We showed that expression of MRJ (DNAJB6) protein is lost in invasive ductal carcinoma, and restoration of MRJ(L) restricts malignant behavior of breast cancer and melanoma cells. However, the signaling pathways influenced by MRJ(L) are largely unknown. Our observations revealed that MRJ(L) expression causes changes in cell morphology concomitant with down-regulation of several mesenchymal markers, viz. vimentin, N-cadherin, Twist, and Slug, and up-regulation of epithelial marker keratin 18. Importantly, MRJ(L) expression led to reduced levels of β-catenin, an epithelial mesenchymal transition marker, and a critical player in the Wnt pathway. We found that MRJ(L) up-regulates expression of DKK1, a well known Wnt/β-catenin signaling inhibitor, that causes degradation of β-catenin. Re-expression of DNAJB6 alters the Wnt/β-catenin signaling in cancer cells, leading to partial reversal of the mesenchymal phenotype. Thus, MRJ(L) may play a role in maintaining an epithelial phenotype, and inhibition of the Wnt/β-catenin pathway may be one of the potential mechanisms contributing to the restriction of malignant behavior by MRJ(L).

The Hsp40/DnaJ family of proteins is known to be a specific client protein provider for the Hsp70/DnaK family of chaperones (1). The important role of the Hsp40 family in a variety of aspects of tumor biology is being described only very recently (2–6). DNAJB6 is a member of the class II Hsp40/DnaJ family of proteins comprising two differentially spliced variants (7). MRJ(S), the shorter variant of DNAJB6, has been widely studied and has been reported to regulate keratin8/k18 organization, assist in NFATc3-mediated transcriptional repression, and play a critical role in Huntington disease (8–10). On the contrary, the role of the long variant, MRJ(L), in various pathophysiologicals has still to be revealed with the exception of a potential role in the nuclear import of human immunodeficiency virus type-2 vpx (11). We and others have reported loss of DNAJB6 in advanced breast cancer patients (7, 12). A detailed functional characterization of DNAJB6 performed by us revealed that MRJ(L) can act as a nuclear protein by virtue of its nuclear localization signal and can considerably reduce the expression of several secreted proteins that promote invasion, migration, and metastasis of cancer cells. Functionally, MRJ(L) was found to reduce tumorigenicity and metastasis of melanoma and breast cancer cells (7). But the mechanistic clues to the downstream signaling regulated by MRJ(L) are still largely unknown.

In the course of our studies, we observed distinct morphological changes in the cells that were restored for MRJ(L) expression. The cells were more epithelial like in appearance. In this study, we describe the role of MRJ(L) in causing partial reversal of epithelial mesenchymal transition (EMT). Our studies show that MRJ(L) inhibits Wnt/β-catenin signaling by up-regulating DKK1, a secreted inhibitor of Wnt signaling, and reverts certain aspects of EMT. We also demonstrate that this inhibition of Wnt/β-catenin signaling is responsible, in part, for the ability of MRJ(L) to restrict malignant behavior.

EXPERIMENTAL PROCEDURES

Cell Culture—MDA-MB-435 cells stably transfected with MRJ(L) and the corresponding vector control cells were cultured in DMEM/F-12 with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% nonessential amino acids, 1.0 mM sodium pyruvate, and 500 μg/ml G418 (Invitrogen). MCF10A, MCF10.DCIS.com, and MCF10CA1d.cl.1 (obtained from Karmanos Cancer Institute) were cultured in DMEM/F-12 with 5% horse serum (Invitrogen), 100 ng/ml cholera toxin (Calbiochem), 10 μg/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma), and 25 ng/ml epidermal growth factor (Sigma). MCF7 cells were cultured in DMEM/F-12 containing 5% horse serum and insulin (10 μg/ml). MCF7 cells stably transfected with MRJ(L) were cultured in medium containing 500 μg/ml G418 (Invitrogen). All cells were maintained at 37 °C with 5% CO2 in a humidified atmosphere. The proteasome inhibitor MG132 was purchased from Sigma, and lactacystin was from Calbiochem.

