Down-regulation of myopodin expression reduces invasion and motility of PC-3 prostate cancer cells

ARIANE DE GANCK[1,2], VEERLE DE CORTE[1,2], ERIK BRUYNEEL[3], MARC BRACKE[3],
JOEL VANDEKERCKHOVE[1,2] and JAN GETTEMANS[1,2]

[1]Department of Medical Protein Research, VIB; [2]Department of Biochemistry, Ghent University, Faculty of Medicine
and Health Sciences, Albert Baertsoenkaai 3; [3]Laboratory of Experimental Cancerology, Department of Radiotherapy
and Nuclear Medicine, Ghent University Hospital (1P7), De Pintelaan 185, B-9000 Ghent, Belgium

Abstract. Enhanced motility of cancer cells by remodelling of the actin cytoskeleton is crucial in the process of cancer cell invasion and metastasis. Although several studies propose a tumor suppressor role for the actin bundling protein myopodin, it was also shown previously that overexpression of mouse myopodin promotes invasion *in vitro*. In the present study, the role of myopodin in human cancer cell motility and invasion was explored using RNA interference with siRNA duplexes designed to down-regulate all human myopodin isoforms currently identified. We show that down-regulation of myopodin expression in human cancer cells significantly reduces the invasive properties of these cells both in collagen type I and in Matrigel®. Furthermore, the motile characteristics of cancer cells are also curbed by reduced myopodin expression whereas cell-cell contacts are reinforced. These results point to a role for myopodin as tumor activator. While these findings are at variance with the suggested tumor suppressor role for myopodin, we hypothesize that the subcellular localization of the protein is involved in its suppressor or activator function in tumorigenesis.

Introduction

Metastasis, a complex process characterized by the spread of tumor cells from the primary tumor to distant sites and organs, is the major cause of death in cancer patients. Although the molecular and cellular mechanism of cancer invasion and metastasis is not fully understood, it is clear that the acquisition of enhanced motility by cancer cells is a major requirement for these events (1). During the process of cell migration, the organization of the actin cytoskeleton is dynamically remodelled through the concerted action of different classes of actin binding proteins. Many of these actin binding proteins display altered expression levels in malignant cells. For example, expression of the actin capping protein CapG is upregulated in several cancer types including glioblastomas (2), ocular melanomas (3), pancreatic ductal adenocarcinomas (4) and oral squamous cell carcinomas (5). Decreased expression of the actin monomer sequestering protein profilin has been observed in breast cancer (6), pancreatic cancer (7) and hepatocarcinomas (8). Furthermore, the group of Condeelis used an *in vivo* invasion assay to collect the invasive cell population from primary mammary tumors in intact animals using chemotaxis of cancer cells to growth factors. Gene expression profiling of these invasive cells compared to the tumor cells that remained behind in the primary tumor revealed that many proteins involved in regulation of the cytoskeleton, including a gelsolin-like actin filament capping protein, cofilin, vinculin and zyxin, are upregulated in the invasive cells indicating the enhanced migratory behaviour of these cells (9,10). Although it is clear that several aspects of tumorigenesis, including migration and invasion of tumor cells, are related to altered expression of the actin binding proteins, the underlying mechanism remains to be elucidated.

The present study focuses on myopodin, the second member of the synaptopodin family. Myopodin is an actin bundling protein that shuttles between the nucleus and the cytoplasm in a differentiation-dependent manner in muscle cells (11). A single nuclear localization sequence mediates nuclear entry of the protein (12). We have recently shown that mammalian cells, including cancer cells, express three myopodin transcripts that differ at their carboxy-terminus (13). Furthermore, expression of myopodin has also been observed in prostate, small and large intestine (14), normal urothelium and tumors of the bladder (15). A number of studies propose that myopodin contains tumor suppressor activity in urothelial carcinoma and prostate cancer (14-16). However, we have previously shown that expression of

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mouse myopodin in non-invading cells promotes invasion of these cells into a collagen type I matrix (17). Such apparent controversial results have also been observed for other actin binding proteins like gelsolin (4,18,19), CapG (2,5,20), cofilin (21-24) and α-actinin 4 (25-27) making it difficult to classify actin binding proteins either as a tumor activator or as a tumor suppressor.

