Specific Myosins Control Actin Organization, Cell Morphology, and Migration in Prostate Cancer Cells

Graphical Abstract

Highlights

- Myo1b, Myo9b, Myo10, and Myo18a are highly expressed in metastatic prostate cancer
- Knockdown of individual myosins distinctly affects the cytoskeleton and cell migration
- Myosins act in concert to directly influence actin organization and cell migration
- Misregulation of myosin expression may drive the metastatic phenotype

Authors

Katarzyna A. Makowska, Ruth E. Hughes, Kathryn J. White, Claire M. Wells, Michelle Peckham

Correspondence

claire.wells@kcl.ac.uk (C.M.W.), m.peckham@leeds.ac.uk (M.P.)

In Brief

Makowska et al. show that several myosin isoforms (Myo1b, Myo9b, Myo10, and Myo18a) are overexpressed in metastatic prostate cancer. Knockdown of each of the myosins resulted in distinct cell phenotypes, showing that they can contribute to metastasis through reorganization of the actin cytoskeleton in addition to motor activity.
Specific Myosins Control Actin Organization, Cell Morphology, and Migration in Prostate Cancer Cells

Katarzyna A. Makowska,1 Ruth E. Hughes,1 Kathryn J. White,1 Claire M. Wells,2,* and Michelle Peckham1,*
1School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK
2Division of Cancer Studies, King's College London, London SE1 1UL, UK
*Correspondence: claire.wells@kcl.ac.uk (C.M.W.), m.peckham@leeds.ac.uk (M.P.)
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SUMMARY

We investigated the myosin expression profile in prostate cancer cell lines and found that Myo1b, Myo9b, Myo10, and Myo18a were expressed at higher levels in cells with high metastatic potential. Moreover, Myo1b and Myo10 were expressed at higher levels in metastatic tumors. Using an siRNA-based approach, we found that knockdown of each myosin resulted in distinct phenotypes. Myo10 knockdown ablated filopodia and decreased 2D migration speed. Myo18a knockdown increased circumferential non-muscle myosin 2A-associated actin filament arrays in the lamella and reduced directional persistence of 2D migration. Myo9b knockdown increased stress fiber formation, decreased 2D migration speed, and increased directional persistence. Conversely, Myo1b knockdown increased numbers of stress fibers but did not affect 2D migration. In all cases, the cell spread area was increased and 3D migration potential was decreased. Therefore, myosins not only act as molecular motors but also directly influence actin organization and cell morphology, which can contribute to the metastatic phenotype.

INTRODUCTION

Myosins are a large and diverse family of molecular motors important for cell migration and motility. The human genome encodes 39 myosin genes, subdivided into 12 different classes (Berg et al., 2001; Peckham and Knight, 2009). Class 2 is the largest (13 genes). Ten of these are found exclusively in muscle. The remaining three encode the non-muscle (NM) myosin isoforms 2A, 2B, and 2C, which contribute to cell shape, adhesion, and cytokinesis (Mogilner and Keren, 2009; Vicente-Manzanares et al., 2009). Myosin isoforms in the remaining classes contribute to a wide range of functions, including organelle trafficking, membrane tethering, Golgi organization, actin organization, and actin polymerization (Hartman and Spudich, 2012). Individual cell types only express a subset of myosin genes. Early studies have shown that ~8–11 different myosin isoforms are co-expressed in epithelial cell lines, leukocytes, liver cells, and myoblasts (Bement et al., 1994; Wells et al., 1997). Some myosin isoforms are expressed widely, whereas others (e.g., Myo7a and Myo3) are restricted to a small tissue subset (Dosé and Burnside, 2000; Hasson et al., 1995).

It has never been determined how variation in myosin expression profile between closely related cell types contributes to a variation in cellular phenotype. Modulating myosin expression could help to drive a cell toward a more migratory phenotype and, therefore, metastasis in cancer. Here we determined the myosin isoform expression profile in a range of prostate cell lines and in silico and then investigated four of the overexpressed myosin isoforms to uncover how each contribute to the more highly metastatic phenotype of PC-3 cells (Pulukuri et al., 2005).

