MSX1-induced neural crest-like reprogramming promotes melanoma progression

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Abstract

Melanoma cells share many biological properties with neural crest stem cells. Here we show that the homeodomain transcription factor Msh homeobox 1 (MSX1), which is significantly correlated with melanoma disease progression, reprograms melanocytes and melanoma cells towards a neural crest precursor-like state. MSX1-reprogrammed normal human melanocytes express the neural crest marker p75 and become multipotent. MSX1 induces a phenotypic switch in melanoma, which is characterized by an oncogenic transition from an E-cadherin-high non-migratory state towards a ZEB1-high invasive state. ZEB1 up-regulation is responsible for the MSX1-induced migratory phenotype in melanoma cells. Depletion of MSX1 significantly inhibits melanoma metastasis *in vivo*. These results demonstrate that neural crest-like reprogramming achieved by a single factor is a critical process for melanoma progression.

Introduction

The neural crest is a transient structure of the vertebrate embryo that gives rise to many types of derivative cells, including neurons and epidermal melanocytes. Neural crest cells migrate extensively to reach distinct sites within the developing embryo and those that migrate along the dorsolateral pathway give rise to melanocytes of the skin. The switch from a multipotent precursor towards a committed melanoblast is made early by expression of the melanocyte-restricted isoform of microphthalmia-associated transcription factor (M-MITF), which is required both for fate determination and maintenance of melanocytic cells (Bentley et al., 1994, Hemesath et al., 1994, Yasumoto et al., 1994). We have recently shown that activation of Notch1-signaling alone is sufficient to reprogram foreskin-derived melanocytes to multipotent stem cells, which are functionally equivalent to neural crest cells (Zabierowski et al., 2011). Others have shown that ectopic endothelin-3 is able to convert clonal cultures of pigment cells to glial cells, putatively through a bipotent glial-melanocytic progenitor (Dupin et al., 2000). These studies imply that the stepwise differentiation from neural crest cells to melanocyte precursors, and then to mature melanocytes, is not a one-way road, but can be reversed by reactivating genetic and/or epigenetic signatures that favor a stem cell-like phenotype.

Msh homeobox 1 (MSX1) is a homeodomain transcription factor that plays an important role in the establishment of the early neural crest (Monsoro-Burq et al., 2005, Ramos and Robert, 2005). During early embryogenesis, a complex gene regulatory network coordinates the formation of the neural crest at the border

of the neural plate and the non-neuralized ectoderm. Proteins expressed by the adjacent paraxial mesoderm determine the expression of transcription factors that act to induce neural crest formation; more specifically, intermediate concentrations of BMP4 induce the expression of MSX1 (Tribulo et al., 2003). The observation that ectopic expression of MSX1 is sufficient to dedifferentiate myotubes to mononucleated precursor cells (Odelberg et al., 2000) led us to hypothesize that the reactivation of MSX1 would dedifferentiate human melanocytes to a similar precursor state.

Malignant melanomas develop from melanocytes. Highly aggressive, therapy-refractory melanomas often lack pigment-related markers, but instead express neural crest-specific genes (Bailey et al., 2012). The various implications of neural crest genes in cancer prompted us to investigate whether MSX1 contributes to an aggressive phenotype in melanoma. Here, we examined the role of MSX1 in melanocytes and in melanoma. MSX1 is highly expressed in multipotent neural crest stem cell-like cells (NCSC-like cells) isolated from human dermis and in a panel of melanoma cell lines and patients' tissues. MSX1-transduced melanocytes lost pigmentation and gained the expression of neural crest markers, suggesting that these cells represent a dedifferentiated phenotype. Furthermore, these melanocytes were able to survive under human embryonic stem cell (hESC) culture conditions and were susceptible to differentiation into neuronal and mesenchymal lineages. Overexpression of MSX1 promoted cell motility in both melanocytes and melanoma cells and induced substantial changes in cell morphology, while silencing MSX1 by shRNA significantly inhibited

melanoma migration *in vitro* and metastasis formation *in vivo*. Taken together, these results suggest that the neural crest-like reprogramming process conferred by MSX1 contributes to the metastatic spread of melanoma.