In this study, RNA interference was used to knock-down myopodin expression in human cancer cells. Subsequently, several assays were performed to determine the effect of myopodin down-regulation on the invasive and motile behaviour of these cancer cells. Our results show that decreased expression of myopodin inhibits motility and invasion of the cells indicating a role for myopodin in the metastasis cascade of cancer cells.

Materials and methods

Cell culture. PC-3 and RT4 cells were maintained at 37°C in a humidified 10% CO_2 incubator and grown in RPMI-1640 (Gibco-BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin.

Transient gene knock down via specific siRNAs. Annealed siRNA duplexes specific for human myopodin and RhoA (28) were obtained from Eurogentec (Seraing, Belgium). Both myopodin siRNAs are targeted to the complementary region of all three human myopodin isoforms.

The following siRNA sequences were used for myopodin: sense siRNA sequence 1: 5'-GCACCUCCUUCUCUGGUA UdTdT-3'; antisense siRNA sequence 1: 5'-AUACCAGAGA AGGAGGUGCdTdT-3'; sense siRNA sequence 2: 5'-GGAG GAUACAUGUGAAGUAdTdT-3'; antisense siRNA sequence 2: 5'-UACUUCACAUGUAUCCUCCdTdT-3'. The Rho siRNA sequences are: sense siRNA sequence: 5'-GACAUGC UUGCUCAUAGUCdTdT-3'; antisense siRNA sequence: 5'-GACUAUGAGCAAGCAUGUCdTdT-3'.

PC-3, PC-3-Myo cells [lentiviral transduced cells that overexpress human myopodin isoform 1 (13)] and RT4 cells were transfected with small interfering RNA (siRNA) duplexes (50 nM) at 80% confluency using lipofectamine 2000 (Invitrogen Life Technologies, Merelbeke, Belgium) according to the manufacturer's instructions.

A negative control siRNA duplex obtained from Eurogentec, consisting of a unique sequence that does not match with any other sequence in the human genome, was used under similar conditions. Seventy-two hours following transfection, the cells were harvested.

Invasion assays. Invasion into collagen type I was performed as described previously (29). Gels were prepared in a 6-well plate from a collagen type I solution (Upstate Biotechnology, Lake Placid, NY) and incubated for at least 1 h at 37°C to allow gelification. Cells (1x10⁵) were incubated on top of the gels for 24 h at 37°C. Cells inside the gel were scored with a phase contrast microscope controlled by a computer program. Invasive and superficial cells were counted in 12 fields of 0.157 mm². Trypan blue staining was performed to check the viability of the cells. The invasion index is the percentage of cells invading the gel over the total number of cells counted. Experiments were performed in triplicate. Mean values and standard deviations were calculated.

Cell invasiveness was also determined by the ability of cells to transmigrate through a basement membrane (ECM) in Matrigel (QCM™ 24-well Invasion assay, Chemicon® International, Temecula, CA). This was performed according to the manufacturer's protocol. Briefly, cells were placed in Matrigel inserts at 1x10⁶ cells/ml in serum-free medium and 10% fetal bovine serum was used in the lower chamber as a chemoattractant. Cells were allowed to migrate for 24 h at 37°C. Cells at the bottom of the insert membrane were dissociated from the membrane by incubation with cell detachment buffer. These cells were subsequently lysed and CyQuant GR® dye was added (Molecular Probes, Eugene, OR, USA). Fluorescence was measured with a 480/520 filter set. Data are expressed as relative fluorescence units (RFU). Assays were performed in triplicate.

