MiR-135 (AAGCCAU) binding site at positions 4278-4284 in the FAK-UTR. By a dual-luciferase assay we demonstrate that MiR-138 and MiR-135 directly bound the FAK untranslated region using FAK-UTR-Target (FAK-UTR) luciferase plasmid and inhibited its luciferase activity. The site-directed mutagenesis of the MiR-138 and MiR-135 binding sites in the FAK-UTR abrogated MiR-138 and MiR-135-directed inhibition of FAK-UTR. Real-time PCR demonstrated that cells transfected with MiR-138 and MiR-135 expressed decreased FAK mRNA levels. Moreover, stable expression of MiR-138 and MiR-135 in 293 and HeLa cells decreased cell invasion and increased sensitivity to 5-fluorouracil (5-FU), FAK inhibitor, Y15, and doxorubicin. In addition, MiR-138 significantly decreased 293 xenograft tumor growth *in vivo*. This is the first report on regulation of FAK expression by MiR-135 and MiR138 that affected invasion, drug sensitivity, and tumor growth in cancer cells, which is important to the development of FAK-targeted therapeutics and understanding their novel regulations and functions.

Keywords

Cancer; expression; Focal Adhesion Kinase; invasion; microRNA; tumor

Introduction

Focal Adhesion Kinase (FAK) is a 125 kDa non-receptor protein kinase that is overexpressed in many types of tumors, including breast, colon, pancreatic and

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neuroblastoma [1-3]. FAK plays a significant role in adhesion, motility, survival, proliferation and invasion. FAK is activated by integrin clustering, growth factor receptor signaling or angiogenesis (VEGFR) signaling [4]. Recently, FAK has been proposed as a target of cancer therapy, and several small molecule inhibitors were developed to block FAK signaling and functions in cancer [5]. Several approaches have been used to down-regulate Focal Adhesion Kinase and block survival signaling, such as dominant-negative C-terminal domain of Focal Adhesion Kinase (FAK-CD or FRNK) [6]; anti-sense oligonucleotides [7]; small interfering RNA (siRNA)[8-10]; and small molecule inhibitors [11-14]. Down-regulation of FAK with dominant-negative FAK, FAK-CD caused breast cancer apoptosis [15], while down-regulation of FAK with anti-sense oligonucleotides to the FAKmRNA in combination with 5-fluorouracil resulted in increased apoptosis in melanoma cancer cells [7]. FAKsiRNA decreased tumor cell growth in breast xenograft and other models [10]. In this report, we defined a novel regulation of FAK expression by microRNA: MiR-135 and MiR138 in different cancer cells that modulated cancer cell functions.

The recent increasing interest in microRNA (miRNA) is caused by the discovery of the novel roles of miRNA in many cellular and pathological processes, such as carcinogenesis. MicroRNAs (miRNAs) are endogenous small non-coding single stranded RNA molecules with a length from 18 to 24 bases that regulate gene expression either by post-transcriptional degradation or translational repression and play a major role in intracellular signaling by targeting important gene expression [16-19]. The mechanism and functions of specific miRNA needs to be explored, as it is a new field of cellular and cancer biology. MicroRNA binds mainly to the 3'untranslated region (UTR) of target mRNA (miRNA binding site) based on complementarity with the 7-8 nucleotides at the 3'untranslated end of target mRNA (3'UTR). The mechanisms of regulation of different proteins that play a significant role in carcinogenesis by miRNA remain to be discovered. In this report, we analyzed with Target Scan software the 3' untranslated region of FAK and found that microRNA: MiR-138 and MiR-135 target the 3' untranslated region of FAK. Both sites of the MiR-135 and 138 binding in FAK-UTR are highly conserved among different species, suggesting their importance in FAK regulation in many species. We expressed the MiR-138 and MiR-135 in different cancer cells and found by a dual-luciferase assay that both MiR-135 and -138 directly bound to the 3' untranslated region of FAK and inhibited FAK-UTR-luciferase activity. The site-directed mutagenesis of the MiR-138 and Mi-135 binding sites reversed MiR-138 and MiR-135-directed inhibition of FAK-UTR luciferase activity. In addition, MiR-138 and MiR-135 inhibited both FAKmRNA and protein levels in different cancer cells. We generated a stable MiR-138 and MiR-135-expressing 293 and HeLa cells and demonstrated that cancer cells became less invasive and more sensitive to chemotherapy. In addition, MiR-138 significantly decreased 293 xenograft tumor growth *in vivo*. Thus, MiR-138 and MiR-135 directly target 3'-FAK-UTR, down-regulate FAK expression in cancer cells that affect cancer cell invasion and chemotherapy response that can be used as a new therapy approach to block tumor growth.

