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Myosin-Va Contributes to Manifestation of Malignant-Related Properties in Melanoma Cells

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TO THE EDITOR

Melanoma is a highly metastatic and therapeutically resistant cancer, whose incidence has more than tripled in the last decades (Smalley *et al.*, 2010). Physiologically, melanocytes produce and store melanin pigments in the melanosomes, which are transported to the cell periphery and transferred to keratinocytes, a process that requires the tripartite complex Rab27a/melanophilin/myosin-Va (Hume and Seabra, 2011). Myosin-Va is an actin-based molecular motor that also serves a multitude of other functions, such as plasma membrane receptor recycling, exocytosis, association with nuclear speckles and the centrosome (see Woolner and Bement, 2010); interaction with PTEN, thereby modulating PI3K pathway (van Diepen *et al*., 2009), interaction with Bcl-xL, proposed to promote invasion of islet-tumor cells (Du *et al*., 2007); as biomarker of invasiveness for nonfunctioning pituitary adenomas (Galland *et al*., 2010). Moreover, myosin-Va was shown to be up-regulated by Snail to promote cancer cell invasion (Lan *et al.*, 2010), and was postulated to control apoptosis by sequestering the pro-apoptotic protein Bmf, which is unleashed upon loss of cell attachment (Puthalakath *et al*., 2001).

Up-regulation of *MYO5A* gene in melanoma and other cancer types was revealed in different microarray studies compiled here (Table S1; Figure S1). However, these data did not clarify whether *MYO5A* up-regulation was associated with melanocyte transformation or simply reflected tissue specificity since comparison was against normal skin and melanocytes are

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minor cells in the skin. Here, we extended this evidence by showing that *MYO5A* is up-regulated in a variety of melanoma cell lines in comparison with primary melanocytes (Figure 1a), as well as in metastatic cells in comparison to paired vertical growth phase cells (Figure 1b and S2), implicating myosin-Va in malignant transformation and/or melanoma progression. Interestingly, in this WM panel, myosin-Va expression correlated with that of the oncogenic transcription factor *MITF* (Sousa and Espreafico, 2008).

To investigate the role of myosin-Va in melanoma cells, we knocked down this protein using three different shRNAs (shMYO5A#1-3) carried by lentiviral vectors (Figure S3 and Qin *et al.*, 2003) and an siRNA (siMYO5A). Once efficient knockdown was attained (Figures 1c-e), functional studies were performed. Upon adhesion to fibronectin-coated glass coverslips, MYO5A-depleted cells showed numerous small blebs on their surface and reduced lamellipodia/filopodia formation (Figure 1f), besides deficient adhesion (Figure 1g) and spreading (Figure 1h).

Next, we examined the role of myosin-Va in adhesion-independent growth. The ability to form colony in soft agar, as analyzed after 25-30 days of incubation, was at least 50% lower for MYO5A-depleted cells than controls, for the three different shRNAs used (Figure 2a). Proliferation rates under adherent conditions were determined by crystal violet staining for WM1617 (Figure 2b) or ATP measurements for UACC-257 (Figure 2f), and no differences were observed, in the time courses analyzed, between MYO5A-knockdown and control cells. Subsequently, we analyzed transwell migration and invasion and found rates 50 to 70% lower for shMYO5A#2/3-transfected WM1617 cells than controls (Figure 2c). Similar decrease in transwell invasion was observed for siMYO5A-transfected UACC-257 cells (Figure 2e). Next, we performed spheroid assays (as in Smalley and Herlyn, 2008) with shMYO5A#1-transduced cells. Compact spheroids with intact appearance were added to a tri-dimensional collagen gel and imaged after 24 and 48 hours of culture. Myosin-Va-depleted cells exhibited migration distances from spheroid margin to invasion front 50 to 60% shorter than controls (Figure 2d). Also, knockdown cells that migrated out of the spheroids looked smaller than controls after 48 hours, suggesting that myosin-Va-depleted cells differ in the sensitivity to microenvironment factors during migration in collagen matrix.