Fast aggregation assay. Cell-cell adhesion was numerically evaluated in an aggregation assay as described previously (30). Briefly, single cell suspensions were prepared with an E-cadherin-saving procedure including detachment by collagenase A (Boehringer Mannheim) treatment followed by trypsin, both in the presence of 0.04 mM Ca^{2+}. The cells were allowed to aggregate on a Gyrotory shaker (New Brunswick Scientific, New Brunswick, NJ) at 80 rounds per minute for 30 min in aggregation buffer containing 1.25 mM Ca^{2+}, 0.1 mg DNase/ml, 10 mM HEPES and 0.1% BSA and equilibrated at physiological pH and osmolarity. Cell aggregation was measured with an LS particle size analyser (LS200, Coulter Electronics) after 0 and 30 min of aggregation. The relative volume as a function of particle size was used as an index of aggregation. Kolmogorov-Smirnov statistics was used to analyze the differences between the cumulative distribution curves obtained in the fast aggregation assay.

Wound healing assays. Equal numbers of PC-3 cells (900000) were seeded into 6-well cell culture plates. Eighteen hours after seeding, cells were transfected with either Myopodin siRNAs or with the siRNA negative control provided by Eurogentec. Seventy-two hours after transfection a wound was made by scratching a line in a confluent monolayer. The debris was removed by washing the cells with serum-free medium. Migration of cells into the wound was then observed at different time-points. To this end, the width of the wound (in μm) was measured at different points across the wound (3 points in each wound, and three wounds for each well of the culture plate) at time-point 0 and this measurement was repeated at hourly intervals at the same locations. Cells were followed for 8 h. Afterwards, the distance that the cells migrated (in μm) was calculated for each point of measurement and each time-point by reducing the width of the wound at time-point 0 with the width of the wound at that time-point. Finally, for each time-point, the average of the distance migrated at the different points of measurement was taken and plotted. Data are means ± SE of three independent experiments.

Lentiviral transduction (generation of PC-3-Myo cells). V5-tagged human myopodin isoform 1 was cloned into the pLVTHM vector (kindly provided by Dr D. Trono, Lausanne,

Switzerland) by excision of GFP cDNA from pLVTHM using the restriction enzymes *Pme*I and *Spe*I. Recombinant lentiviruses were produced by triple transfection of HEK293T cells. HEK293T cells (8×10^5) were transfected using calcium phosphate precipitation with 3 μg of the pLVTHM-myopodin lentiviral vector, 3 μg of pMD2G-VSVG packaging plasmid and 1.5 μg of the psPAX2 envelope plasmid. 24 h before transduction, 10^5 PC3 cells were seeded onto 24-well tissue culture dishes. The medium was replaced with 800 μl of the lentivirus-containing medium to which 5 μg/ml polybrene was added. Cells were then centrifuged for 90 min at 700 rpm and 32˚C. Subsequently, 800 μl RPMI with 10% FBS was added and the cells were maintained at 37˚C in 5% CO_2. The next day, transduction was repeated.

Western blotting. Cells were disrupted in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF, and a protease inhibitor cocktail mix). Insoluble material was removed by centrifugation (20000 x g for 10 min at 4˚C). Protein concentrations were determined by the method of Bradford (31) using bovine serum albumin as a standard. Western blotting was performed as described (32) with anti-V5 antibody (Invitrogen). Proteins were visualized by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK).

Results

Down-regulation of myopodin expression counteracts tumor cell invasion. It was previously demonstrated that over-expression of mouse myopodin in human endothelial kidney cells (HEK293T) or in mouse C2C12 myoblast cells induces invasion of these cells into a collagen type I matrix (17). In the current study, we investigated whether reduced expression of myopodin in cancer cells could also modulate the invasive properties of these cells. To this end, we designed two myopodin siRNA duplexes to down-regulate myopodin expression in human cancer cells. As no reliable myopodin antibodies are available to detect endogenous myopodin on Western blotting (13) we devised an alternative way to show the reliability of the myopodin siRNA duplexes. PC-3 cells were transduced with the lentiviral pLVTHM vector encoding V5-tagged human myopodin isoform 1 (13) to create a stable cell line that overexpresses V5-tagged myopodin. Subsequently, this cell line, called PC-3-Myo, was transiently transfected with one of the myopodin siRNA duplexes. Western blot analysis using a monoclonal anti-V5 antibody revealed that both duplexes reduced the expression of V5-tagged myopodin indicating that the two myopodin siRNA duplexes can be used to efficiently knock-down endogenous myopodin expression (Fig. 1).