Materials and Methods

Cells

HeLa cells were obtained from ATCC and cultivated in DMED medium with 10% fetal bovine serum (FBS) with addition of 1 µg/ml penicillin/streptomycin. SW480 cells were cultivated in DMEM medium with 10% FBS Human epithelial kidney 293T cells grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1 µg/ml. A375 and PANC-1 cells were cultivated in DMEM with 10% serum.

Plasmids

The control pCMV-GFP-MiR (called pCMV-Control); pCMV-GFP-MiR-138; called pCMV-MiR-138 (#SC400164), (containing MiR-138-2 sequence); pCMV-GFP-MiR-135, called pCMV-MiR135 (#SC400164, containing MiR135-B sequence); the pMIR-Target luciferase, containing the 3'UTR of FAK transcript (called FAK-UTR) plasmids were obtained from *Origene Inc.* All plasmids were sequenced in both forward and reverse directions in Roswell Park Sequencing Facility.

Antibodies and Reagents

FAK monoclonal antibody (FAK 4.47) was obtained from (*Upstate Biologicals*). beta-Actin antibody was obtained from Sigma. Lipofectamine was obtained from *Invitrogen*. FAK inhibitor Y15, described in [13], 5-FU, and doxorubicin were obtained from Sigma.

Transfection

The plasmids were transfected into cells with lipofectamine according to the manufacturer's protocol. In brief, the day before transfection, cells were plated at 70% confluency and DNA with lipofectamin was added in serum-free medium for 6-24 hours and then incubated in serum-containing medium for 24-48 hours. The stable clones of Control and pCMV-MiR-138 and 138 were generated by growing cells in the presence of G418 at 500 µg/ml for 2 weeks.

RNA Isolation

Total cellular RNA and miRNA were isolated from cultured cells with a NucleoSpin RNA II Purification Kit and with NucleoSpin miRNA Purification Kit, respectively (Clontech Laboratories, Inc.) according to the manufacturer's protocol.

Real-time PCR

For FAK cDNA amplification was performed by Real time RT-PCR using TaqMan One-Step RT-PCR Master Mix (Applied Biosystems). RT-PCR Primers and Taq Man fluorescent probes and PCR conditions were described previously in [20]. The forward FAK primer was: 5'-GTGCTCTTGGTTCAAGCTGGAT-3' and the reverse: 5'-ACTTGAGTGAAGTCAGCAAGATGTGT-3' primers and the FAK probe was: 5'-FAM-TTACCTAACGGACAAGGG CTGCAATCC-TAMRA-3'. For detection of MiR-135 and MiR-138 RNA level in the cells the probes and forward and reverse primers were used from TaqMan MircoRNA Assay 135 and 138 kit, respectively (*Applied Biosystems Inc*). The RT-PCR reaction was performed with MiR primers according to the manufacturer's protocol. For normalization purposes, we used GAPDH primers: forward: 5'-GAAGGTGAAGGTCCGAGTC-3', and reverse 5'-GAAGATGGTGA TGGGATTTC-3' and the GAPDH probe 5'-FAM-CAAGCTTCCCGTTCTCAGCCT-3'-TAMRA. The ABI PRISM 7700 cycler's software calculated a threshold cycle number (Ct) at which each PCR amplification reached a significant threshold level.

Dual-luciferase Assay

The dual-luciferase assay was performed with the Dual-Luciferase Reporter Assay System kit (*Promega Inc*). For normalization of luciferase activity, the pRL-TK control vector, encoding Renilla luciferase was co-transfected with FAK-promoter-PGL3 luciferase plasmids, as described in [21]. Luminescence was measured on a Luminometer in three independent experiments.