MDA-MB-435 has now been confirmed to be of melanoma origin (13–15). However, there still are convincing arguments about the lineage infidelity of this cell line (16). However, the work presented here uses this cell line as a model system for signaling studies. MCF10A is a spontaneously immortalized breast epithelial cell line (17, 18). MCF10DCIS.com (locally aggressive) and metastatic variants MCF10CA1a.cl.1 and MCF10CA1d.cl.1 are isogenic cell lines derived from in vivo passages of MCF10AT.

This work was supported, in whole or in part, by National Institutes of Health Grant 1R01CA140472-01A1 (to R. S. S.).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

1 Recipient of Grant BCRP-W81XWH-07-1-0400 from the Department of Defense.

2 To whom correspondence should be addressed: Dept. of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604. E-mail: rsamant@usouthal.edu.

The abbreviations used are: EMT, epithelial mesenchymal transition; shRNA, small interfering RNA; siRNA, small interfering RNA; QRT, quantitative reverse transcription-PCR; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; HSP, heat shock protein.
MCF10AT cells are transfected with T24 Ha-ras, and generate carcinomas in ~25% of xenografts in nude mice (17–20). These cell lines were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI).

SUM159 cells were cultured in Ham’s F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM1315 was cultured in Ham’s F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and epidermal growth factor (10 ng/ml). Both SUM lines were bought from Asterand plc, Detroit, MI.

**RNA Isolation and Real Time Quantitative RT-PCR—**TRIzol reagent (Invitrogen) was used to isolate total RNA from cultured cells. RNA was treated with DNase I (Promega, Madison, WI). cDNA synthesis was carried out using a cDNA synthesis kit (Applied Biosystems Inc., Foster City, CA) with 1 μg of total RNA as the template and random primers. Real time quantitative RT-PCR (QRT-PCR) analysis was performed on the experimental mRNAs. The PCR primers and probes for Dickkopf1 (DKK1), DNAJB6 (MRJ(L)), c-myc, E-cadherin, and keratin 18 and normalization control gene glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems Inc. QRT-PCR was performed on an ABI 7500 HT instrument (Applied Biosystems). The gene expression ΔCt values of mRNAs from each sample were calculated by normalizing with the template control and glyceraldehyde-3-phosphate dehydrogenase, and relative quantitation values were plotted using GraphPad Prism® (La Jolla, CA). QRT-PCR analysis was performed on the experimental mRNAs in triplicate, and the experiment was repeated once from an independent passage to confirm the findings.

**Plasmids and Constructs—**pDKK-707, a luciferase construct encompassing −707 to +43 bp of human DKK1 promoter was a gift from the Varmus laboratory (21). M50 TOPFlash luciferase construct was a generous gift from Dr. Randall Moon (22). shRNA Knockdown—Oligo Design Tool™ (Oligoengine, Seattle, WA) was used to design two shRNAs, NM_807 and NM_808, predicted to silence the expression of MRJ(L). The heteroduplexes supplied as asymmetric oligomers were annealed following the manufacturer’s instructions and cloned into pSUPERIOR.neo+GFP (Oligoengine) at the BglII and HindIII sites. Recombinants were analyzed by restriction digestion using EcoRI and HindIII (Promega), and recombinant DNA was prepared using the QIA Maxiprep kit (Qiagen, Valencia, CA).

MCF10A cells were transfected with shRNA targeting MRJ(L) (NM_808) or a control nontargeting shRNA using Lipofectamine 2000 according to the manufacturer’s instructions. The extent of knockdown was assessed using QRT-PCR, using RNA isolated 32 h after transfection.

**siRNA Knockdown—**Cells were transiently transfected with SMARTpool® siRNA (Dharmacon, Chicago) designed to target DKK1 or nontargeting siRNA control using Dharmafect1 (Dharmacon) following the manufacturer’s protocol. Conditioned serum-free medium and lysates were collected from both control and DKK1-siRNA-treated cells 38 h post-transfection. The extent of knockdown was assessed using Western blot analysis.