Several assays were used to assess the effect of myopodin knock down on cell motility and invasion. Initially, we studied the effect of myopodin down-regulation on the invasive capacity of cancer cells into a collagen type I matrix. The invasive bladder cancer cell line RT4 was transiently trans-fected with a myopodin siRNA duplex and these cells were subsequently seeded on top of a collagen type I layer. We could observe dramatically reduced invasive properties of these cancer cells in a collagen type I matrix (for both siRNA
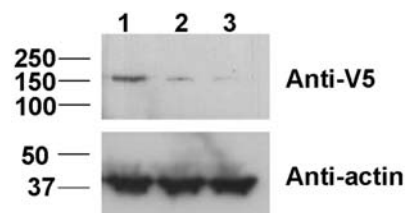

Figure 1. Knock down of myopodin expression in PC-3-Myo cells by myopodin siRNA duplexes 1 or 2. A PC-3 cell line stably overexpressing V5-tagged human myopodin isoform 1 (PC-3-Myo) was transiently trans-fected with the myopodin siRNA duplexes 1 or 2. Western blotting on PC-3 cell extracts with anti-V5 antibody shows that both duplexes efficiently reduce myopodin expression. Protein markers (in kDa) are shown on the left. The expression of actin is shown as a control for equal loading. Lane 1, negative control siRNA; lane 2, myopodin siRNA 1; lane 3, myopodin siRNA 2.
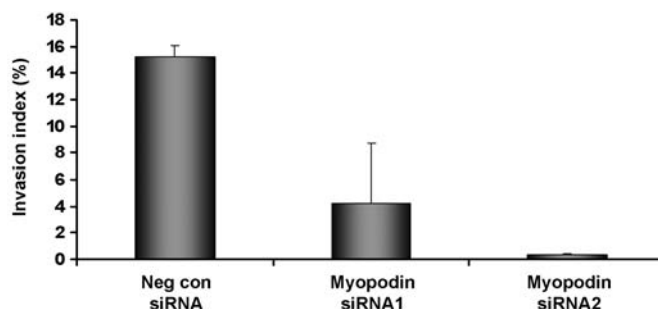

Figure 2. Myopodin RNA interference in RT4 cells inhibits collagen type I invasion. RT4 cells were transiently transfected with myopodin siRNA duplexes 1, 2 or a negative control duplex. Both myopodin siRNA 1 and 2 reduce the invasion of RT4 cells in collagen type I. Data are means ± standard deviation of three independent experiments.
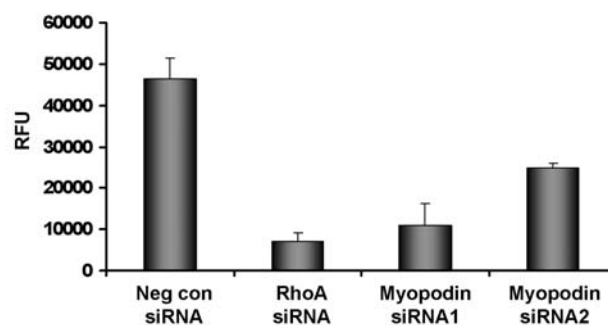

Figure 3. Down-regulation of myopodin expression reduces invasion of PC-3 cells through Matrigel®. Myopodin siRNA 1 and 2 inhibit invasion of PC-3 cells into Matrigel. RhoA siRNA was used as a positive control. RFU, relative fluorescence units are shown in the y-axis. Data are means ± standard deviation of three independent experiments.

duplexes) in comparison to the negative control siRNA duplex (Fig. 2).