Site-directed Mutagenesis

Site-directed mutations of the MiR 138 and MiR-135 binding sites in FAK UTR sequence were generated with QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) using FAK-UTR-Target plasmid as a template, according to the manufacturer's protocol. To generate MiR-138 site mutation, we used the following oligonucleotides (mutated base is underlines): forward: 5'-GA TGTTCCTAGCCTTCCACAAGCAGCGAGGAATTAA CCC-3' and reverse: 5'GGGTAAATTCCTCGCTGCTTGTGGAAGGCTAGA GAACATC-3'. To generate the MiR-135 mutation, we used the following oligonucleotides: 5'-GAAAGATTAAA GGCGTGTTGA CTATTTTACAGCCAC TGG-3' and reverse 5'-CCAGTGGC TGTAATAATAG TCAACACGCCTTTAATCTTTC-3'. All mutant plasmids were sequenced at the Automated DNA Sequencing Facility at the Roswell Park Cancer Institute (Buffalo, NY).

Western Blotting

Western blot analysis was performed as described previously [20]. In brief, cells were washed twice with cold 1xPBS and lysed on ice for 30 min in a buffer containing: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X, 0.5% NaDOC, 0.1% SDS, 5mM EDTA, 50 mM NaF, 1mM NaVO₃, 10% glycerol and protease inhibitors: 10 µg/ml leupeptin, 10 µg/ml PMSF and 1 µg/ml aprotinin. The lysates were cleared by centrifugation at 10,000 rpm for 30 min at 4°C. The protein concentrations were determined using a Bio-Rad Kit. The boiled samples were loaded on Ready SDS-10% PAGE gels (*Bio Rad, Inc*) and used for Western blot analysis with the protein- specific antibody. Immunoblots were developed with chemiluminescence reagent. The membranes were stripped in a stripping solution (BioRad) at 37°C for 15 min and then re-probed with the primary antibody to check equal loading of proteins.

Immunostaining

Immunostaining was performed with FAK primary antibody as described in [22] and slides were analyzed using Zeiss Axiovert microscope and AxioVision 4 software.

Cell Viability Assay

Cells were treated with different concentrations of FAK inhibitor Y15, 5-FU, and doxorubicin for 24 hours. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-(4-sulfophenyl) - 2H-tetrazolium compound from Promega Viability kit (Madison, IL) was added, and the cells were incubated at 37°C for 1-2 hours. The optical density on 96-plate was analyzed with a microplate reader at 490 nm to determine cell viability.

Invasion Assay

The invasion assay was performed on Boyden chambers with the invasion kit from Chemicon International Inc according to the manufacturer's protocol.

Tumor Growth in Nude Mice *in vivo*

Female nude mice, 6 weeks old, were purchased from Harlan Laboratory. The mice were maintained in the animal facility and all experiments were performed in compliance with NIH animal-use guidelines and IACUC protocol approved by the Animal Care Committee. The cells (2×10^6 cells/mice) were injected subcutaneously into the left and right side of the same mice. Tumor volume in mm³ was calculated using this formula = (width)² × Length/2.

Statistical Analyses

The Student's t-test was performed to determine significance. The difference between data with p-value <0.05 was considered significant.

Results

The 3' Untranslated Region of FAK Contains Conservative Sites for Binding MiR-135 and MiR-138

To define the regulation of FAK expression by miRNA, we analyzed the 3'-untranslated region of FAK, FAK-UTR with Target Scan software (<http://www.targetscan.org/>) and MicroCosm Targets (formerly miRBase target software) (<http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/targets/v5/>). Fig. (1A) shows FAK sequence (NM_005607, length 1-4550 bp), which was used for the analysis. It contained the 5'-untranslated: 1-234 bp (5'UTR); coding FAK cDNA: 234-3458 bp; 3'UTR: 3458-4500 bp and poly-A-tail: 4500- 4550 bp regions (Fig. 1A). The TargetScan software identified two sites in the 3'-untranslated region of FAK that were conserved in at least 12 different species: 8-base site for MiR-138 at positions 56-63, and 7-base site for MiR-135b (called MiR-135) at positions 820-826 of the 1042 base pair long 3'FAK-UTR (Fig. 1A). The sequence of the conserved MiR-138 binding site is shown in Fig. (1B); and the sequence of the conserved binding site for MiR-135 in the FAK-UTR is shown in Fig. (1C). Thus, the binding sites for MiR-138 and MiR-135 were conserved inside the 3'FAK-UTR sequence among different species suggesting their important role in FAK regulation.