**Immunocytochemistry—**Cells cultured on coverslips were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin. F-actin was stained with phalloidin-TRITC. Cells were viewed using a Zeiss Axiocover 200 M microscope equipped with an Axiocam camera and Axiowizard software (Carl Zeiss, Inc., Thornwood, NY).

**Luciferase Assay—**Cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Total protein was harvested and luciferase activity measured using a Turner 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). The luciferase reading was normalized to the total protein concentration. Data are expressed as relative luciferase activity, where control is 100%.

**Western Blot—**The cell monolayer was washed twice with calcium- and magnesium-free PBS and lysed with cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, and protease and phosphatase inhibitors). The lysates were kept on ice for 1 h and centrifuged at 10,000 rpm for 30 min at 4 °C. Protein concentration was measured using Bradford reagent (Bio-Rad). Proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (0.2 μm). The membranes were blocked with 5% skimmed milk in TBST (1 M Tris, pH 7.5, 9% NaCl, 0.5% Tween 20) and incubated with primary antibodies overnight at 4 °C. The membranes were then washed three times with TBST, incubated with respective secondary antibodies for 1 h at room temperature, and then developed using SuperSignal™ (Pierce) following washes for 1 h with three changes of TBST.

For detection of MRJ (DNAJB6(MO1) 1:5000) was used with 5% milk in PBS containing 0.2% Tween 20 after bloting, as per the manufacturer’s instructions. DNAJB6 rabbit polyclonal (MO1) and SNAI2 (Slug) antibodies were purchased from...
Abnova Corp. (Taipei City, Taiwan). Anti-vimentin (1:500) was from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA), and horseradish peroxidase-β-actin (1:50,000) was from Sigma.

Colony Formation Assay—MDA-MB-435 cells stably expressing MRJ(L) were plated in 6-well plates and transfected with DKK1 siRNA and control siRNA using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. After 24 h of transfection, the cells were plated into three 10-cm plates in selection medium and were allowed to grow for 12 days. Crystal violet staining was used for visualization of foci. Six different fields were counted for the number of foci, and data are represented as average ± S.E.

Soft Agar Colonization Assay—MDA-MB-435 cells (2 × 10⁴) treated with DKK1 siRNA or control siRNA suspended in 0.35% agar were plated onto a layer of 0.75% BactoAgar in DMEM/F-12 (10% fetal bovine serum) in 6-well tissue culture dishes. Visible colonies (>50 cells) were counted after 15 days with the aid of a dissecting microscope.

Wound Healing Assay—Cells were cultured to confluence on 6-well plates, which were previously externally marked with parallel lines to use as guides for the subsequent photography. The cells were transfected with DKK1 siRNA and control siRNA using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. After 24 h of transfection, a central linear wound (perpendicular to the guide lines) was made with a 200-µl sterile pipette tip. Media were changed gently to remove any floating cells. Phase micrographs of the wound cultures were taken at 0 and 16 h (average doubling time is about 20 h). The photographs were analyzed by measuring the distance from the wound edge of the cell sheet at the end of 16 h to the original wound site. Wound healing activity was calculated as the mean distance between edges in 12 independent fields per well. Each test group was assayed in triplicate, and the results are expressed relative to vector control cell migration.

Migration Assay—Migration assays were conducted using 8-µm polyethylene terephthalate filters (BD Pharmingen), as described previously (23). Cells (treated with DKK1 siRNA or control siRNA), which migrated to the lower sides of the transwell, were stained using 0.05% crystal violet, and the cell number was counted. Each test group was assayed in triplicate. Four different fields of each insert were photographed at 10× magnification using a Zeiss AxioCam 200 M microscope (Zeiss AxioCam, Carl Zeiss Microimaging Inc.). Each field was divided into quadrants, and cells in diagonally opposite quadrants were counted.

