MR-1 Modulates Proliferation and Migration of Human Hepatoma HepG2 Cells through Myosin Light Chains-2 (MLC2)/Focal Adhesion Kinase (FAK)/Akt Signaling Pathway*

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The key of cell migration process on solid substrates is phosphorylation of myosin light chain-2 (MLC2), which is implicated in a variety of intracellular functions. The previous data show that MLC2 interacts with a novel human gene, myofibrillogenesis regulator 1 (MR-1). Here, we reported that MR-1 was specially overexpressed in human hepatoma HepG2 cells. Transient treatment of cells with small interfering RNA (siRNA) against MR-1 or stable transfection of cells with plasmid expressing MR-1-siRNA led to inhibitions of cell proliferation, migration, and adhesion. Following down-regulation of MR-1, the phosphorylations of MLC2, focal adhesion kinase (FAK), and Akt were dramatically decreased, and the formation of stress fiber was destroyed by MR-1-siRNAs in hepatoma HepG2 cells. In addition, exogenous MR-1-induced as well as inherent phosphorylations of FAK and Akt were decreased by MLC kinase (MLCK) inhibitor, and F-actin polymerization inhibitor also decreased phosphorylations of FAK and Akt. Correspondingly, MR-1-enhanced migration of cells was also inhibited by these two inhibitors. These indicated that MLC2 activation and intact actin cytoskeleton were pivotal for MR-1 function. In vivo data showed that MR-1-siRNA markedly inhibited growth of human HepG2. This study suggested that overexpression of MR-1 was associated with cancer cell proliferation and migration through MLC2 and that MR-1 might be a potential cancer therapeutic target.

Cancer progression from a primary tumor to secondary metastasis is a highly complex process involving alteration of gene expression, acquisition of cell motility, interaction with extracellular matrix (ECM), and change in cell adhesion, and expression of ECM-degrading protease (1). Cell migration is a critical step in tumor metastasis. Cancer cells move within tissues during invasion and metastasis by their own motility, and cell migration involves multiple processes that are regulated by various signaling molecules (2). It results from a dynamic interplay between the substrate and cytoskeleton protein located at the focal adhesion complex (3).

It is known that cells exert force propelling the cell forward by contraction of the actin cytoskeleton through activation of myosin II (4). The actin-myosin II interaction in non-muscle cells is regulated by the phosphorylation of MLC2 at serine-19 (5). MLC2 dephosphorylation can induce apoptosis (6), and inhibitor of MLCK can abrogate MLC2 phosphorylation, cell polarization, and migration (7). MLC2 is also involved in the activation of mid-G1 phase cyclin D1 expression (8, 9). It has been reported that hyperphosphorylated MLC2 induces stress fiber formation and integrin clustering that link cell surface cytoskeletal proteins such as FAK to actin (10, 11). FAK is a member of the focal adhesions that mediates integrin-mediated signal transduction associated with a variety of cellular functions including cellular proliferation, migration, and adhesion (12). Downstream signaling pathways implicated in FAK signaling include the Jun N-terminal kinases (JNK) survival pathway that inactivates the tumor suppressor p53 regulating cell death pathway (13), the death-associated protein kinase (14), and the phosphoinositide 3-kinase/Akt pathway regulating cell viability (15, 16).

Previously, we have identified a novel human gene, MR-1, from a human skeletal muscle cDNA library (GenBank™ accession number AF417001) (17). MR-1 is located on human chromosome 2q35 (GenBank accession number AC021016), and there are three alternatively spliced forms of MR-1 encoding three isoforms (18). MR-1 is composed of three distinct exons, in which exon 3 is unique when compared with other two genes, and encodes a 142-amino acid protein with a hydrophobic transmembrane structure between amino acids 75 and 92. Yeast two-hybrid screening and in vitro GST pulldown revealed that MR-1 protein interacts with three proteins involved in muscle contraction such as MLC2 (17), indicating that MR-1 might be associated with cell migration and growth process through MLC2.

In this study, we investigated whether MR-1 was related to the proliferation and migration of human cancer cells. We observed that MR-1 was overexpressed in human cancer cells.
Our data demonstrated for the first time that the inhibitions of proliferation, migration, and adhesion of hepatoma HepG2 cells by siRNA against MR-1 were associated with the interdiction of MLC2/FAK/Akt signaling pathway.

