

Review

MIA - a new target protein for malignant melanoma therapy

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Summary. Malignant melanoma, a malignancy of pigment producing cells, causes the greatest number of skin cancer-related deaths worldwide. The tumor is characterized by its aggressive phenotype and can metastasize at very early stages of the disease. Since metastatic lesions are usually characterized by an intrinsic resistance to standard radiation and chemotherapy, the prognosis of this tumor remains very poor in advanced stages.

Melanoma inhibitory activity (MIA), an 11 kDa protein expressed and secreted by melanoma cells after their malignant transformation, is known to play a key role in melanoma development, progression and tumor cell invasion. After its secretion, which is restricted to the rear pole of migrating cells, MIA protein directly interacts with cell adhesion receptors and extracellular matrix molecules. By this mechanism, MIA protein actively facilitates focal cell detachment from surrounding structures at the cell rear and strongly promotes tumor cell invasion and formation of metastases. It has further been demonstrated that MIA contributes to immunosuppression frequently seen in malignant melanomas by binding to integrin $\alpha_4\beta_1$ expressed by leukocytes and thus inhibiting cellular antitumor immune response. Analyses at the molecular level revealed that MIA protein reaches functional activity by self assembly. Functional inactivation of MIA protein by dodecapeptides that directly bind to the dimerization interface leads to a strongly reduced tumor cell invasion in an *in vivo* mouse melanoma model. The molecular understanding of the contribution of MIA protein to formation of metastases provides an excellent starting point for the development of a new strategy in malignant melanoma therapy.

Key words: Malignant melanoma, MIA, Peptide inhibitor, Metastasis, Immunosuppression

Malignant melanoma – incidence and risk factors

Malignant melanoma is the most aggressive type of cutaneous cancer originating from pigment producing cells in the skin, the melanocytes. This highly invasive tumor is known for its uncontrollable growth and for its ability to give rise to metastases into several tissues even at early stages of the disease (Varney et al., 2006; Gray-Schopfer et al., 2007; Greinert, 2009). The incidence of malignant melanoma is currently 18.000 newly diagnosed cases per year in Germany alone and it is rising in caucasian populations, with the highest recorded incidence occurring in Australia, where the annual rates are 10 and over 20 times the rates in Europe for women and men, respectively. Exposure to UV radiation appears to be a significant risk factor for the development of malignant melanoma (de Gruijl et al., 2001; Cleaver and Crowley, 2002; Abdel-Malek et al., 2008; Besaratinia and Pfeifer, 2008). From an initial phase of radial growth, the tumor may evolve into the more dangerous vertical growth phase. In advanced cases, tumor cells acquire a strong potential to disseminate. Metastatic lesions are usually characterized by an intrinsic resistance to both standard radiation and chemotherapy, thus representing one of the major causes of the very poor prognosis of the tumor (Scheier et al., 2011).

The subsequent promotion from radial to vertical growth phase through the basement membrane or matrix proteins serves as a progression mechanism for melanoma. This exceptionally high migratory potential is probably inherent in these cells, since it is also observed during or shortly after neurulation, an embryological event marked by neural tube closure. In this process, neural crest cells, including the later

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pigment producing cells, quickly migrate after migratory stimuli by inhibitory or attractive signals to their destination and proliferate there. Nowadays, it has been described that many of the genes expressed in melanoma cell lines and melanocytic tumors are required specifically during melanoma development, and similar categories of genes are expressed in metastatic melanoma as well as in migratory neural crest cells (Clark et al., 2000; Gammill and Bronner-Fraser, 2002; Segal et al., 2003). At present, it is discussed whether melanoma cells reactivate their strong migratory ability during cancer development.

However, the molecular mechanisms that are involved in melanoma growth and progression are still poorly understood. All previous attempts at targeted therapies, although the targets chosen were relevant, did not lead to success in the treatment of melanoma patients. New target molecules in melanoma therapy are therefore urgently needed.

Facilitation of tumor cell detachment from the pericellular matrix promotes invasion and formation of metastases

To build up metastases in healthy tissues and organs distant from their origin, cancer cells, released from the primary tumor spread through blood vessels, lymphatic ducts, or cavities to form colonies. It is a highly dynamic process that essentially depends on the ability of cancer cells to detach from the pericellular matrix, migrate, invade intact tissue structures, and finally to overcome physiological barriers such as basement membranes upon intra- and extravasation.