RESULTS

MRJ(L) Expression Leads to Change in Cell Shape—We compared the morphology of the cells that were engineered to constitutively express MRJ(L) to the corresponding vector control. As seen in Fig. 1, A and B, MRJ(L) stable transfectants of MDA-

FIGURE 2. MRJ(L) expression leads to loss of mesenchymal markers and gain in epithelial markers. Total protein (20 μg) from MDA-MB-435-MRJ(L), MCF10CA1d.c1.1-MRJ(L), and MCF7-MRJ(L) and their corresponding vector controls was immunoblotted and analyzed for different levels of a variety of EMT markers. V, vector control; M, MRJ(L) expressor.

FIGURE 3. Degradation of β-catenin by MRJ(L) is proteasome-dependent. MDA-MB-435MRJ(L) (A) and MCF7-MRJ(L) (B) and the corresponding vector controls were treated with MG132 or lactacystin or vehicle (DMSO) control. Protein extract (40 μg) from the cells was analyzed for the level of β-catenin. β-Tubulin was used to verify equal loading for MDA-MB-435, and β-actin was used to verify equal loading for MCF7.
MRJ(L) Suppresses Wnt Signaling

MRJ(L) up-regulates DKK1—DDK1 is a secreted inhibitor of Wnt/β-catenin signaling that blocks Wnt signaling by binding to the Wnt co-receptor Lrp5/6. QRT-PCR analysis of different breast cancer cell lines showed that all of them express lower levels of DKK1 mRNA as compared with nontumorigenic MCF10A cells. SUM1315, SUM159, and MCF10.DCIS.com express very low levels of DKK1, whereas MCF7 and MDA-MB-231 cells seem to express slightly higher levels. The levels of DKK1 in MCF10CA1d.cl.1 cells were intermediate (Fig. 5A). We have also evaluated DKK1 mRNA levels in MDA-MB-435 and found it to be very low. Hence, we examined the MRJ(L) expressors for expression of DKK1. As seen in Fig. 5B, we observed a substantial increase in the levels of secreted DKK1 in serum-free conditioned medium from 435-MRJ(L) and MCF10CA1d.cl.1-MRJ(L) cells. These results corroborate with the increased DKK1 transcript by QRT-PCR (supplemental Fig. 1).

To determine whether the increase in DKK1 is at a transcriptional level, we assessed the activity of the luciferase reporter construct pDKK1–707 in MCF10CA1d.cl.1-MRJ(L), MCF7-MRJ(L), and MDA-MB-435-MRJ(L) and compared it with the corresponding vector control. As seen in Fig. 5C, we observed a 2.5-fold up-regulation of DKK1 promoter activity in MCF7-MRJ(L), and MCF10CA1d.cl.1-MRJ(L), and 6-fold up-regulation in MDA-MB-435.

Silencing of DKK1 from MRJ(L) Expressors Leads to Restoration of β-Catenin—DKK1 binding to the Lrp5/6 receptors directs β-catenin to phosphorylation and subsequent proteosome-mediated degradation. If the degradation of β-catenin in MRJ(L) expressing cells is due to the elevated DKK1 levels,
silencing DKK1 expression in MRJ(L)-expressing cells should lead to increased /H9252-catenin levels. We treated 435-MRJ(L) with DKK1 siRNA and compared their /H9252-catenin levels with 435-MRJ(L) cells independently treated with control siRNA. We found that silencing DKK1 from MRJ(L) expressors can restore /H9252-catenin levels (Fig. 6A). MCF10CA1d.cl.1 MRJ(L) expressors also show comparable restoration of /H9252-catenin levels upon DKK1 silencing (Fig. 6B). For reasons not known to us, we always noticed a slight elevation of /H9252-catenin in the control siRNA transfectants of MCF10CA1d.cl.1 MRJ(L). Because most of the elevated mesenchymal markers observed by us in Fig. 2 are downstream targets of /H9252-catenin transcription, we hypothesized that DKK1 silencing will increase the /H9252-catenin protein levels and result in changes in the transcript levels of the mesenchymal markers. We explored the effect of DKK1 silencing, in MDA-MB-435-MRJ(L), on the EMT markers (supplemental Fig. 2). We observed that there was a noticeable up-regulation of mesen-