RESULTS

Myo1b, Myo9b, Myo10, and Myo18a Are Overexpressed in More Highly Metastatic Cells

We analyzed myosin expression for all 26 of the non-muscle myosin genes in the three most widely used prostate cancer cell lines: PC-3, DU145, and LNCaP (Weber et al., 2004). PC-3 cells are considered to have a higher metastatic potential than LNCaP cells (Aalinkeel et al., 2004). Class 2 muscle myosin isoforms were excluded because they are not expressed in non-muscle cells. We also analyzed a matched pair of normal (1535NP) and cancerous (1535CT) cell lines derived from the prostate of the same patient (Bright et al., 1997).

A core of 12 myosin genes were expressed in all cell lines tested, as demonstrated by RT-PCR (Table S1). However, DU145 cells additionally expressed two myosin isoforms, Myo7a and Myo3, normally only expressed in the cochlea, retina, testis, lung, and kidney (Hasson et al., 1995) or in the retina and pancreas (Dosé and Burnside, 2000) respectively, and, therefore, we did not use these cells in further experiments, although, for completeness, the qPCR analysis on these cells is included (Figure S1).

Expression levels of MYO1B, MYO1D, MYO1E, MYO9B, MYO10, and MYO18A were significantly higher in PC-3 than in LNCaP cells by qPCR (Figure 1A). MYO1B and MYO10 expression levels were also significantly higher in 1535CT than in 1535NP cells (Figure 1B). An in silico analysis (Figure 1C) showed that MYO1B,
MYO1D, and MYO10 levels were significantly higher in metastatic tumors than in benign tissue, suggesting that this trend is also found in vivo. MYO1E and MYO18A expression levels were also higher in 1535CT cells compared with 1535NP cells, although this difference was not significant, and the in silico analysis did not show any significant differences in expression (Figure 1C). However, the expression of MYO18A or MYO10 may be upregulated in some tumors. MYO6 expression levels were significantly lower in PC-3 cells compared with LNCaP (Figure 1A), lower in LNCaP cells are p53 wild-type, and PC-3 cells are p53-null (Carroll et al., 1993), suggesting that, in this case, there is no link between Myo10 overexpression and expression of mutant p53. In DU145 cells, which do express mutant p53, Myo10 expression is slightly higher, and numbers of filopodia are increased compared with LNCaP cells (Figures S1A and S1B), but both are lower compared with PC-3 cells.

Myo1b localized to organelles in both cell types (Figures 2D and 2F), as expected from its roles in trafficking of endosomes,
Multivesicular bodies, and lysosomes (Cordonnier et al., 2001; Raposo et al., 1999; Salas-Cortes et al., 2005). Higher Myo1b expression in PC-3 cells was associated with an additional localization of Myo1b to actin-rich structures at the plasma membrane and filopodia (Figure 2D), consistent with an earlier study (Tang and Ostap, 2001). Myo9b and Myo18a were both enriched in membrane ruffles/lamellipodia in PC-3 cells (Figure 2D), consistent with Myo18a's role in modifying actin organization in the lamellipodium (Hsu et al., 2010) and Myo9b's role in cell polarity and recruitment of RhoGAP to the lamellipodium (Hanley et al., 2010). Staining for both was diffuse in LNCaP cells.

The higher endogenous expression levels of Myo1b, Myo9b, and Myo10 in more metastatic cell types/tissue suggested that they all contribute to the cellular phenotype of metastatic cells. We therefore used siRNA knockdown (KD) to determine the effects of reducing their expression levels in PC-3 cells on cell morphology and cell migration. We also investigated Myo18a because the interaction of Myo18a regulates NM2A filaments (Billington et al., 2015) and, therefore, may also influence cell migration and phenotype.

Knockdown of Myo1b, Myo9b, Myo10, and Myo18a resulted in isoform-specific changes in cell morphology, cell migration, and actin bundle organization in PC-3 cells.

siRNA-mediated KD for 72 hr significantly reduced expression levels of each myosin isoform in PC-3 cells (Figure 3A) and altered their morphology (Figure 3B). The spread area of the cells increased up to 3-fold (Figure 3C). KD of Myo10, but not Myo1b, Myo9b, or Myo18a, also significantly reduced the numbers of filopodia (Figure 3D). Although the increase in cell area in Myo10 KD cells could be explained by the reduction in filopodia, as reported for COS-7 and HeLa cells (Bohil et al., 2006), it does not explain the increased cell area for Myo1b, Myo9b, and Myo18a KD cells, where filopodia are still present.