Collagen type I represents only one component of the interstitial stroma. However, a critical event in the metastatic cascade is the invasion of cancer cells through the basement membrane. A more suited model for invasion of cells through this basement membrane is a matrigel invasion assay (33). PC-3 cells transfected with the myopodin siRNA duplexes 1 or 2 showed a substantially reduced invasion through Matrigel compared to the negative control siRNA treated cells (Fig. 3). These results are in line with the
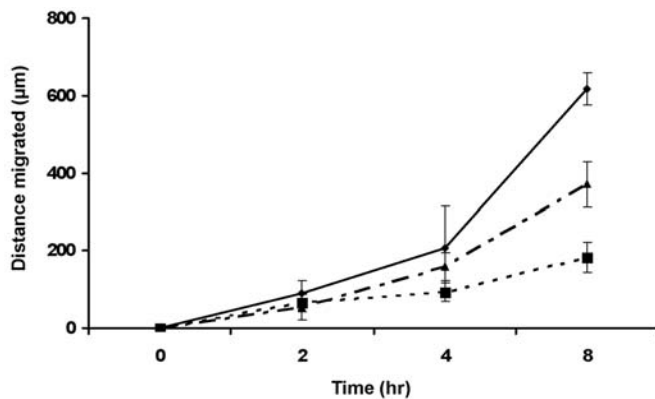
Figure 4. Down-regulation of myopodin offsets motility of PC-3 cells. A wound healing assay was used to determine the effect of myopodin down-regulation on the motility of PC-3 cells. Plotted curve of distance migrated (y-axis) as a function of time (x-axis) are shown for PC-3 cells transfected with the negative control siRNA duplex (solid line), with myopodin siRNA1 (dashed line) or myopodin siRNA2 (dashed-point line).
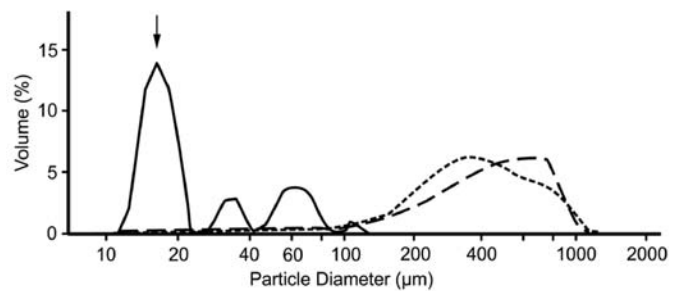

Figure 5. Reduced myopodin expression positively affects cell-cell contacts. The effect of reduced myopodin expression using RNAi on the formation of cell aggregates was investigated in a fast aggregation assay. Plotted curves of relative volume distribution (y-axis) as a function of particle diameter (x-axis) are shown after 30 min for PC-3 cells transfected with a negative control siRNA duplex (solid line), PC-3 cells transfected with myopodin siRNA1 (small-dashed line) or myopodin siRNA2 (large-dashed line). The arrow indicates the peak position of the negative control siRNA-treated cells after 0 min of aggregation.

collagen type I invasion assay. PC-3 cells transfected with a RhoA siRNA duplex were used as a positive control. Over-expression of this small GTPase has been observed in several human cancers (34,35) and down-regulation of RhoA using siRNA in MDA-MB231 breast cancer cells inhibited cell invasion effectively (28). The level of decreased invasion induced by myopodin RNA interference was of the same order to that observed after RhoA down-regulation.

Down-regulation of myopodin expression affects cell migration and cell-cell contact formation. Both *in vitro* invasion assays indicate that the expression levels of human myopodin influence the invasive capacity of cancer cells but the underlying mechanism is not clear. Different cellular activities are involved in the process of invasion including loss of cell-cell adhesion, changes in the cell-matrix interaction, activation of extracellular proteases and increased motility. An important factor in the process of cell movement is the dynamic remodelling of the actin cyto-skeleton, regulated by actin binding proteins. As myopodin is an actin bundling protein, we addressed the question whether myopodin has a direct effect on the motility of cancer cells. To this end, we investigated the effect of myopodin down-regulation on cell motility by using a wound healing assay. In this assay, a wound is made in a confluent layer of cells and the time required for wound closure is measured. We observed that PC-3 cells treated with the myopodin siRNA duplexes 1 or 2 showed a significant decrease in wound closure efficiency in comparison to control siRNA treated cells within a time span of 8 h (Fig. 4).