Overexpression of MiR-138 and MiR-135 Decreased FAK Protein Expression in Cancer Cell Lines

To test if MiR-138 and MiR-135 overexpression in cancer cell lines affected FAK protein expression, we transfected control pCMV-MiR and pCMV-MiR-138 and MiR-135 plasmids into different cancer cell lines and performed Western blotting with FAK antibody (Fig. 2A). Overexpression of MiR-138 in 5 different cell lines (HeLa cervical carcinoma; SW480 colon cancer; A375 melanoma; PANC-1 pancreatic cancer and 293 epithelial kidney cells, transformed with SV40 cells) decreased FAK expression compared with untreated or with cells transfected with pCMV-Control plasmid, pCMV-MiR-Control cells (Fig. 2A). The same was observed in cancer cells that overexpressed MiR-135 (Fig. 2B, SW40 cells with decreased FAK expression by MiR-135 is shown). We controlled expression of MiR by analyzing the expression of GFP in GFP-containing pCMV-Control, pCMV-MiR-135 and pCMV-MiR-138-transfected cells (Materials and Methods) (Fig. 3A) and RT-PCR with MiR-138 (Fig. 3B) and MiR-135 primers (Fig. 3C). Both pCMV-MiR Control and pCMV-MiR-138 cells expressed GFP (Fig. 3A, PANC-1 cells are shown). The cells that expressed MiR-138 decreased FAK level and changed FAK distribution from the cytoplasmic to the perinuclear area (Fig. 3A, lower panel). The MiR-138 cells changed morphology, became rounded and were less spread (Fig. 3A). Fig. (3B) shows that 293 and HeLa cells, transfected with pCMV-MiR-138 plasmid express a high level of MiR-138 compared with Control pCMV-MiR-transfected cells, which did not express MiR-138 (Fig. 3B). The same result was obtained with MiR-135-transfected cancer cells, where overexpression of MiR-135 decreased FAK expression (Fig. 3C; SW480 cells are shown). Thus, expression of MiR-135 and MiR-138 decreased FAK expression in different cancer cells.

MiR-138 and MiR-135 Directly Bound FAK-UTR

To test the direct binding of MiR-138 to the 3' FAK untranslated region, FAK-UTR, we co-transfected cells with FAK-UTR-Target luciferase plasmid together with either pCMV-MiR Control or pCMV-MiR138 plasmid, and performed a dual-luciferase assay. The FAK-UTR

was cloned down-stream of luciferase sequence in FAK-UTR Target plasmid, where direct binding of MiR-138 to FAK-UTR results in decreased luciferase activity due to decreased translation of luciferase. The dual-luciferase assay showed that MiR-138 directly bound to FAK-UTR, because the FAK-UTR-Target luciferase activity was significantly decreased by pMiR-138 compared to control pCMV vector in 293 cells (Fig. 4A). The same result was obtained in HeLa cells (Fig. 4B). Thus, MiR-138 directly bound FAK-UTR.

To test that pMiR-138 specifically bound the pMiR-138 site in the FAK-UTR, we performed site-directed mutagenesis of the MiR-138 binding site, generated a mutant MiR-138 site, and performed a dual-luciferase assay with either wild type MiR-138 site (FAK-UTR WT-138) or with mutant MiR-138 site (FAK-UTR Mut-138) luciferase plasmids (Fig. 4C). The wild type FAK-UTR-WT site and mutant site FAK-MUT-UTR-138 are shown in (Fig. 4C), right panel. The dual-luciferase assay showed that MiR-138 decreased luciferase activity of FAK-wild type UTR, but it did not decrease the luciferase activity of mutant FAK-MUT-UTR 138 plasmid (Fig. 4C, left panel). Thus, MiR-138 directly and specifically bound to the MiR-138 site in the FAK UTR.

To test the direct binding of MiR-135 to the FAK-UTR, we co-transfected FAK-UTR-Target luciferase plasmid together with pCMV-MiR135 plasmid and found that binding of MiR-135 decreased FAK-UTR luciferase activity compared to control 293 and HeLa cells (Fig. 4A, E, respectively). To test that pMiR-135 specifically bound the pMiR-135 site in FAK-UTR, we performed site-directed mutagenesis of the MiR-135 binding site, generated mutation of the MiR-135 site (Fig. 4F, left panel) and performed a dual-luciferase assay with wild type (FAK-UTR WT-135) and mutant 135 site (FAK-UTR Mut 135) (Fig. 4F, right panel). The dual-luciferase assay showed that MiR-135 bound wild type FAK-UTR and decreased FAK UTR WT-135 activity, but it did not bind FAK-UTR with mutant 135 site and did not decrease luciferase activity of FAK-UTR Mut-135 (Fig. 4F, right panel). Thus, MiR-135 directly and specifically bound to the MiR-135 site in the FAK-UTR. These data show that FAK is a direct target of MiR-138 and MiR-135.