**EXPERIMENTAL PROCEDURES**

**SiRNA Preparation and Treatment**—21-Nucleotide siRNAs were synthesized by Ribo Technology Company (Beijing, China) using 2′-ACE protection chemistry. Two siRNA sequences targeting MR-1 were 5′-ACC GUG UGA AGC AGA UGA AdTdTT-3′ and 5′-CUU AGG CUA UUG ACU GUU AdTdTT-3′, and the mock siRNA sequence was 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′. Human hepatoma HepG2 cells were cultured in the MEM-EBSS medium (Invitrogen) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2. A t2 4h to test for MR-1 level of HepG2/MR-1 cultured for experiments. RT-PCR and Western blot were used.

**Experimental Procedures**

**Transfection and Selection of Stably Transfected HepG2/MR-1 Cells**—HepG2 cells at 70 – 80% confluence were transfected with 2 μg of pCD-MR-1, pCD-mock, pcDNA3.1-MR-1, and pcDNA3.1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For stable transfection, the HepG2 cells were treated with MLCK inhibitor ML-7 (Cell Signaling Technology, Beverly, MA) or F-actin polymerization inhibitor cytochalasin D (Sigma) for 24 h.

**Construction of siRNA-expressing and MR-1-expressing Plasmid**—The DNA sequence to knock down expression of MR-1 was 5′-CCT AGG CTA TGT ACT GTT A TTCAA-3′ and the mock sequence was 5′-TTT TTC TCC GAA GTT CGT ACG T TTCAA-3′. pcDNA3.1 (Invitrogen). was reconstructed from pcDNA3.0 (Invitrogen) in our laboratory, in which cytomegalovirus promoter was cut off and the expression was based on H1 promoter. The above mentioned DNA sequences were ligated into pCD-shRNA. The above mentioned DNA sequences were ligated into pCD-shRNA. The above mentioned DNA sequences were ligated into pCD-shRNA.

**RT-PCR Analysis**—Total mRNA was extracted from the cells by TRIzol reagent (Invitrogen) with an extra step of acid phenol extraction. RT-PCR was carried out using a SuperScript™one-step RT-PCR kit (Invitrogen) as described previously (19). Oligonucleotide primers used were as follows: MR-1 P1, 5′-TAT CCT CCT CTT CAT CCT CAC C-3′; MR-1 P2, 5′-AGG CAC GAA CTG GAA TCT GG-3′; GAPDH P1, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′; GAPDH P2, 5′-GTC TTC ACC ACC ATG GAG AAG GCT G-3′; β-actin P1, 5′-CCC AGG AAC CAG GGC GTG ATG GT-3′; β-actin P2, 5′-GGA CTC CAT GCC CAG GGA A-3′. GAPDH and β-actin mRNA was analyzed as internal control. A measure of 1 μg of total RNA was reverse-transcribed to synthesize cDNA at 50 °C for 30 min, and then the cDNA was subjected to PCR amplification with specific primers in 25-μl mixtures. PCR comprised 30 cycles with denaturing at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 40 s in each cycle using an MJ PCR system (Bio-Rad). The PCR products were then subjected to 2% agarose gel electrophoresis.

**Quantitative Real-time RT-PCR Analysis**—Quantitative real-time RT-PCR was performed using specific sense and anti-sense primers in a 25-μl reaction volume containing 12.5 μl of Absolute™QPCR SYBR Green mix (Invitrogen), 0.25 pmol of each primer, and 0.5 μg of mRNA. Oligonucleotide primers used were as follows: for MR-1 P1, 5′-cag cgg gga cga tga tga ga-3′; MR-1 P2, 5′-ctg ggc ccc tga gga c-3′; CLCA1 P1, 5′-TCA TCA GGA AAT GGA GCT GTC-3′; CLCA1 P2, 5′-TCA TCA GGA AAT GGA GCT A-3′; KIAA1486 P1, 5′-GCT GAG CAC CTC ATC AGA G-3′; KIAA1486 P2, 5′-GGG TCT TGG TTG AGC TGA CG-3′; IMMT P1, 5′-GGA TAT AAA TAC TGC TCA TGC CAG A-3′; IMMT P2 5′-CTT CCT CTG CAG CAA CTG CAT-3′. The amplification number of cycles was 40, and the reaction took place for 3 min at 50 °C, 15 s at 95 °C, and 30 s at 63 °C, with an initial step of 95 °C for 3 min.