Myo9b and Myo10 were most important for PC-3 cell migration in 2D. Knockdown of Myo9b and Myo10 both significantly reduced cell speed (~2-fold in a 2D random migration assay (Figures 3E and 3F). Directional persistence was increased slightly for Myo9b KD cells but unaltered for Myo10. In contrast, knockdown of Myo1b and Myo18a did not affect speed in 2D random migration assays (Figures 3E and 3F). Knockdown of Myo18a significantly reduced directional persistence in 2D (Figures 3E and 3G), indicating that these cells are less able to polarize. However, cell migration was inhibited for each myosin in a circular invasion assay (Figure 3H) that closely mimics 3D invasion (Yu and Machesky, 2012). Staining for F-actin in circular migration
assays (Figure 3) showed an increase in actin stress fibers for cells at the border for each myosin knockdown compared with controls.

We also observed distinct changes in the acto-myosin organization following KD of each myosin. Control PC-3 cells (Figures 4A and 4B) contained few F-actin stress fibers, and NM2A staining was mostly localized to the lamellae. A marked increase in centripetal F-actin fibers running parallel to the plasma membrane in the lamella associated with NM2A filaments was characteristic of Myo18a KD (Figures 4A and 4B). The appearance of sparse, long stress fibers, associated with NM2A and extended along the length of the cells, was characteristic of Myo1b KD (Figures 4A–4C). A line profile analysis of the frequency of actin bundles in the lamellae of KD cells showed that the frequency of bundles was reduced significantly (2.1 ± 0.1 bundles/μm, mean ± SEM, n = 9) compared with controls (2.6 ± 0.2 bundles/μm, mean ± SEM, n = 9, p < 0.05) (Figures 4A and 4B), suggesting that the actin cytoskeleton is being reorganized. Myo9b KD cells contained a distinctive actin-rich area at the cell periphery from which NM2A was largely absent, in addition to an increase in stress fibers (Figures 4A and 4B).
Myo10 KD cells showed loss of filopodia and the appearance of distinctive actin bundles in the central region of the cell (Figures 4A and 4B).

Changes to the F-actin organization were associated with formation of focal adhesions at the edges of the cells, consistent with a more spread cell phenotype (Figure 4C). Knockdown of Myo1b, Myo10, or Myo18a did not change phosphorylation levels of myosin light chain (MLC) (Figure 4D), suggesting that NM2A is re-organized rather than activated as a result of their knockdown. Myo9b KD did increase MLC phosphorylation ~2-fold in cells, and this is likely to contribute to the actomyosin re-organization observed (Figure 4D).

DISCUSSION

These data show that Myo10, Myo9b, and Myo1b are overexpressed in more highly metastatic cell lines and in metastatic tissue. High levels of Myo10 in PC-3 cells are linked to high numbers of filopodia, and high levels of Myo9b are linked to low levels of stress fibers. Both isoforms contribute to a more migratory phenotype, as shown by immunostaining, cell migration, and KD experiments. Myo1b and Myo18a influence cell morphology and actin organization but have little effect on migration in 2D, whereas all four isoforms inhibit cell migration in invasion assays. Therefore, changes in expression of several myosin isoforms may contribute to metastasis in prostate cancer.