Cell migration, physiologically exhibited during wound healing, angiogenesis, embryonic development, and immune function, is initiated by migratory stimuli triggered by attractants like chemokines and ECM gradients (Lauffenburger and Horwitz, 1996). Generally, cells respond by local activation and amplification of signaling events facilitating localized actin polymerization leading to morphological polarity and establishment of a dominant-leading pseudopodium and rear cell body compartment (Lauffenburger and Horwitz, 1996; Parent and Devreotes, 1999). Integrin cell adhesion receptors tether the extending membrane to the substratum by the formation of new focal complexes at the leading front of the extending membrane. This adhesion process provides the necessary signals to fine-tune and maintain directional movement, while retraction mechanisms are suppressed. Cell movement then commences as the cell undergoes repeated cycles of membrane extension and integrin ligation at the front to provide traction points, and cell body retraction at the rear (Lauffenburger and Horwitz, 1996; Schwab et al., 2007). Changes in cell shape occurring during migratory processes are further supported by ion channels and aquaporins regulating cell volume by fluctuations (Schwab et al., 2007). Intracellular Ca^{2+} plays a crucial

role in almost all cellular processes, including regulation of membrane-fusion as well as cell migration (Brundage et al., 1991; Hahn et al., 1992; Schwab et al., 1994; Komuro and Kumada, 2005). By modulating turnover of actin filaments, involvement of calpain and recycling of integrins, Ca^{2+} coordinates different components of the cellular migration machinery (Pettit and Fay, 1998; Franco and Huttenlocher, 2005).

Clinical relevance and structural analysis of MIA protein

MIA (melanoma inhibitory activity) protein has been described to play a key role in melanoma development and progression (Bosserhoff et al., 1997). In order to identify autocrine growth-regulatory factors secreted by melanoma cells, MIA, an 11 kDa protein, strongly expressed and secreted by melanocytic tumor cells, was purified from tissue culture supernatant of the human melanoma cell line HTZ-19 (Blesch et al., 1994; Bosserhoff et al., 1997). MIA protein is not detectable in normal skin. Physiologically, it is expressed in cartilage and plays a role in chondrocyte differentiation (Bosserhoff and Buettner, 2003; Schmid et al., 2010). Interestingly, MIA expression is induced by UV irradiation, which resembles a link between the role of UV in melanoma induction and MIA expression (Marr et al., 2004). MIA protein strongly contributes to the invasive and migratory potential of melanoma cells and promotes the formation of tumor metastases (Bosserhoff et al., 1997, 2003). Increased MIA protein plasma levels directly correlate with progressive malignancy and a more advanced state of melanocytic tumors (Guba et al., 2000; Bosserhoff et al., 2001). Thus, MIA protein serves as a reliable clinical tumor marker to detect and monitor metastatic diseases in patients suffering from malignant melanoma (Dreau et al., 1999; Stahlecker et al., 2000). In 2001, the three-dimensional structure of the protein was solved by multidimensional nuclear magnetic resonance (NMR) and X-ray crystallography techniques (Stoll et al., 2000; Loughheed et al., 2001). Structural analysis revealed that MIA protein comprises an SH3 domain-like fold in solution, a structure with two perpendicular, antiparallel, three- and five-stranded beta-sheets (Stoll et al., 2001, 2003). MIA represents a novel class of secreted proteins comprising an SH3 domain (Stoll and Bosserhoff, 2008). The MIA protein family consists of MIA and the homologous proteins OTOR, MIA-2 and TANGO (MIA-3). In contrast to the other MIA family members MIA protein has a tendency to form dimeric assemblies. By sequence alignment of MIA family proteins it has also been demonstrated that amino acids that are crucial for dimerization are not conserved in the other MIA family members and that a charge inversion at the dimerization interface and even deletions of these structural domains have been found (Schmidt et al., 2012).