**Cell Migration Assays**—Cell migration was measured as the ability of cells to migrate through a Transwell filter (8-μm pores, Costar, Cambridge, MA). Cells suspended in serum-free MEM-EBSS containing 0.1% bovine serum albumin were applied to the upper chamber. MEM-EBSS containing 20% fetal bovine serum and 10 μg/ml fibronectin (FN) was added to the lower chamber. After the cells were incubated for 37 °C for 3 h, the cells that migrated to the lower side of the upper chamber were stained with hemotoxylin, and the number of cells per microscopic field (×300) was counted under microscope.

**Cell Adhesion Assay**—Cells were washed in serum-free MEM-EBSS containing 0.2% trypsin inhibitor and resuspended in culture medium. 100 μl of suspended cells was added to each well of 96-well plates coated with 10 μg/ml FN and blocked with 1 μg/ml bovine serum albumin. The plates were incubated for the appropriate periods of time at 37 °C in CO2 incubator.
Non-adherent cells were removed by washing with phosphate-buffered saline, and attached cells were analyzed by 3-(4,5-di-
methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

3-(4,5-di-
methylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assay was processed as described previously (20). For Western blot assay, cells were allowed to adhere to FN-coated plates for 2 h, and then proteins were collected.

Analysis of Cell Spreading Morphology—Cells were suspended for 1 h in serum-free MEM-EBSS containing 1% bovine serum albumin and then replanted on 10 μg/ml FN (Merck)-coated 96-well plates. Cells were allowed to spread for the indicated times at 37 °C and then photographed using a Nikon upright scope equipped with a camera.

F-actin Cytoskeleton Organization Detection—Cells were planted in six-well plates on glass coverslips. After the indicated treatment, cells were fixed in 3.7% paraformaldehyde for 20 min. Fixed cells were washed twice with phosphate-buffered saline, permeabilized by treatment with 0.5% Triton X-100 in phosphate-buffered saline for 5 min, and then stained with 5 μg/ml rhodamine-conjugated phalloidin (Merck) in phosphate-buffered saline for 10 min in the dark. Pictures were taken with Nikon fluorescence microscope.

Cell Proliferation and Tumorigenicity Assay—For cell proliferation assay, 1 ml of cells was seeded at a density of 1000/ml in 24-well plates with 10% fetal bovine serum medium. The medium was changed regularly. The cell number was counted every 24 h for 6 days. For tumorigenesis assay, various cells (5 × 10^3) were injected subcutaneously into the right flank of nude mice. Bidimensional tumor measurements were made every 4 days, and the average of these measurements was used to calculate tumor volume.

Statistical Analysis—Data are expressed as the arithmetic mean ± S.D. Statistical analysis was performed using the t test. p < 0.05 was considered statistically significant.

RESULTS

Overexpression of MR-1 in Human Cancer Cells—To examine whether MR-1 was particularly expressed in human cancer cells, we first measured mRNA levels of MR-1 by RT-PCR assay in various human cancer cell lines, including liver cancer (HepG2, SMMC-7721, and BEL-7402), breast cancer (MCF-7), colon cancer (HT-29 and HCT116), fibrosarcoma (HT1080), and lung cancer (A549, PG, and PAA) cells, and in normal human cell lines, including liver L02 cells, lung fibroblast 2BS cells, and human umbilical vein endothelial cells. As shown in Fig. 1A, mRNA levels of MR-1 in human cancer cells were higher than in human normal cells, indicating that MR-1 might be an oncogene. Furthermore, a Western blot assay showed that protein levels of MR-1 were higher in hepatoma cells when compared with normal cells (Fig. 1B). Both mRNA and protein levels of MR-1 were superlatively high in hepatoma HepG2 cells. In the rest of this study, we chose hepatoma HepG2 cells to validate MR-1 as a therapeutic oncogene.