Overexpression of MiR-138 and MiR-135 Decreased FAK mRNA Level in Cancer Cell Lines

To test if MiR-138 and MiR-135 overexpression affected FAKmRNA level, we transfected 293 cells with MiR135 and MiR138, isolated RNA and analyzed FAKmRNA by Real-time PCR. Overexpression of MiR-138 and MiR-135 decreased FAK mRNA in 293 cells (Fig. 4G). The same effect was observed in PANC-1 cells, where MiR-138 and MiR-135 decreased FAKmRNA (Fig. 4H). Thus, Mir-138 and Mir-135 decreased FAKmRNA expression in cancer cells.

Stable Expression of MiR-138 and MiR-135 Decreased Cancer Cell Invasion

To test the functional significance of MiR-138 and MiR-135, we analyzed the effect of MiR-138 and MiR-135 on cell invasion. First, we generated stable 293 (Fig. 5A) and HeLa cell lines (Fig. 5B), transfected with Control pCMV plasmid and with pCMV-MiR-138. The 293 and HeLa cell lines stably transfected with MiR-138 decreased expression of FAK mRNA (Fig. 5A, B, left panels) and protein (Fig. 5A, B, right panels). Both 293 and HeLa cells with stable MiR-138 expression had significantly less invasion compared with pCMV-Control cells (Fig. 5C and D, respectively).

The same effect on cell invasion was observed with stably transfected with MiR-135 293 and HeLa cells. Both stably expressing MiR-135 293 cells (Fig. 5E) and HeLa cells (Fig. 5F) expressed less FAK mRNA (Fig. 5E and F, left panels) and less FAK protein levels (Fig. 5E and F, right panels). Both MiR-135 stable 293 and HeLa cells had significantly less

cell invasion (Fig. 5G and H, respectively). Thus, stable expression of MiR-138 and MiR-135 decreased FAK expression and decreased cancer cell invasion.

MiR-138 and MiR-135 Increased Cancer Cell Sensitivity to Chemotherapy Drugs

To test the effect of MiR-138 and MiR-135 on the chemotherapy drug response in cancer cells, we analyzed the response of 293 and HeLa Control and MiR-138 stably expressing cells to 5-fluorouracil (5-FU) and FAK inhibitor, Y15. The MiR-138 expressing 293 cells treated with 5-FU and Y15 had increased sensitivity to both chemotherapy drugs compared to pCMV-Control-treated cells (Fig. 6A). The same effect was observed in HeLa cells transfected with MiR-138 and treated with FAK inhibitor (Fig. 6B) or doxorubicin (Fig. 6C). The MiR-138 stably expressing HeLa cells with down-regulated FAK by MiR-138 had less viability in response to doxorubicin than Control cells, and the increased sensitivity to chemotherapy was dose-dependent (Fig. 6B, C). MiR-135 expressing HeLa cells also had significantly increased sensitivity to Doxorubicin, 5-FU and FAK inhibitor Y15 (Fig. 6D). Thus, MiR-138 and MiR-135 significantly increased the sensitivity of cancer cells to different chemotherapy drugs.

MiR-138 Decreased Xenograft Tumor Growth *in vivo*

To test the effect of MiR-138 on tumor growth *in vivo*, we injected subcutaneously pCMV-MiR-138 and pCMV-Control 293 cells into the left and right flanks of the same animals and measured xenograft tumor growth. MiR-138 significantly decreased 293 xenograft tumor growth (Fig. 6E, left panel). The MiR-138 xenograft tumors expressed less FAK than pCMV-Control xenografts (Fig. 6E, right panel). The same effect was observed with HeLa xenografts (not shown). Thus, MiR-138 was able to significantly decrease xenograft tumor growth *in vivo*.

Discussion

This report for the first time shows regulation of FAK expression by MiR-135 and MiR-138. FAK mRNA and FAK protein expression were decreased by overexpression of MiR-135 and MiR-138 in different cancer cells. FAK-UTR had one conserved binding site for MiR-138 and MiR-135 at 5' and 3' of the FAK-UTR, respectively. Dual-luciferase assay demonstrated that MiR-138 and MiR-135 directly bound to the FAK-UTR and decreased luciferase activity of FAK-UTR plasmid and that mutation of these binding sites did not decrease FAK-UTR activity due to the absence of binding. In addition, expression of MiR-138 and MiR-135 decreased FAK mRNA and protein level. The 293 and HeLa cells with stable expression of MiR-138 and MiR-135 had significantly less invasion. The 293 and HeLa cells with stable expression of MiR-138 and MiR-135 had increased sensitivity to chemotherapy: 5-FU, doxorubicin, and FAK inhibitor Y15. In addition, MiR-138 significantly decreased xenograft tumor growth *in vivo*.