**FIGURE 5.** MRJ(L) up-regulates DKK1. A, quantitative RT-PCR analysis was performed to measure DKK1 transcript level from different breast cancer cell lines as well as MDA-MB-435. Real time quantitative RT-PCR analysis was performed on the experimental mRNAs in triplicate, and the experiment was repeated once from an independent passage. All the breast cancer cells showed significant down-regulation compared with MCF10A (p < 0.05). Significance was not calculated for MDA-MB-435.8, MDA-MB-435MRJ(L), MCF10CA1d.cl.1-MRJ(L), and their corresponding vector (V) controls were analyzed for the level of secreted DKK1. The lysate was immunoblotted and probed for MRJ(L). /H9252-Actin was used as a loading control for the cell lysate. The serum-free cell-free supernatant (SFM) was harvested from an equal number of cells, concentrated to 10% of its original volume, and immunoblotted for DKK1. C, pDKK1–707 was transfected into MCF10CA1d.cl.1-MRJ(L), MDA-MB-435-MRJ(L), and MCF7MRJ(L) and their corresponding vector controls. The luciferase activity was compared with the corresponding vector controls. The experiment was performed in triplicate and repeated twice. The error bars represent means ± S.E. Statistical significance was determined if it reached 95% confidence. All the assays had p values <0.05.

**FIGURE 6.** Knockdown of DKK1 from MRJ(L) expressors leads to restoration of /H9252-catenin. MDA-MB-435MRJ(L) and MCF10CA1d.cl.1 MRJ(L) cells were transiently transfected with siRNA designed to target DKK1 or nontargeting siRNA control using DharmaFECT1 (Dharmacon). The serum-free cell-free supernatant (SFM) was harvested from equal number of cells, concentrated to 10% of its original volume, and immunoblotted for DKK1. Total protein (35 μg) was immunoblotted and analyzed for the levels of /H9252-catenin. /H9252-tubulin was used to verify equal loading.
MRJ(L) Suppresses Wnt Signaling

We observed that MRJ(L) expressors have a distinct epithelial-like appearance and increased expression of filopodia on the surface (Fig. 1). Recent studies have indicated that distinctive filopodia differing in length, positioning, and dynamics are seen in different cells (43). Filopodia can act as sensors of the environment and change to lamellipodia when the cell decides to migrate (44). Besides their role in cell migration, filopodia also have been reported to play a role in formation of adherens junctions between epithelial cells (45). MRJ(L)-expressing cells show numerous filopodia indicative of epithelial transformation that may be a prerequisite for the cells to search for an adjacent cell with extended filopodia leading to interdigitation of filopodia and formation of adherens junctions.

DISCUSSION

Heat shock proteins (HSPs) are a group of proteins whose expression is increased when cells are exposed to stress; however, HSPs are also present in cells under normal conditions (26–28). They are popularly called “chaperones” and are implicated in protein folding and destruction. However, they also have roles in immunological processes, cell cycle regulation, transcriptional activation, and signal transduction (29–31). Heat shock proteins are overexpressed in a wide range of human cancers, conferring resistance to cytotoxic therapies (29, 31). HSP90 and HSP70 are widely studied for their involvement in molecular signaling events that have direct bearing on understanding cancer biology (32–36). However, the role of HSP40 family members is only recently described (2, 3, 37–42). DNAJB6 is an HSP40 family member whose involvement in cancer has been recently realized (2, 7, 12). Expression of this protein is lost in aggressive breast cancer. The large spliced variant of DNAJB6, MRJ(L) shows reduced expression in aggressive cancer cells. Restoration of MRJ(L) in aggressive breast cancer cells reduces tumorigenicity and metastasis (7). However, the signaling pathways impacted by MRJ(L) were not known.