Inhibition of Cell Proliferation by Down-regulation of MR-1—RNA interference technology has been successfully used to identify gene functions. To avoid the off-target effects, we designed and synthesized two siRNAs against MR-1, MR-1-siRNA-1 and MR-1-siRNA-2, to investigate the function of MR-1. RT-PCR and Western blot analysis showed that transcription and expression of MR-1 in HepG2 cells were dramatically down-regulated by both MR-1-siRNAs after 36 h transfection (Fig. 2A). In the next experiment, we found that following down-regulation of MR-1, the ability of cell proliferation was obviously decreased in MR-1 siRNA-treated cells when compared with control and mock siRNA-treated cells (Fig. 2B).

Furthermore, MR-1-siRNA-2 silence sequence were ligated into an expression plasmid to create an expressing MR-1-siRNA plasmid pCD-MR-1, and at the same time, a negative control plasmid pCD-mock was also created. Here, we investigated whether MR-1-siRNA-2 sequence expressed by pCD-MR-1 has off-target effects on the other genes. A BLAST search of MR-1-siRNA-2 sense chain and antisense chain in the National Center for Biotechnology Information (NCBI) was performed. The results showed that there were two transcripts, chloride channel regulator (CLCA1) and KIAA1486, complementary to the MR-1-siRNA-2 sense chain with query coverage of 73%, and three transcripts of inner membrane protein, mitochondrial (IMMT) complementary to the MR-1-siRNA-2 antisense chain with query coverage of 63%. The real-time RT-PCR assay showed that mRNA level of MR-1 was significantly decreased after a 36-h treatment of MR-1-siRNA-2, whereas CLCA1, KIAA1486, and IMMT were no changed (Fig. 2C), indicating that MR-1-siRNA-2 was specific for MR-1 knockdown without off-target effects and was suitable for the following experiments.

To generate HepG2/MR-1 cells with stable knockdown of MR-1, pCD-MR-1 and pCD-mock were stably transfected into HepG2 cells, respectively. RT-PCR and Western blot assays showed that transcription and expression of MR-1 were knocked down in both HepG2/MR-1-sc and HepG2/MR-1-mc cells (Fig. 2D). It was observed that the ability of cell proliferation was markedly reduced in HepG2/MR-1-sc. In addition, HepG2/MR-1-mc cells also showed decreased proliferation ability when compared with parental cells (Fig. 2E). These data suggested that MR-1 might play an essential role in cancer cell growth.

Inhibition of Cell Migration, Adhesion, and Spreading by Down-regulation of MR-1—It is well known that contraction of the actin cytoskeleton through activation of myosin II exerts force to propel cells to move. The actin–myosin II interaction is regulated by the phosphorylation of MLC2 at Ser-19 (4). Based on the interaction of MR-1 and MLC2, we suppose that MR-1

FIGURE 1. Analysis of MR-1 mRNA and protein levels. The logarithmic growth cells were harvested, and the mRNA and protein levels were measured by RT-PCR (A) and Western blot (B) in the indicated cell lines. GAPDH and β-actin served as loading controls.
probably affect cell migration. The results showed that MR-1-siRNA-1 and MR-1-siRNA-2 both significantly reduced the migration of HepG2 cells with inhibitory rate of 52.9 and 62.7% (Fig. 3A). Similarly, the ability of the migration of MR-1 knockdown HepG2/MR-1\(^{-}\)-sc and HepG2/MR-1\(^{-}\)-mc cells was retarded when compared with that of parental and mock cells with inhibitory rates of 56.6 and 39.5% (Fig. 3B).

Cell adhesion and spreading on ECM is a key step in cell migration process. As the cells adhere and spread, they generate tractions and thereby migration on the substrate. As shown in Fig. 3C, the cell adhesion ability on FN was decreased in MR-1-siRNA-treated HepG2 cells. Similarly, HepG2/MR-1\(^{-}\)-sc and HepG2/MR-1\(^{-}\)-mc cells also partially lost adhesion ability (Fig. 3D). To test cell spreading efficiency, cells were plated on FN-coated cell culture dishes. A striking difference in the appearance of cell spreading on FN was observed between HepG2/ MR-1\(^{-}\)-sc and parental cells. HepG2 and HepG2/mock-sc cells began to spread at 60 min and were normally flattened at 120 min, whereas HepG2/MR-1\(^{-}\)-sc cells exhibited a delayed spreading and a limited extension on FN (Fig. 3E). These results confirmed that MR-1 was important for cell migration, adhesion, and spreading.