Our finding that Myo10-dependent filopodia are likely to be important in prostate cancer agrees with recent similar findings for breast cancer metastasis (Arjonen et al., 2014; Cao et al., 2014) and non-small lung cell cancer (Sun et al., 2015). Filopodia are important but not absolutely required for cell migration because Myo10 KD cells can migrate in 2D but with a reduced speed, and other cells lacking filopodia can migrate (Lundquist, 2009). The increased cell area resulting from Myo10 KD agrees with previous findings (Bohil et al., 2006). The central actin bundles in Myo10 knockdown cells are reminiscent of actin bundles in filopodia. Fascin is also required for filopodial formation (Vignjevic et al., 2006), its overexpression results in multiple filopodia (Vignjevic et al., 2006), and fascin levels are also upregulated in prostate cancer (Darnel et al., 2009). Myo10 KD may lead to actin bundling in the cell body by excess (non-phosphorylated) fascin. The role of two filopodial proteins, Myo10 and fascin, in prostate (and other)
cancers suggest that filopodium formation is key for metastasis. Myo10 has also been implicated in integrin-mediated adhesion, and any reduction in adhesion resulting from its KD could disrupt signaling to the actin cytoskeleton and, therefore, indirectly result in changes in actin organization.

High levels of Myo9b expression in PC-3 cells are likely to contribute to their lack of stress fibers and, therefore, to enhanced migration. The RhoGTPase-activating domain in Myo9b inhibits Rho, reducing the downstream activity of ROCK (RhoKinase), thereby increasing MLC phosphatase activity, reducing MLC phosphorylation (Reinhard et al., 1995; Wirth et al., 1996), and, therefore, reducing actin stress fiber formation. Knockdown of Myo9b is therefore expected to increase MLC phosphorylation and stress fiber formation, as we observed. In agreement with our findings, a previous report has shown that cell migration was reduced and MLC phosphorylation increased in macro-phages isolated from Myo9b knockout mice (Hanley et al., 2010). Myo9b has also been implicated in an increased risk of esophageal cancer (Menke et al., 2012).

The high levels of Myo1b in PC-3 cells and effects of knockdown on 3D invasion, cell shape, and morphology suggest that it, too, has a role in prostate cancer. Myo1b has also been implicated in non-small-cell lung cancers (Ohmura et al., 2015). Myo1b (Myr1/MM1α; Gillespie et al., 2001) regulates actin assembly in vesicular transport (post-Golgi carriers [Almeida et al., 2011] and endocytic organelles [Cordonnier et al., 2001; Raposo et al., 1999]), and it maintains cortical tension at the plasma membrane, where it specifically associates with dynamic, non-tropomyosin-containing actin filaments (Coluccio and Geeves, 1999; Tang and Ostap, 2001). High endogenous levels of Myo1b in more highly metastatic cells might therefore increase cortical tension, allowing cells to move through stiff extracellular matrices in vivo, perhaps explaining why knockdown of Myo1b only affects migration in 3D but not 2D.

Myo18a could contribute to metastasis in prostate cancer. The re-organization of actin and NM2A in Myo18a KD cells may arise from its interaction with non-muscle myosin 2 (NM2). NM2 forms short filaments (~300 nm long) containing ~20 molecules (Billington et al., 2013). The assembly/disassembly of non-muscle myosin 2 filaments is dynamic (Shutova et al., 2014) and regulated by many different pathways (Vicente-Manzanares et al., 2009). Myo18a and NM2A can form mixed bipolar filaments in vitro that are smaller that pure NM2A filaments (Billington et al., 2015). The re-organization of NM2A we observed after knocking down Myo18a, without a change to levels of light chain phosphorylation, supports the idea that an interaction between Myo18a and NM2A modulates NM2A filament formation and organization in PC-3 cells.

Therefore, Myo1b, Myo9b, Myo10, and Myo18a each contribute to the morphology and migration of more highly metastatic PC-3 cells, with each myosin having a specific effect on actin organization. Misregulation of their expression in cells with metastatic potential may allow them to work in concert to generate a torpedo-shaped cell with multiple protrusions that is better able to migrate through a 3D matrix and, therefore, more able to metastasize. Many different drugs have now been developed that can inhibit specific myosin isoforms, including those in classes 1, 2, 5, and 6 (Bond et al., 2015). Developing drugs to block specific myosin functions could be useful in preventing metastasis. Importantly, these results emphasize that myosin not only uses actin as tracks to walk along but that it is able to actively drive actin organization in cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

LNCaP, DU145, and PC-3 cells (from the ATCC) were grown in RPMI 1640 medium with GlutaMAX (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin. 1535NP and CT cells (Bright et al., 1997) were a gift from Suzanne Topalian (Johns Hopkins University School of Medicine). They were grown in keratinocyte medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated FBS, 1% L-glutamate, antibiotics, bovine pituitary extract, and epidermal growth factor.