We observed that MRJ(L) expressors have a distinct epithelial-like appearance and increased expression of filopodia on the surface (Fig. 1). Recent studies have indicated that distinctive filopodia differing in length, positioning, and dynamics are seen in different cells (43). Filopodia can act as sensors of the environment and change to lamellipodia when the cell decides to migrate (44). Besides their role in cell migration, filopodia also have been reported to play a role in formation of adherens junctions between epithelial cells (45). MRJ(L)-expressing cells show numerous filopodia indicative of epithelial transformation that may be a prerequisite for the cells to search for an adjacent cell with extended filopodia leading to interdigitation of filopodia and formation of adherens junctions.
Based on known phenotypes, MCF7 is more epithelial-like and MCF10CA1.d.cl.1 is much more mesenchymal-like than MCF7, but MDA-MB-435 is the most mesenchyme-like. At the molecular level, we observed that protein levels of mesenchymal markers like CTNNB1 (β-catenin), Vim (vimentin), CDH2 (N-cadherin), Twist1, and Slug (SNAI2) were down-regulated in MRJ(L) expressors, whereas the epithelial marker, KRT18 (keratin 18) was up-regulated (Fig. 2). We did not see any noticeable changes in E-cadherin levels (tested in MCF7 and MCF10CA1.d.cl.1), but interestingly, MCF10A cells did show a loss of E-cadherin transcript (CDH1) upon MRJ(L) knockdown (Fig. 8). N-cadherin expression has been reported to increase motility of breast cancer cells regardless of their E-cadherin status (46). Vimientin is an important cytoskeletal protein that controls the formation of focal adhesions for efficient migration and invasion (47, 48). Buhler and Schaller (49) have shown that forced expression of keratin 18 in aggressive breast cancer cells reduces vimentin expression, alters cell shape, and reduces invasion. Thus, down-regulation of vimentin, N-cadherin levels, and up-regulation of keratin 18 in MRJ(L) cells suggests a shift toward a less malignant phenotype. Taken together, MRJ(L) is capable of down-regulating mesenchymal markers and up-regulating the expression of epithelial marker keratin 18. MRJ(L) overexpression led to increased expression of keratin 18 consistently in three different cell lines as observed in Fig. 2. However, this change may be independent of DKK1; when we silenced DKK1 from MDA-MB-435-MRJ(L) cells, we did not see an effect on keratin 18 (data not shown). This could mean that keratin 18 up-regulation by MRJ(L) may be independent of the suppression of the Wnt/β-catenin pathway. Also, we did not see keratin 18 transcript levels down concomitant with MRJ(L) knockdown from MCF10A (Fig. 8C). We think that keratin 18, an epithelial marker, may be under multiple checks and controls, and merely silencing MRJ(L) may not be changing its levels. miR-200 family and Zeb1/2 have a critical role in regulating EMT through Slug and Twist levels (50–53). To see if these signaling pathways affecting mesenchymal phenotype may also be altered by MRJ(L), we tested miR-200 and Zeb1/2 levels in MDA-MB-435-MRJ(L) and found a 10-fold up-regulation of miR-200b and a several fold (C value >40, practically not detected) reduction in Zeb2 levels (supplemental Fig. 4). Zeb1 levels were unaltered. Effectively, expression of MRJ(L) appears to be necessary, in part, to maintain the epithelial phenotype. It is tempting to speculate that loss of MRJ(L) may be one of the triggers in tipping cells toward the metastable phenotype (54, 55).