The data on increased sensitivity of HeLa cancer cells with down-regulated FAK to chemotherapy is consistent with the data on melanoma cancer cells treated with anti-sense FAK oligonucleotides in combination with 5-fluorouracil, where cancer cells were more sensitive to combination therapy [7]. Inhibition of FAK with FAKsiRNA sensitized cells to gemcitabine *in vitro* and *in vivo* in pancreatic adenocarcinoma [8]. Treatment of cells with FAKsiRNA plus docetaxel or platinum inhibited tumor growth more effectively than each agent alone in an ovarian xenograft tumor model [23]. Thus, inhibition of FAK in combination with chemotherapy can be an effective therapy approach to block tumor growth.

The decreased xenograft tumor growth by MiR-138 is consistent with the data on decreased MCF-7 xenograft tumor growth by FAK siRNA [10] or with data on inhibition of breast, neuroblastoma and pancreatic xenograft tumor growth by FAK small molecule inhibitor [13, 24, 25]. While MiR-138 was able to significantly decrease xenograft tumor growth, the effect of MiR-135 was not significant (not shown) suggesting different *in vivo* mechanisms that will be interesting to study. It can indicate that targeting the 5' end of FAK-UTR by MiR-138 is more effective than more distant 3' end of FAK-UTR by MiR-135 (Fig. 1A). Based on increased sensitivity to chemotherapy *in vitro*, the combination therapy can be applied with MiR-135 to more effectively decrease tumor growth *in vivo*.

This report shows the novel regulation of FAK in cancer cells and reveals a new biological function of two microRNAs: MiR-135 and MiR-138. MiR-138 is one of the most frequently down-regulated miRNA's in cancer [26]. The 293 and HeLa cells expressed a low amount of endogenous MiR-138 and MiR-135 RNAs, while they expressed a high FAK level, and overexpression of MiR-138 and MiR-135 in cells caused decreased FAK mRNA and protein levels in these cells. MiR-135b gene was shown to be altered (either amplified or deleted) in 23% of medulloblastomas that could affect gene expression in cancer [27]. The 293 and HeLa cancer cells with overexpressed MiR-138 and MiR-135 had decreased invasion, which is consistent with a recent report on the role of MiR-138 in the suppression of cell invasion in cell and neck squamous cell carcinoma cell lines [28]. It was also shown that Micro RNA-138 suppressed epithelial mesenchymal (EMT) transition in these cells [29]. In addition, MiR-138 decreased xenograft tumor growth *in vivo*. FAK is known to play an important role in cell motility, invasion and angiogenesis [5] and targeting FAK with MiR-138 and MiR-135 leading to decreased cell invasion can be important for cancer cell therapy and inhibition of metastases.

The cancer cells with overexpressed MiR-138 and MiR-135 had increased sensitivity to chemotherapy. The recent report demonstrated that MiR-138 inhibited homologous recombination and enhanced cell sensitivity to cisplatin, camptothecin and ionizing radiation [30]. Our report provides a novel mechanism of increased drug sensitivity by down-regulation of FAK that is critical for cell survival. Thus, MiR-138 and MiR-135 can be additional therapeutic agents to decrease cancer survival pathways.

In conclusion, this report demonstrates targeting and down-regulation of FAK expression by MiR-135 and MiR-138 that provides a new mechanism and function of MiR-135 and 138 in cancer cells that is important for the fields of microRNA and FAK biology and for developing anti-cancer therapeutic approaches.

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Abbreviations

MiR	microRNA
RNA	ribonucleic acid
FAK	Focal Adhesion Kinase
RT-PCR	Real-time PCR
PCR	polymerase chain reaction
UTR	untranslated region

Fig. (1). A. The scheme of FAK 5'UTR, coding and 3'UTR sequences

The FAK sequence from NCI GeneBank database NM_005607 is shown. The Target Scan software shows that FAK 3'UTR contains two binding sites for MiR-138 and MiR-135. The miR-138 binds 8 bases: (56-63) and MiR-135 binds 7 bases (820-826) in the 5' and 3' end of FAK 3'UTR sequence, respectively. **B.** The conservative MiR-138 (B) and MiR-135b (C). sites are shown. The MiR-135b and MiR-138 sites are conserved in at least 12 different species. Several species are shown.