The multifunctional character of myosin-Va makes us believe that this molecular motor, in addition to its role in cell adhesion/motility by promoting focal adhesion dynamics and filopodia/lamillipodia growth (supported by work in progress from our group, Nader *et al.*), may also perform a role in extracellular matrix proteolysis, mediating surface exposure and positioning of matrix metalloproteinases. Indeed, the alignment of metalloproteinases along the cytoskeleton seems to be a prerequisite for cell invasion in melanoma. Also, co-localization of metalloproteinases with myosin-Va (Sbai *et. al.*, 2011) in astrocytes, and a role for RAB27A (Bobrie *et al.*, 2012) in the release of metalloproteinase-9 to promote metastasis of mammary carcinoma cells have been shown. Moreover, evidence that RAB27A (Akavia *et al.*, 2010) functions as a driver of cancer supports the hypothesis that, likewise, myosin-Va promotes malignancy by functioning in vesicular trafficking. Indeed, endocytosis and recycling of plasma membrane receptors require Rab GTPases and molecular motors with reflexes in adhesion dynamics, cell signaling and metabolism in

many instances shown to drive oncogenic transformation and invasion (Mosesson *et al.*, 2008). Furthermore, the relevance of our findings is supported by recent report demonstrating that the formation of filopodia is a critical step in the metastasis cascade (Shibue, *et. al.*, 2013).

Additionally, we cannot rule out the possibility that some of the effects observed could be due to an increase in the rates of apoptosis in the MYO5A knockdown cells. Although we have not observed alteration of viability after myosin-Va depletion in short term culturing under regular conditions, increase of apoptosis rates under adhesion blockage and poor recovery of frozen stocks were noted. In fact, recent independent findings reinforce participation of myosin-Va in the control of apoptosis. Bmf sequestration to the actin cytoskeleton, presumably in complex with myosin-Va/DLC2, promotes resistance to MEK-inhibitors (Van Brocklin *et al.*, 2009). Accordingly, overexpression of myosin-Va tail fragments harboring the binding site for DLC2 leads to apoptosis in melanoma cells likely by disrupting Bmf and probably also Bim anchorage (Izidoro-Toledo and Borges *et. al*., 2013). Finally, miR-145, which is a transcriptional target of p53 and known to act as a tumor suppressor, was recently shown to target myosin-Va (Dynoodt *et. al.*, 2012). Therefore, myosin-Va may integrate mechanisms that interconnect invasion/migration machinery and resistance to apoptosis. Interdependencies between these processes are reviewed in Alexander and Friedl (2012).

In summary, the data presented here show that myosin-Va promotes adhesion dynamics, anchorage-independent survival, migration and invasion *in vitro*. Therefore, up-regulation of myosin-Va during melanoma progression may be part of a general mechanism that promotes malignant properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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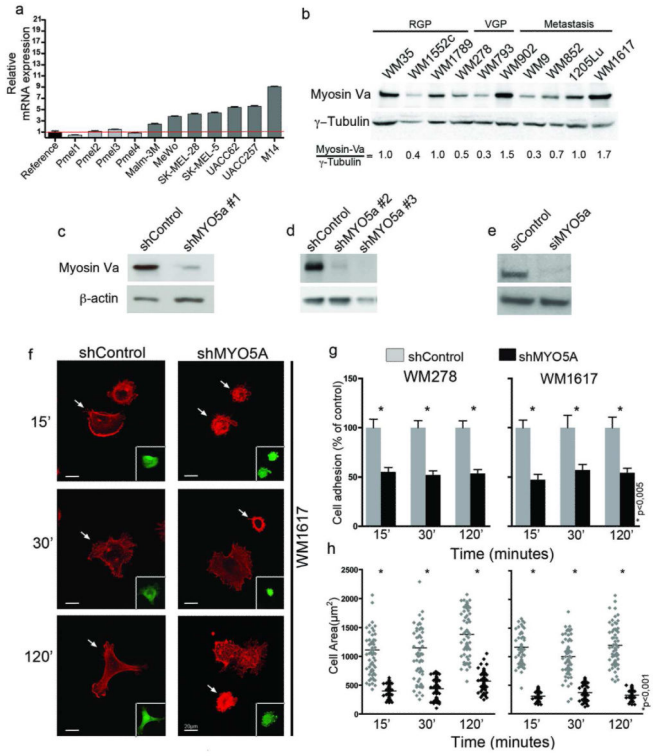