EMT is one of the important series of molecular changes in cancer progression, which may affect prognosis of cancers such as breast cancer (56–58). Several signaling pathways have been implicated to play a role in EMT of cancer cells, and Wnt/β-catenin is one of the important ones (59, 60). Of all the EMT-related signaling changes that accompanied MRJ(L) overexpression, reduced levels of β-catenin protein were most consistent across the different cell types examined by us. This reduction led to significant inhibition of the Wnt/β-catenin signaling in the MRJ(L) expressors (Fig. 4). β-Catenin is a major player in the Wnt signaling pathway, as well as an important
regulator of EMT in cancer cells and possibly in maintaining a metastable phenotype (54, 61, 62). It is important to note that β-catenin is a molecule that has multiple functions based on its location and partners. At the cell membrane, it binds to α-catenin and E-cadherin contributing to the formation of cadherin-catenin junctions. These junctions are contributors to cell polarity and the epithelial phenotype, whereas nuclear β-catenin binds to Tcf/Lef transcription factors and promotes transcription of target genes that among other phenotypes are responsible for regulating mesenchymal markers (63). Our observations indicate that MRJ(L) expression promotes the degradation of β-catenin in a proteasome-dependent manner (Fig. 3). The known pathways that drive the degradation of β-catenin are initiated by inhibition of Wnt ligands or their receptors by their inhibitors like sFRPs, DKKs, etc. (64, 65). We observed that breast cancer cells show relatively lower mRNA levels of DKK1 as compared with nontumorigenic MCF10A cells (Fig. 5A); moreover, the expression profiles of DKK1 paralleled that of MRJ(L) (supplemental Fig. 3). Analysis of MRJ(L) overexpressors showed increased levels of secreted DKK1, due to the transcriptional up-regulation of DKK1 (Fig. 5), which possibly caused reduced β-catenin protein levels (Fig. 5B). Knockdown of up-regulated endogenous DKK1 by treatment of 435-MRJ(L) with DKK1-siRNA restored β-catenin levels in MRJ(L). This confirmed the role of MRJ(L) up-regulated DKK1 in degrading β-catenin (Fig. 6). Enhancement in motility, increased foci formation, as well as increased soft agar colonization were seen in DKK1-silenced MDA-MB-435 cells indicating that the MRJ(L) up-regulated DKK1 contributed to the reduction of malignant activity. Importance of MRJ(L) and DKK1 is also evident in Fig. 8B and supplemental Fig. 5 as silencing the expression of either of these genes leads to increased invasion and motility in MCF10A cells.

DKK1 loss has been correlated with aggressive breast cancer and is reported to be frequently inactivated by epigenetic inactivation (66). DKK1 is implicated as a player in inhibiting malignant behavior of breast cancer (67–71). Stable expression of MRJ(L) Suppresses Wnt Signaling in inhibition of metastasis suppressors and activation of EMT (76). Our observations demonstrate a role for MRJ(L) in up-regulating DKK1, leading to inhibition of the Wnt/β-catenin signaling pathway. This inhibition could be one of the ways by which MRJ(L) reduces tumorigenicity and metastasis of aggressive cancer cells.