Cellular processing of MIA protein during migration

The MIA protein transport was described to take the conventional secretion pathway, including COPI and COPII mediated vesicle sorting in the endoplasmic reticulum and Golgi apparatus, to exit the cell (Schmidt et al., 2010). By N-terminal labeling of MIA protein using a HisTag 3' of the N-terminal secretion sequence, the intracellular protein transport was followed. Interestingly, the transport along the microtubule system to the cell periphery is restricted to the rear of migrating cells (Fig. 1). The final release of MIA protein is a triggered event that depends on an increase in intracellular Ca^{2+} concentration. It was further shown that secretion of MIA protein is significantly augmented by KCNN4 potassium channel activity. This channel type was found to be aberrantly expressed in various tumor types and implicated in the promotion of cell migration and cell proliferation (Jäger et al., 2004; Tajima et al., 2006; Wang et al., 2007; Lallet-Daher et al., 2009). In migrating cells, KCNN4 channel activity was detected predominantly at the rear cell pole, which may be due to the intracellular Ca^{2+} gradient in polarized, migrating cells (Schwab et al., 1995). As illustrated in Fig. 2, MIA protein strongly contributes to the invasive and migratory potential of melanoma cells by inhibiting attachment to extracellular matrix structures, including fibronectin, laminin and tenascin *in vivo* (Bossertoff et al., 1998; Stoll et al., 2001; Bauer et al., 2006). Following localized protein secretion, a direct interaction of MIA protein with cell adhesion receptors integrin $\alpha_4\beta_1$ and $\alpha_5\beta_1$ results in locally reduced cell adhesion contacts. Formed MIA-integrin-complexes are subsequently internalized into the cell at the rear

(Schmidt and Bossertoff, 2009). This localized uptake of MIA protein results in focal cell detachment at the cell rear and allows a directed cellular movement. Once internalized, these complexes are dissociated and MIA protein is degraded in lysosomes, while integrins are recycled and transported to the cell front in order to form new adhesion contacts. Changes in metastatic behavior in correlation with the expression level of MIA provide evidence that upregulation of MIA expression during malignant transformation of melanocytic cells is causally involved in acquisition of the malignant cancer cell phenotype (Guba et al., 2000).

The cell adhesion molecule integrin $\alpha_4\beta_1$ (VLA-4) is expressed by human blood leucocytes and plays a key role in the immune response by governing the infiltration of T-cells into the tumor tissue mediating cellular antitumor immune reaction. By binding of MIA protein to integrin $\alpha_4\beta_1$ (VLA-4) molecules on the cell surface of leucocytes it competitively masks the binding site and as a consequence leukocyte attachment is inhibited and cell migration is decreased. Further, integrin-mediated molecular crosstalk is inhibited and the activation of immune cell response and their antitumor cytotoxicity is suppressed. On this account, tumor-derived MIA protein has been discussed to enforce immune escape mechanisms commonly found in melanoma patients (Jachimczak et al., 2005).

Functional inhibition of MIA protein

NMR spectroscopy and Western blot analysis revealed that MIA protein has a tendency to form dimeric assemblies with head-to-tail linkages. By functionally analyzing MIA protein mutants *in vitro* it

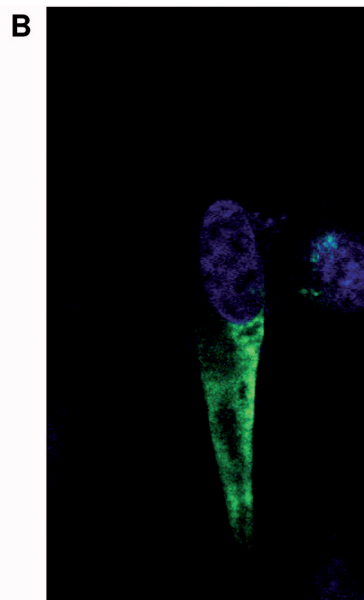
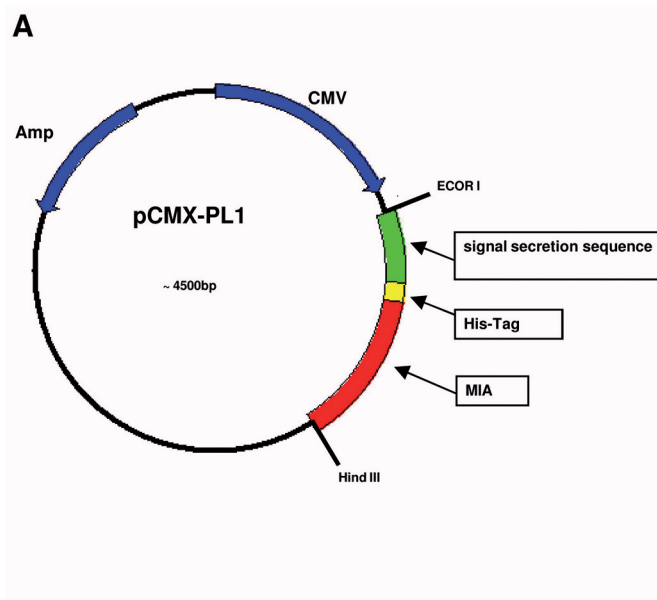