Figure 1. Myosin-Va is highly expressed in melanoma cells and its knockdown impairs cell adhesion and spreading on fibronectin-coated surface

(a) Relative *MYO5A* mRNA expression detected by qPCR in melanocytes (pMel1 to 4) versus melanoma cells, using β-actin for normalization and mean of all melanocytes as reference value. **(b)** Western-blot of myosin-Va in a panel of melanoma cell lines of radial growth phase (RGP), vertical growth phase (VGP) and metastasis (M), including two genetic pairs (WM793 and 1205Lu; WM278 and WM1617). **(c-e)** Western-blots for myosin-Va in **(c, d)** WM1617 and **(e)** UACC-257. WM1617 cells were lysed 3 days after transduction with lentiviral vectors carrying shRNAs targeted to bacterial Lac-Z (shControl) or MYO5A (shMYO5A#1), or after stable selection with antibiotics for about 2-3 weeks in the case of shMYO5A#2-3 and respective shControl (Figure S3). UACC-257 cells were lysed 3 days after transfection with siRNA against myosin-Va or control. **(f)** Confocal images of F-actin stained cells adhered to fibronectin-coated coverslips for the indicated times. Arrows indicate transduced cells visualized by GFP expression (inserts). Scale bar = 20μm. **(g)** Cells allowed to adhere on fibronectin-coated surface for the indicated times were counted and data were plotted as mean ± SD from 3 independent experiments. **(h)** Cell spreading. Imaged as in (f) and the areas for 60 cells/time point were measured using Image[J].

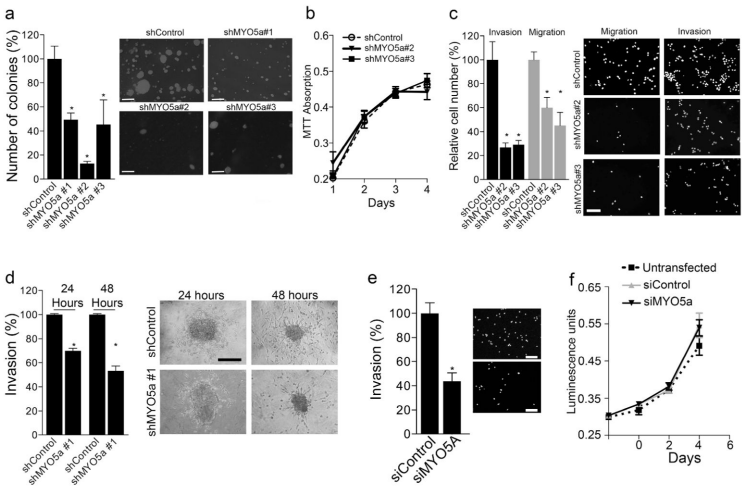

Figure 2. Ablation of myosin-Va inhibits colony formation, migration and invasiveness of metastatic melanoma cells without affecting cell proliferation

(a-c) Lentiviral transduced WM1617 cells, using three independent shMYO5A or shControls, were used to assess: **(a)** Colony formation in soft-agar after 30 days of growth. Scale bar = 500μm; **(b)** Proliferation rates by absorbance measurement of crystal violet staining; **(c)** Migration in transwell and invasion in transwell-matrigel assay. Cells were kept in starvation conditions 24 hour prior the assay and were then allowed to migrate/invade for 24 hours. Scale bar = 50μm. **(d)** Migration in 3D collagen. After 24 or 48 hours of incubation - distance from spheroid edge to invasive front was measured and the data from three independent experiments were plotted as a percentage of control. Scale bar = 100μm. **(e-f)** Transwell invasion and proliferation rates of UACC257 cells transiently transfected with duplex siRNA targeted to MYO5A and irrelevant siRNA. Invasion assay was done as described in **c**, and proliferation rates were estimated based on ATP measurements. Data were plotted as mean ± SD from 3 independent experiments. Scale bar = 50μm.