Phosphorylation of MLC2, FAK, Akt, and Stress Fiber Formation by MR-1—To clarify the mechanism of action of MR-1, we examined whether the phosphorylation of MLC2 at Ser-19 was affected by MR-1. As shown in Fig. 4A, after treatment of MR-1-siRNAs, MLC2 phosphorylation was reduced. It is known that phosphorylation of MLC2 controls myosin II activity (21) and subsequent organization of the actin stress fibers
Therefore, rhodamine-conjugated phalloidin was used to stain the cells for measurement of F-actin (23). The results of the fluorescent image in Fig. 4B showed abundances of organized stress fibers in control and mock siRNA-treated cells. By contrast, MR-1-siRNA-treated HepG2 cells displayed mass spots of F-actin staining and no organized stress fiber formation, and there were numerous microspikes at the cell periphery (Fig. 4B). The finding indicated that MR-1 could strengthen the formation of actin stress fibers.

Based on the above results, we further detected phosphorylation of FAK, a key kinase in the formation of focal adhesions, which is dependent upon activation of myosin II (24). The data showed that the phosphorylation of FAK at Tyr-925, a representation of FAK activity, were both dramatically decreased in MR-1-siRNA-1-treated and MR-1-siRNA-2-treated cells (Fig. 4A), indicating the stimulating function of MR-1 on FAK activity. Our results also showed that Tyr-473 phosphorylation of Akt, a survival signaling factor activated by FAK (25), was decreased in MR-1-siRNA-treated cells (Fig. 4A). At the same time, the levels of total MLC2, FAK, and Akt were kept invariant.

As shown in Fig. 4C, the phosphorylations of FAK and Akt were dramatically down-regulated in the suspension cells. Cell adhesion on FN stimulated the phosphorylations of FAK and Akt, which were also significantly inhibited by MR-1-siRNA-1 and MR-1-siRNA-2. The levels of total FAK and Akt were kept invariant in various cells (Fig. 4C). These results showed that MLC2, FAK, and Akt might be involved in MR-1-regulated adhesion and migration.

Initiation of MLC2-FAK-Akt Pathway by MR-1—To investigate the MR-1-mediated signaling pathway, we further tested the key molecules to be involved. The results showed that exogenous expression of MR-1 by transfection of pcDNA3.1-MR-1 enhanced phosphorylations of MLC2, FAK, and Akt, which were blocked by MLCK inhibitor ML-7. Here, MR-1 levels were not affected by ML-7 treatment, indicating that MLC2 was downstream of MR-1 and upstream of FAK and Akt (Fig. 5A).

Furthermore, to test whether the formation of stress fibers stimulated the activation of FAK, we examined the activations of FAK and Akt using specifically the actin polymerization inhibitor cytochalasin D. Fig. 5B showed that the phosphorylations of FAK and Akt were inhibited by cytochalasin D. However, MLC2 phosphorylation was increased. These data indicated that the integrity of the actin cytoskeleton was an essential upstream factor for FAK activation and subsequent Akt activation in the MR-1-mediated signaling pathway. In addition, exogenous MR-1 enhanced the migration ability of cells by 28.77%, which was blocked by ML-7 and cytochalasin D (Fig. 5C). This result also supported the fact that MLC2 and stress fibers functioned as key downstream factors of MR-1.