Fig. (2). MiR-138 and MiR-135 decreased FAK protein expression in cancer cell lines

A. MiR-138 decreased FAK expression. Different cancer cell lines were transfected with pCMV-MiR-Control and pCMV-138 plasmids for 24 hours and Western blotting with FAK antibodies was performed. The MiR-138 decreases FAK expression in pCMV-MiR-138-transfected cells compared with pCMV Control cells. HeLa, SW480, A375, PANC-1 and 293-T cells are shown. Beta-Actin was used as a control of equal loading. **B. MiR-135 decreased FAK expression.** SW480 cancer cells were transfected with MiR-135 and FAK expression was analyzed by Western blotting after 24 hours, as above. FAK is decreased by MiR-135 overexpression in SW480 cells.

Fig. (3). (A) Mir-138 was expressed in cells transfected with pCMV-138 plasmid and decreased FAK expression and localization in PANC-1 cells

To control expression of MiR-138, we monitored it by GFP expression, as pCMV-MiR plasmid contained GFP sequence (A) and by RT-PCR with MiR-138 primers. Both cells pCMV-MiR and pCMV-MiR-138 expressed GFP. FAK expression was decreased by MiR-138 and localization was changed from the cytoplasm to the perinuclear region in PCMV-MiR-138 transfected cells compared with pCMV-Control cells.(B). MiR-138 was expressed in cells transfected with pCMV-MiR-138 plasmid. Real-time PCR with MiR-138 primers was performed in 293 and HeLa cells, transfected with pCMV-138 plasmid, as described in Materials and Methods. RT-PCR showed a high expression of MiR-138 in the cells transfected with pCMV-MiR-138 compared with pCMV-Control-transfected or untransfected cells. The bars represent the average of three independent experiments $\pm$

standard errors. $P < 0.05$, pCMV-MiR-138 versus pCMV-Control cells, Student's t-test. (C). **MiR-135 was expressed in cells transfected with pCMV-MiR-135 plasmid.** The same RT-PCR experiment as in Fig. (3B) with MiR-135 primers was performed and showed a high expression of MiR-135 in 293 cells transfected with pCMV-MiR-135 plasmid in contrast with untreated (cells with no plasmid transfection) and pCMV-Control cells. The bars represent the average of three independent experiments $\pm$ standard errors. $P < 0.05$, CMV-MiR-138 versus pCMV-Control cell, Student's t-test.

Fig. (4). Dual-luciferase assay and site-directed mutagenesis showed that MiR-138 and MiR-135 directly and specifically targeted FAK-UTR. A, B. MiR-138 directly bound FAK-UTR The 293 (A) and HeLa (B) cells were co-transfected with FAK-UTR-Target luciferase plasmid and pCMV-MiR 138 plasmid or pCMV-Control plasmid with Renilla plasmid for normalization. The dual-luciferase assay showed that MiR-138 directly bound FAK-UTR and decreased luciferase activity of the FAK-UTR-Target luciferase in 293 and HeLa cells. **C. The site-directed mutagenesis of MiR-138 site in FAK-UTR sequence blocked binding of MiR-138 to FAK-UTR.** Left panel: Mutation (underlined) is shown in FAK-MU-UTR-138 sequence and compared with wild type sequence. Right panel. The mutation of the binding site for MiR-138 reversed inhibition of FAK-UTR activity by MiR-138. The bars represent the average of three independent experiments. The data are expressed relative to pCMV-Control cells. *, $p < 0.05$, MiR-138 versus pCMV-Control. Student's t-test. **D, E. MiR-135 directly and specifically bound FAK-UTR.** The pCMV-MiR135 was co-transfected with FAK-UTR Target luciferase plasmid into 293 (D) or HeLa cells (E) with Renilla plasmid and dual-luciferase assay was performed, as described in Materials and Methods. The dual-luciferase assay shows that MiR-135 directly bound FAK-UTR and the luciferase activity of FAK-UTR-Target luciferase was significantly decreased in 293 and HeLa cells compared with Control cells. *, $p < 0.05$, pCMV-MiR-138 versus pCMV-Control with FAK-UTR WT-138. * $p < 0.05$, Student's t-test. **F. The site-directed mutagenesis of MiR-135 site in FAK-UTR sequence blocked binding of MiR-135 to FAK-UTR.** Left panel: Mutations (underlined) are shown in FAK-MU-UTR-135 sequence and compared with wild type sequence. The mutation of the binding site for MiR-135 reversed inhibition of FAK-UTR activity by MiR-135 in 293 cells (right panel). The data are normalized to pCMV-Control. The bars represent the average of three independent experiments $\div$ standard errors. * $p < 0.05$, MiR-135 versus pCMV- Control with FAK-UTR WT-135. Student's t-test. **Fig. 4 G, H. MiR-138 and MiR-135 inhibited FAKmRNA expression in cancer cells.** The