Fig. 1. MIA protein transport is restricted to the cell rear of migrating cells. **A.** Cloning strategy of the MIA-His-tag construct. MIA (red) with a His-tag (yellow) and a preceding N-terminal secretion sequence (green) was cloned into the pCMX-PL1 vector. **B.** After cleavage of the N-terminal secretion sequence, His-tagged MIA protein (green) is transported along the microtubule system to the rear of migrating cells. The cell nucleus is stained with DAPI (blue).

was demonstrated that MIA protein achieves functional activity by self assembly (Schmidt et al., 2012). Monomeric species are inactive, suggesting that the binding site for integrins, probably generated by protein assembly, could be located at the surface involving both MIA subunits. Inactivation of MIA protein by peptidic dimerization inhibitors provides an excellent starting point for the development of a new inhibitory strategy to reduce tumor cell invasion and formation of metastases. To selectively screen for substances that directly bind to the MIA protein dimerization domain and thus generate

inactive monomers, a transition metal based fluorescence polarization assay was established (Riechers et al., 2009). AR71, a dodecapeptide identified in this screening assay, was analyzed for its MIA inhibitory potential *in vitro* and *in vivo* studies. Binding to the dimerization domain was confirmed by NMR-spectroscopy. Interestingly, inhibition of MIA protein function in an *in vivo* mouse melanoma model of hepatic metastasis led to strongly reduced numbers of metastases in mice being treated with daily iv injections of peptide AR71 as shown in Fig. 3 (Schmidt et al., 2012). Further,

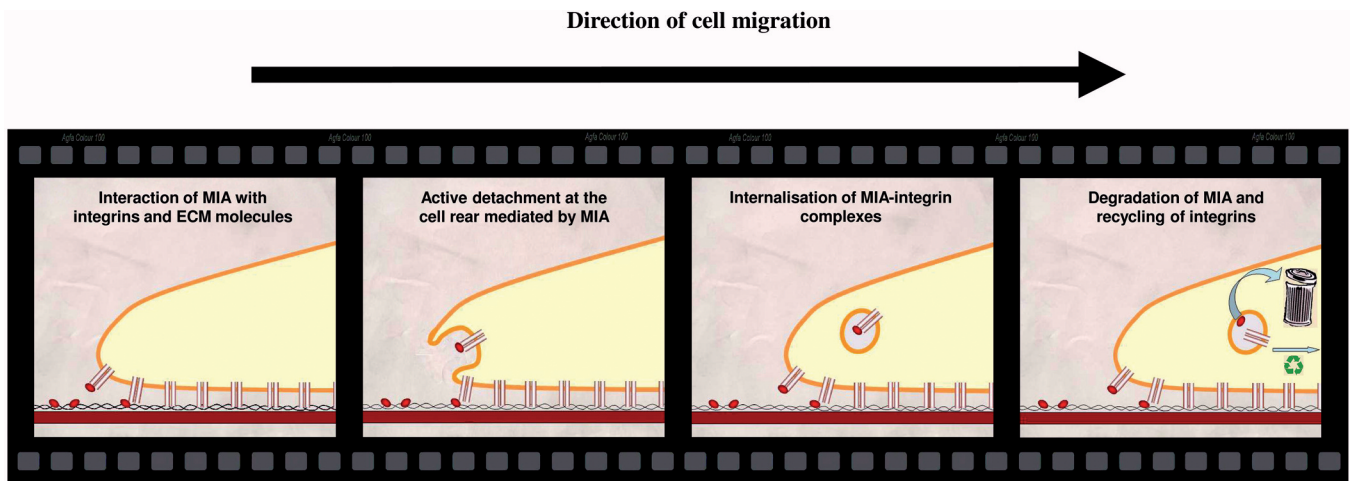


Fig. 2. Processing of MIA protein during cellular migration. MIA protein promotes localized cell detachment at the rear cell pole by modulating integrin activity. By directly binding to these cell adhesion receptors and extracellular matrix structures it thus strongly contributes to the invasive and migratory potential of melanoma cells. Formed MIA-integrin-complexes are subsequently internalized into the cell at the rear pole. This focal cell detachment allows a directed cell movement. After internalization, MIA-integrin complexes are dissociated, MIA protein is degraded in acidic vesicles while integrins are recycled and transported to the cell front in order to form new adhesion contacts.