Another important characteristic of invading cancer cells is loss of cell-cell contacts. Because the key molecule in cell-cell adhesion, E-cadherin, is connected to the actin cyto-skeleton, we examined the effect of myopodin down-regulation on the E-cadherin-dependent formation of cell-cell contacts and thus on the formation of cell aggregates in a fast cell aggregation assay (36). A single cell suspension of PC-3 cells treated with the negative control siRNA duplex did not aggregate into large particles. The aggregates that were formed had a particle size of 10-100 μm after 30 min of aggregation. After transient transfection of the cells with myopodin siRNA duplexes 1 or 2, the PC-3 cells were able to form aggregates with a larger particle diameter than the particle diameter of the negative control siRNA treated PC-3 cells (Fig. 5). Particle diameters of 100-1000 μm were observed after myopodin down-regulation showing that reduced myopodin expression levels promote cell-cell adhesion. These findings suggest that myopodin has a negative effect on cell-cell contacts possibly by disturbing the inter-action between the cell-cell adhesion complex and the actin cytoskeleton.

Discussion

In this study we have demonstrated that siRNA-induced down-regulation of the myopodin expression level reduces invasion of human cancer cells in both collagen type I and Matrigel. As such these findings corroborate earlier studies showing that enhanced expression of myopodin in cells elicits invasion (17). Furthermore, we provide evidence that reduced invasion is linked to defects in cell motility and to reinforcing E-cadherin mediated cell-cell contacts induced by decreased myopodin levels. Taken together, this suggests a potential role for myopodin as a tumor activator. Our data further extend the concept that modulation of the expression level of actin binding proteins affect cell motility and invasion. For instance, overexpression of α-actinin 4, an F-actin cross-linking protein, induces the formation of filopodia and increases cell motility (27). Moderate overexpression of green fluorescent protein-tagged CapG (a ubiquitous nuclear-cytoplasmic F-actin capping protein) in epithelial cells induces invasion into collagen type I and in chick heart fragments. However, CapG tagged with a nuclear export sequence, preventing nuclear accumulation of the protein, is unable to promote invasion, indicating that nuclear CapG is responsible for the invasive phenotype (37). Overexpression of the F-actin severing protein gelsolin promotes invasion of cells into collagen type I and in chick heart fragments (18) whereas down-regulation of gelsolin or CapG expression in various types of cancer cells reduces invasion (38). As these proteins
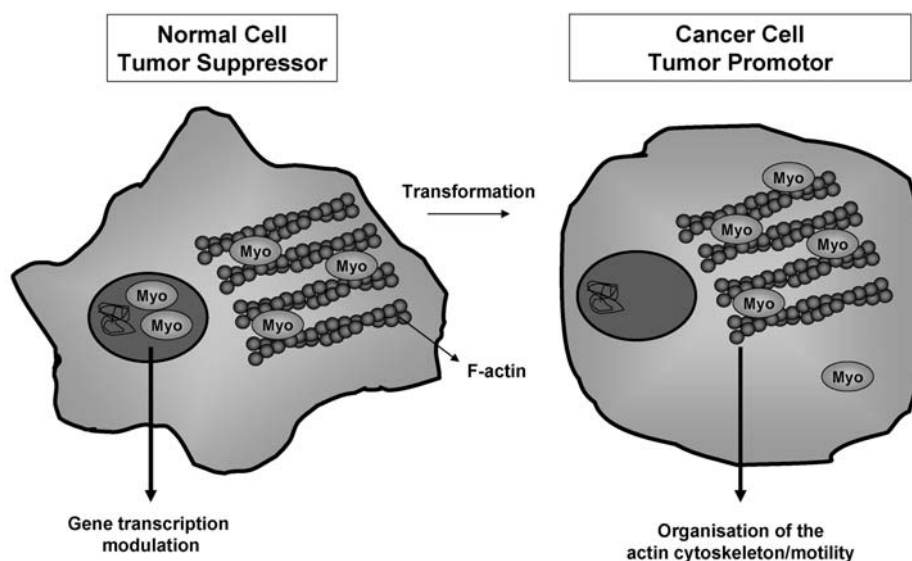
Figure 6. Model showing the relation between the subcellular localization of myopodin and its role in tumorigenesis. In normal cells, myopodin is localized in both the cytoplasm and in the nucleus. Upon cell transformation, the nuclear-cytoplasmic equilibrium shifts in favor of the cytoplasmic localization. We propose that the tumor suppressor role of myopodin is linked to its function in the nucleus (left), i.e. a putative function in gene transcription modulation. The tumor promoter role of myopodin would be associated with its function in the cytoplasm, i.e. regulation of the organization of the actin cytoskeleton (right) and cell motility. Myo, myopodin.