MIR-138 and MiR-135 were overexpressed in 293 (G) and PANC-1 cells (H) and Real-time-PCR with FAK primers was performed with RNA from the cells transfected with MiR-138 or with CMV-Control plasmids cells. MiR-138 and MiR-135 significantly inhibited FAK mRNA expression in 293 (G) and PANC-1 (H) cells. * $p < 0.05$ MiR-138 or MiR-135 versus pCMV-Control cells, Student's t-test.

Fig. (5). MiR-138 decreased FAK mRNA and protein expression in stable 293 and HeLa cells
 A. The stable 293 (A) and HeLa (B) cells with pCMV-MiR Control or pCMV-MiR-138 expression were generated with G418, as described in Materials and Methods. 293 and HeLa cells with stable MiR-138 expression down-regulated FAK mRNA (left panels) and protein levels (right panels) in 293 and HeLa cells (A,B). Beta-actin was used as a normalization control of equal protein loading. **C, D. MiR-138 expression decreased cancer cell invasion.** The stably MiR-138 expressing 293 (C) and HeLa (D) cells were analyzed on a Boyden chamber with an Invasion Kit, as described in Materials and Methods. Both cell lines with stable MiR-138 expression had decreased invasion. Blank shows control with no

cells. * $p < 0.05$, pCVM-MiR-138 versus pCMV- Control cells. Student's t-test. **E, F. MiR-135 decreased FAK mRNA and protein levels in stable 293 and HeLa cells.** The stable 293 (E) and HeLa (F) cells were analyzed by RT-PCR and Western blotting to detect FAK expression, as described in 5A,B. **G, H. MiR-135 decreased cell invasion.** The experiment was performed, as described in Fig. (5C, D). * $p < 0.05$, pCVM-MiR-135 versus pCMV- Control cells. Student's t-test.

Fig. (6). MiR-138 and MiR-135 increased sensitivity to chemotherapy

A. The pCMV-Control and pCMV-MiR-138 and MiR-135 stable 293 cells were treated with 5-FU or with FAK inhibitor Y15 [13] (Materials and Methods) and an MTT viability assay was performed 24 hours after treatment. The MiR-138 and MiR-135 stable 293 cells were more sensitive to 5-FU and Y15 than control vector cells. The structures of 5-FU and Y15 is shown below treatments. *, $p < 0.05$, Student's t-test. **B, C.** MiR-138 increased sensitivity to FAK inhibitor Y15 and doxorubicin in a dose-dependent manner in HeLa cells. The pCMV-Control and pCMV-MiR-138 cells were treated with different doses of Y15 or doxorubicin for 24 hours and an MTT assay was performed. The structure of doxorubicin is shown.

* $p < 0.05$, pCVM-MiR-138 versus pCMV- Control cells. Student's t-test. **D.** MiR-135 increased sensitivity to FAK inhibitor Y15, 5-FU or doxorubicin. Bars are averages $\pm$ standard errors of three independent experiments. * $p < 0.05$, pCVM-MiR-135 versus pCMV- Control cells Student's t-test. **E. MiR-138 significantly decreased 293 xenograft tumor growth.** Left panels: pCMV-MiR-138 and pCMV-Control 293 stable cells were injected into the left and right flanks subcutaneously and xenograft tumor growth was measured, as described in Materials and Methods. MiR-138 significantly decreased 293 xenograft tumor growth compared with Control cells. * $p < 0.05$, Student's t-test. Right panels. Right panel: MiR-138 decreased FAK expression in xenograft samples. The representative tumors showed decreased FAK expression in pCMV-MiR-138 xenograft tumors compared with pCMV-Control xenograft tumors.