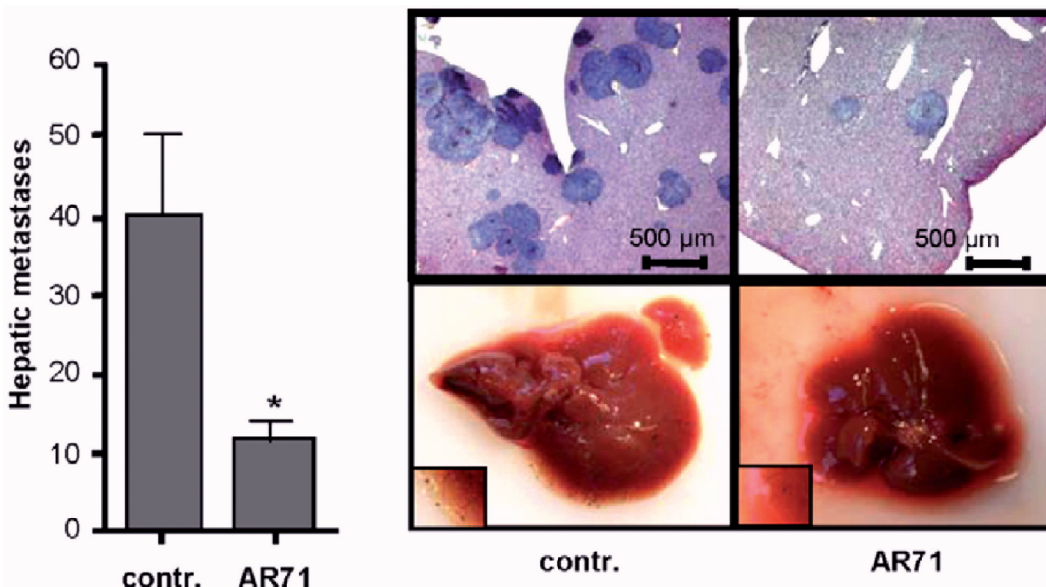


Fig. 3. Dodecapeptide AR71 strongly reduces formation of melanoma metastasis *in vivo*. Wild type murine B16 melanoma cells were injected into the spleen of B1/6N mice with the mice being subsequently treated with i.v. injections of AR71. Histological analyses revealed a significant reduction of metastases in the livers of mice treated with AR71 compared to the livers of untreated control mice. This figure was adapted from our own previously published work (Schmidt et al., 2012).

it has been shown that MIA-induced immunosuppression was also inhibited; increased numbers of CD3⁺ cells and activated caspase 3 were detected in the metastases of mice by immunohistochemistry analyses. Based on these data, the rational design and development of a novel pharmacophore which inhibits MIA protein dimerization may provide a key element in malignant melanoma therapy (Schmidt et al., 2012).

Antimetastatic agents as a new therapy strategy

Conventional chemotherapy treatments target all fast dividing cells and thus indiscriminately kill cancer cells, as well as hair follicle cells and cells in the mucous membranes in the gastrointestinal tract, for example. Targeted melanoma treatments include Vemurafenib and Imatinib, both targeting specific mutations like BRAF V600E and activating c-kit mutation, respectively. By the use of MIA inhibitory compounds the expected side effects of treatment should be minimal because healthy normal cells do not express MIA protein and thus will not be affected; MIA protein is only expressed in malignant melanoma cells and in early-phase differentiating chondrocytes. Even MIA-deficient mice show only minor phenotypic changes in cartilage (Moser et al., 2002). Further, therapeutic intervention by targeting of MIA protein does not only affect a mere subset of tumors comprising specific mutations, but all melanomas. Next to cytotoxic compounds and angiogenesis inhibitors, a MIA inhibitor will present a novel antimetastatic therapeutic strategy of cancer treatment by targeting both the metastatic spread and the immunosuppression of malignant melanoma. Only in rare cases is the primary tumor fatal for patients suffering from malignant melanoma. Mostly, the cause of death is the failure of vital organs due to the formation of metastases. By targeting MIA, the development of antimetastatic agents in combination with reduced immunosuppression in malignant melanoma could be of great importance and a milestone in the treatment of this disease.

Acknowledgements. Funded by: DFG, German Cancer Aid.

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