In Vivo Reduction of HepG2/MR-1−/−Tumorigenicity—The above data raised a possibility that hepatoma HepG2/MR-1−/−sc was not easily formed and developed in the animal model. To validate this possibility, human hepatoma HepG2, HepG2/
HepG2/mock-sc and HepG2/MR-1 grew quickly (Fig. 6A). Tumor masses resected from mice on day 20, the average size of HepG2, HepG2/mock, and HepG2/MR-1 cells were significantly decreased by the inhibition of MR-1 expression and stress fiber formation (Figs. 4, 5A, and 5B), which we think is attributed to the inhibition of MLC2 phosphorylation, consistent with other reports that MLCK inhibition can prevent the formation of focal adhesions in human umbilical vein endothelial cells (30), and the inhibition of myosin II function can suppress PC cell migration and adhesion (31). When MR-1 is knocked down stably by the transfection of MR-1-siRNA-expressing plasmid and cell spreading on FN are reduced (Fig. 3E), suggesting a defect in extending forward to form surface contacts with matrix. These results are noticeable since during migration, cells undergo morphologic changes involving extension of lamellipodia along the extracellular matrix, the formation of focal adhesion complexes, and translocation of the cell body (32). It is detected that MLC2 phosphorylation is also decreased in HepG2/MR-1 cell (data not shown), which is consistent with the report that MLC2 phosphorylation is critical for efficient spreading on fibronectin (50). It has been reported that myosin II is one of the most important cytoskeletal components and can generate force of cell motility as an intracellular motor (32). Here, we also find that the formation of stress fibers is broken by MR-1-siRNAs (Fig. 4B), indicating that cell contraction force is impaired.

The phosphorylation of MLC2 is regulated by two classes of enzymes: MLCK, which phosphorylates MLC2 and promotes its activity, and MLC2 phosphatase, which dephosphorylates MLC2 and inhibits its activity (33, 34). Several kinases have been shown to affect MLC2 phosphorylation: MLCK, P21-activated kinase (PAK), myotonic dystrophy kinase-related cdc 42-binding kinase (MRCK), citron kinase, zipper-interacting protein (ZIP) kinase, integrin-linked kinase, and Rho kinase (ROCK) (35–37). In our study, ML-7 can block the stimulatory effect of MR-1 on phosphorylation of MLC2, suggesting that function of MR-1 on MLC2 may be dependent on MLCK activity. Recent clinical study of non-small cell lung cancer patients found a significant positive correlation between expression levels of MLC2 and likelihood of disease recurrence and metastasis (38), indicating that excessive myosin II activation could be a contributing factor to metastasis. These data suggest that MR-1 might be a novel oncogene.

We furthermore provide MR-1-mediated signal factors involved in cell migration and proliferation process. In various human cancers, FAK signaling is a key pathway related to cancer cell invasion and migration (39). FAK can serve as a scaffold or adaptor to recruit a number of structural and signaling molecules to promote cell adhesion (40). The lack of phosphorylation on FAK Tyr-925 is related to an inability of cells to extend their cellular protrusions associated with cell-matrix adhesion (41). In our study, the phosphorylation of FAK at Tyr-925 is

**FIGURE 6. Tumorigenicity of HepG2/MR-1−/−sc is reduced in nude mice.** A, xenograft tumors were derived from HepG2, HepG2/mock-sc, and HepG2/MR-1−/−sc cells. The mice were sacrificed at 20 days after injection. B, tumor volume was measured for the indicated times after subcutaneous implantation of various hepatoma cells. Each point represents the mean of six mice with S.D.

**DISCUSSION**

After the completion of human genome sequencing and significant advances in genomics and proteomics, discovering the oncogene for therapeutic intervention of cancer remains as a future challenge. The present study demonstrates that MR-1 may be a novel human oncogene overexpressed in human cancer cell lines (Fig. 1). In vitro study showed that the proliferation, migration, and adhesion of hepatoma HepG2 cells are strongly inhibited by down-regulation of MR-1 either stably or transiently, using RNA interference technology. Furthermore, in vivo study further proves that MR-1 is essential for tumorigenicity. These results suggest that MR-1 is a potential tumor biomarker and therapeutic target.