**Antibodies and Reagents**

The antibodies used were as follows: Myo6 (H-215, Santa Cruz Biotechnology); Myo10 (HPA024223, Sigma); total ERK (p44/42 mitogen-activated protein kinase [MAPK], Cell Signaling Technology); Myo18a, a gift from Prof. Yu and Dr. Hsu (Chang Gung University, Taiwan; Hsu et al., 2010) or from GenScript; NM2A (PRB-440P, Covance); paxillin (SA4502553, Sigma); Myo1b (HPA013607, Sigma); phospho-myosin light chain (Cell Signal); Myo9b (Proteintech); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam). HRP-conjugated secondary antibodies and fluorescent secondary antibodies were from Molecular Probes.

**Transfections**

siGENOME SMARTpool siRNA (GE Healthcare, Dharmacon) was used to silence myosins in PC-3 cells. Cells were seeded at a density of 20,000 cells/ml in growth media and allowed to adhere and grow overnight. Lipofectamine RNAiMAX reagent (Invitrogen, Life Technologies) was used for transfection. Maximum KD was achieved after 72 hr.

**PCR**

The RNAeasy mini kit (QIAGEN) was used to extract cellular RNA. cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase. RT-PCR was used to detect which myosin isoforms were expressed (Table S1). Real-time PCR using SYBR Green was used to investigate the expression levels of expressed myosins (see Table S2 for primer sequences). Data analysis was performed using a Bio-Rad system and software.

**Immunoblotting**

Cells were lysed (30 min, 4°C) in lysis buffer (150 mM NaCl, 0.05 M Tris [pH 8], 1% Triton X-100, and 1 mM EDTA [pH 8]) with protease inhibitor cocktail (Thermo Scientific). Lysates were clarified by centrifugation, protein content was quantified by bicinchoninic acid (BCA) assay, and then samples were mixed with 2x Laemmli buffer for use in protein gels (4%–20% or 7.5%) and blots. Chemiluminescence detection (Supersignal West Pico, Thermo Scientific) used multiple exposures to ensure signal linearity. If required, membranes were stripped using Restore western blot stripping buffer (Thermo Scientific) and re-probed.

**Immunostaining**

Cells were grown on glass coverslips, fixed with 2% paraformaldehyde in PBS, and stained using standard procedures (Swales et al., 2006). Cells were imaged using a DeltaVision deconvolution microscope or Zeiss880 Airyscan.

**Migration Assays**

For 2D assays, cells were plated onto a glass-bottomed 96-well plates, transfected with non-targeting siRNA or with myosin KD siRNA (three replicates each), serum-starved 48 hr later for 24 hr, and then treated with hepatocyte growth factor (HGF) (25 ng/ml) for filming. A minimum of three fields from each replicate was selected for imaging, over 14 hr at 5-min intervals using...
differential interference contrast (DIC) optics, and a 20× lens (512 × 512 total pixel size, 2 × 2 binning) on a DeltaVision system. Cell migration was analyzed using ImageJ software (MTrackJ plugin). To perform the 3D-like circular invasion assay (Yu and Machesky, 2012), cell-free space was created using cell stoppers (ibidi). After removing the stopper, cells were covered with a thin layer of Matrigel (4 mg/ml) and normal medium and allowed to grow and migrate for another 24–48 hr. Cells were then fixed in 2% paraformaldehyde (PFA) and stained.

Data Analysis

Immunoblotts and digitized images of immunostained cells were analyzed using ImageJ. GraphPad Prism 5.0 was used to analyze data. Data are presented as mean ± SD for at least three separate experiments (n ≥ 3). A two-way ANOVA was used to compare differences between groups, and statistical significance was accepted for p ≤ 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and two tables and can be found with this article online at http://dx.doi.org/10.1016/j_cebrep.2015.11.012.

AUTHOR CONTRIBUTIONS


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