are directly involved in cell motility by virtue of their ability to reorganize the actin cytoskeleton in migrating cells it seems likely that the observed effect on cancer cell invasion is linked to defects in motility.

Our findings diverge from earlier studies proposing a role for myopodin as tumor suppressor (14-16,39). In those studies however, a human myopodin variant was used that differs from the human myopodin isoforms that we have identified (13). Moreover, the myopodin variant has a different subcellular localization than the three human myopodin isoforms used in our studies (13). Whereas the 3 human myopodin isoforms localize along actin filaments in the cytoplasm of various cell lines, the myopodin variant used by others (16) is detected both in the cytoplasm and in the nucleus. Several lines of evidence support a model where the nuclear function of myopodin is involved in the tumor suppressor activity of myopodin and that cytoplasmic myopodin has a tumor activator role (Fig. 6). For example, immunohistochemistry on paired normal urothelium and bladder tumors showed that in normal urothelium, myopodin is localized in both the nucleus and the cytoplasm. In contrast, nuclear myopodin was lost depending on the stage of bladder tumors (15). Tumors with higher stage showed lower nuclear myopodin localization. Furthermore, large-scale analysis of a prostatic tissue microarray led to the observation that cytoplasmic myopodin was notably higher in cancer cells than in normal cells (39). These observations propose that the cytoplasmic myopodin pool gains importance during cancer progression. Moreover, in vitro and in vivo functional studies proposing a tumor suppressing function for myopodin were carried out with a variant that localizes predominantly in the nucleus of these cells (16,40). Interestingly, actin binding proteins residing temporarily in the nucleus, including gelsolin, filamin A, supervillin, cofilin and others, have been shown to act as modulators of gene transcription controlled by nuclear receptors (reviewed in ref. 41). Importantly, alignment of all mouse and human myopodin isoforms known to date shows that they contain the LXXLL signature, a motif frequently encountered in co-regulators of nuclear receptors. This α-helical LXXLL motif, also called nuclear receptor box, is believed to mediate the initial contact between nuclear receptor and co-regulator to facilitate or impede transcription activation (42-44). Loss of nuclear myopodin, as demonstrated by several immunohistochemical studies, could thus lead to changes in the gene transcription profile accompanying and/or promoting cell transformation. Further experiments are needed to investigate this hypothesis as well as the nature of the putative nuclear receptors involved.

Several precedents have been documented showing changes in the subcellular localization of actin binding proteins during cancerogenesis. These include cofilin (45), α-actinin 4 (26,46) and gelsolin (4). Whether the loss of nuclear myopodin localization is due to changes in the nucleo-cytoplasmic transport mechanism or the outcome of changes in myopodin isoform expression remains to be determined.

Another explanation for the apparently contradictory findings relating to the role of myopodin in cancer progression may be that myopodin expression levels oscillate during tumorigenesis. Such a biphasic pattern has also been suggested for other actin binding proteins including gelsolin. Shieh et al (47) described low gelsolin expression during early stages of tumorigenesis followed by an increased expression in invasive tumors, and concluded that different functions of the protein are involved in different stages of carcinogenesis. Further studies on the role of myopodin in tumorigenesis taking the subcellular localization of the protein and the different isoforms into consideration are therefore warranted.

Acknowledgements

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