REFERENCES

1. Ohtsuka, K., and Hata, M. (2000) Cell Stress Chaperones 5, 98–112
2. Deleted in proof
3. Tsai, M. F., Wang, C. C., Chang, G. C., Chen, C. Y., Chen, H. Y., Cheng, C. L., Yang, Y. P., Wu, C. Y., Shih, F. Y., Liu, C. C., Lin, H. P., Jou, Y. S., Lin, S. C., Lin, C. W., Chen, W. J., Chan, W. K., Chen, J. J., and Yang, P. C. (2006) J. Natl. Cancer Inst. 98, 825–838
4. Chen, H. W., Lee, J. Y., Huang, J. Y., Wang, C. C., Chen, W. J., Su, S. F., Huang, C. W., Ho, C. C., Chen, J. J., Tsai, M. F., Yu, S. L., and Yang, P. C. (2008) Cancer Res. 68, 7428–7438
5. Wang, C. C., Tsai, M. F., Dai, T. H., Hong, T. M., Chan, W. K., Chen, J. J., and Yang, P. C. (2007) Cancer Res. 67, 4816–4826
6. Cheng, H., Cenciarelli, C., Nelkin, G., Tsan, R., Fan, D., Cheng-Mayer, C., and Fidler, I. J. (2005) Mol. Cell. Biol. 25, 44–59
7. Mitra, A., Fillmore, R. A., Metge, B. I., Rajesh, M., Xi, Y., King, J., Ju, I., Pannell, L., Shevde, L. A., and Samant, R. S. (2008) Breast Cancer Res. 10, R22
8. Dai, Y. S., Xu, J., and Molkentin, J. D. (2005) Mol. Cell. Biol. 25, 9936–9948
9. Izawa, I., Nishizawa, M., Ohtakara, K., Ohtsuka, K., Inada, H., and Inagaki, M. (2000) J. Biol. Chem. 275, 34521–34527
10. Chuang, J. Z., Zhou, H., Zhu, M., Li, S. H., Li, X. J., and Sung, C. H. (2002) J. Biol. Chem. 277, 19831–19838
11. Cheng, X., Belsham, M., and Ratner, L. (2008) J. Virol. 82, 1229–1237
12. Abba, M. C., Drake, J. A., Hawkins, K. A., Hu, Y., Sun, H., Notovitch, C., Gaddis, S., Sahin, A., Baggery, K., and Aldaz, C. M. (2004) Breast Cancer Res. 6, R499–R513
13. Rae, J. M., Creighton, C. J., Meek, J. M., Haddad, B. R., and Johnson, M. D. (2007) Breast Cancer Res. Treat. 104, 13–19
14. Rae, J. M., Ramus, S. J., Waltham, M., Arnes, J. E., Campbell, I. G., Clarke, R., Barndt, R. J., Johnson, M. D., and Thompson, E. W. (2004) Clin. Exp. Metastasis 21, 543–552
15. Xie, X., Brunner, N., Jensen, G., Albrechtsen, J., Gotthardsen, B., and Rygaard, J. (1992) Clin. Exp. Metastasis 10, 201–210
16. Chambers, A. F. (2009) Cancer Res. 69, 5292–5293
17. Strickland, L. B., Dawson, P. J., Santner, S. J., and Miller, F. R. (2000) Breast Cancer Res. Treat. 64, 235–240
18. Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Elaison, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H., and Miller, F. R. (2001) Breast Cancer Res. Treat. 65, 101–110
19. Tait, L. R., Pauley, R. J., Santner, S. J., Heppner, G. H., Heng, H. H., Rak, J. W., and Miller, F. R. (2007) Int. J. Cancer 120, 2127–2134
20. Miller, F. R., Santner, S. J., Tait, L., and Dawson, P. J. (2000) J. Natl. Cancer Inst. 92, 1185–1186
21. Chamorro, M. N., Schwartz, D. R., Vbonica, A., Brivanlou, A. H., Cho, K. R., and Varmus, H. E. (2005) EMBO J. 24, 73–84
22. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H., and Moon, R. T. (2003) Curr. Biol. 13, 680–685
23. Shevde, L. A., Santam, R. S., Paik, J. C., Metge, B. I., Chambers, A. F., Casey, G., Frost, A. R., and Welch, D. R. (2006) Clin. Exp. Metastasis 23, 123–133
24. Mironchik, Y., Winnard, P. T., Jr., Vesuna, F., Kato, Y., Wildes, F., Pathak, A. P., Kominsky, S., Artemov, D., Van Diest, P., Burger, H., Glackin, C., and Raman, V. (2005) Cancer Res. 65, 10801–10809
25. Sarrió, D., Rodriguez-Pinilla, S. M., Hardisson, D., Cano, A., Moreno-Bueno, G., and Palacios, J. (2008) Cancer Res. 68, 989–997
26. Rylander, M. N., Feng, Y., Bass, J., and Diller, K. R. (2005) Ann. N.Y. Acad. Sci. 1066, 222–242
27. Schafer, C., and Williams, J. A. (2000) J. Gastroenterol. 35, 1–9
28. Pirkkala, L., Nykänen, P., and Sistonen, L. (2001) FASEB J. 15, 1118–1131
29. Jäättelä, M. (1999) Ann. Med. 31, 261–271
30. Matijasevic, Z., Snyder, J. E., and Ludlum, D. B. (1998) Oncol. Res. 10, 605–610