Understanding the signaling molecules involved in MR-1 bioactivity is important for targeting therapeutic purposes. Previous data have shown that MR-1 protein interacts with MLC2 (17). Our data show that phosphorylation of MLC2 is significantly decreased by the inhibition of MR-1 expression and stimulated by exogenous MR-1 (Figs. 4A and 5A). Moreover, the effect of MR-1 on migration appears to be mediated by MLC2, based on the ability of the MLCK inhibitor ML-7 to block the stimulatory effect of exogenous MR-1 on HepG2 migration (Fig. 5A). Therefore, we identify MLC2 as a key mediator of MR-1 bioactivity. It has been known that phosphorylation of MLC2 controls the activity of myosin II, which has multiple functions in cells, including stimulation of cell motility as a key component of focal adhesion formation and stress fiber formation (24, 26–28) and prevention of apoptosis as a cell survival regulator (29). Moreover, our data suggest that the inhibition of MR-1 expression suppresses HepG2 cell adhesion (Fig. 3, C and D), which we think is attributed to the inhibition of MLC2 phosphorylation, consistent with other reports that MLCK inhibition can prevent the formation of focal adhesions in human umbilical vein endothelial cells (30), and the inhibition of myosin II function can suppress PC cell migration and adhesion (31). When MR-1 is knocked down stably by the transfection of MR-1-siRNA-expressing plasmid and cell spreading on FN are reduced (Fig. 3E), suggesting a defect in extending forward to form surface contacts with matrix. These results are noticeable since during migration, cells undergo morphologic changes involving extension of lamellipodia along the extracellular matrix, the formation of focal adhesion complexes, and translocation of the cell body (32). It is detected that MLC2 phosphorylation is also decreased in HepG2/MR-1 cell (data not shown), which is consistent with the report that MLC2 phosphorylation is critical for efficient spreading on fibronectin (50). It has been reported that myosin II is one of the most important cytoskeletal components and can generate force of cell motility as an intracellular motor (32). Here, we also find that the formation of stress fibers is broken by MR-1-siRNAs (Fig. 4B), indicating that cell contraction force is impaired.

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significantly decreased in HepG2 cells treated with two MR-1-siRNAs. Previous studies have shown that the spreading ability on FN of FAK-deficient cells is impaired (42–44). Our data also suggest that alterations of HepG2/MR-1-sc cells spreading on ECM are related to dephosphorylation of FAK induced by MR-1 knockdown.

Cell adhesion to ECM can generate transmembrane signals originating from focal adhesions. It is known that the phosphoinositide 3-kinase/Akt pathway is involved in FAK signaling and regulates cell viability (15, 16). Activated Akt is a key downstream survival factor that stimulates cell proliferation and inhibits apoptosis, whereas aberrant activation of the phosphoinositide 3-kinase/Akt pathway by any number of mechanisms may contribute to increased tumor invasiveness and cancer progression. After the phosphorylation of Akt at Ser-473 is inhibited by LY294002, cell proliferation is reduced (45–48). Our results show that the phosphorylation of Akt at Ser-473 is significantly decreased in MR-1-siRNA-treated HepG2 cells, which we think is responsible for the inhibition of cell proliferation. Moreover, FN-stimulated phosphorylations of FAK and Akt in adhesion cells is obviously increased, consistent with the report that FAK is upstream of Akt in the proliferative response (25, 49). This process is significantly inhibited by MR-1-siRNAs, indicating that MR-1-siRNAs block the ECM-stimulated FAK/Akt signaling pathway through the inhibition of the ability of cells to adhere to ECM.

Our data here show the MR-1-mediated signaling pathway involved in cancer cell adhesion, migration, and proliferation. Firstly, MR-1-siRNA-induced inhibition of cell migration and activation of FAK/Akt can be mimicked by MLCK inhibitor ML-7 that causes a significant decrease of FAK/Akt phosphorylation and cell migration induced by exogenous and inherent MR-1, indicating that MLC2 is downstream of MR-1 and upstream of FAK/Akt. Secondly, actin polymerization inhibitor cytochalasin D significantly reduces FAK/Akt phosphorylation and conversely increases MLCK2 phosphorylation, which might be a negative feedback of F-actin depolymerization, suggesting that the formation of stress fiber is upstream of FAK/Akt when MR-1 functions its bioactivity and might be a key factor in MR-1 signaling pathway.

In conclusion, our study demonstrates that MR-1 is overexpressed in human cancer cells and plays an important role in the proliferation, migration, and adhesion. Knockdown of MR-1 in human hepatoma HepG2 cells blocks cell migration and proliferation both in vitro and in vivo. The mechanism of action is that MR-1 induces MLCK2 activation, subsequently stimulates stress fiber formation, and indirectly activates the FAK/Akt signaling pathway to promote cell migration and proliferation. Although additional functional analyses are required, MR-1 might be a potential therapeutic target for treatment of human cancers.

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