Results

MSX1 attenuates pigmentation and alters the expression of adhesion molecules in melanocytes

Melanoma is considered to begin in transformed melanocytes. However aggressive melanoma cells share many biological properties such as cell plasticity and invasion with the neural crest, which is the embryonic origin of melanocytes. To investigate the molecular mechanisms that dedifferentiate melanoma cells to a neural crest-like state, we utilized NCSC-like cells as a model. NCSC-like cells existing in human skin display the capacity for self-renewal and are able to differentiate into multiple neural crest-derived lineages, including melanocytes (Li et al., 2010, Toma et al., 2005). We have recently shown that differentiated melanocytes can be fully reprogrammed to multipotent NCSC-like cells by the reactivation of Notch1-signaling. These reprogrammed cells were devoid of pigment and lost expression of E-cadherin (Zabierowski et al., 2011). MSX1, which is an essential molecule for neural crest specification, was one of the most significantly up-regulated genes in Notch-induced NCSC-like cells (Tribulo et al., 2003, Zabierowski et al., 2011). Thus we hypothesized that MSX1 is a major player in the reprogramming process. NCSC-like cells expressed high

endogenous levels of MSX1, whereas differentiated melanocytes barely expressed MSX1 (Figure 1A). The difference in expression of MSX1 between NCSC-like cells and somatic pigment cells suggested a role for MSX1 in maintaining the neural crest-like state. We stably expressed MSX1 in melanocytes using an MSX1-coding lentiviral vector (pLU-EF1αL-MSX1-IRES-BLAST). After 2 to 3 weeks of stable expression, we detected a dramatic decrease in pigmentation in MSX1-melanocytes (Figure 1B). Most melanocytes (5 of 7 lines) that were transduced with MSX1 lost pigmentation macroscopically, even though the level of pigmentation varied between donors. Consistent with the overt decrease in pigmentation, M-MITF was strongly reduced at the protein level (Figure 1C). Furthermore, *M-MITF* mRNA levels were decreased (Figure 1D), suggesting that M-MITF was regulated at the transcriptional level and not by diverse post-translational modifications, which can also regulate its activity (Murakami and Arnheiter, 2005, Wu et al., 2000). During development, expression of tyrosinase is induced under the control of M-MITF and is directly correlated with the amount of pigment in primary melanocytes. The expression of tyrosinase in MSX1-melanocytes decreased by approximately 80% compared to basal levels of control cells (Figure 1D). In contrast, MSX1 overexpression induced only a slight decrease in MITF expression and no change in tyrosinase expression in the melanocyte line, which did not macroscopically lose pigmentation (Figure S1). This suggests that the rate of MITF reduction by MSX1 is correlated with the impact in pigmentation loss in melanocytes. MSX1-melanocytes grown as an adherent monolayer lost the classical melanocytic morphology of long and smooth dendrites, and instead, gained a mesenchymal appearance with shorter dendrites and a triangular shape (Figure 1E). In addition to the loss of

pigmentation, ectopic expression of MSX1 in melanocytes had a significant impact on the expression of adhesion molecules, as the expression of E-cadherin was strongly reduced in MSX1-melanocytes (Figure 1F). Ectopic expression of MSX1 in melanocytes consistently increased the expression of L1 cell adhesion molecule (L1CAM), a transmembrane glycoprotein that plays a central role in axon guidance and cell migration in the nervous system (Maness and Schachner, 2007) (Figure 1F). Altogether, exogenous expression of MSX1 alone was sufficient to impede the melanogenic activity of melanocytes and to alter the expression of cellular adhesion molecules.

MSX1 elicits stem cell-like properties in melanocytes

The depigmentation potential of MSX1 in melanocytes, similar to the Notch1-reprogrammed NCSC-like cells, led us to hypothesize that MSX1 may dedifferentiate epidermal melanocytes towards a neural crest-like state. An important hallmark of neural crest cells is their ability to migrate to distant sites throughout the developing embryo. To address whether MSX1 regulated cell migration, we subjected MSX1-melanocytes to Boyden Chamber assays. The migratory potential of MSX1-melanocytes was significantly higher compared to control vector-infected melanocytes (Figure 2A). When cultured in melanocyte-specific medium (254CF), we observed that MSX1-cells had a tendency to cluster together and form spheres, which is the classic growth pattern of neural precursor cells *in vitro* (Fernandes et al., 2004, Toma et al., 2001) (Figure 2B, upper left panel, white arrows). These spheres were morphologically similar to dermis-derived NCSC-like spheres and to Notch1-

reprogrammed melanocytes (Figure 2A, middle left panel) (Li et al., 2010). The formation of spheres was more evident under hESC growth conditions (StemPro®). After 2 weeks of exposure to StemPro® in low attachment plates, MSX1-melanocytes formed tight round spheres, whereas GFP-transduced control cells died off in necrotic clusters (Figure 2B, top and bottom right panels). Notch1-reprogrammed melanocytes served as positive control (Figure 2B, middle right panel). Next, we evaluated cell death in StemPro® using a low-attachment surface. After 7 days, we observed that control cells formed irregular cell clusters while MSX1-spheres appeared round and regular in form and size (Figure 2C). The necrotic clusters that formed from control cells broke apart over time, and cells were almost all dead within 2 weeks. In contrast, throughout the experimental time of 3 weeks, MSX1-cells formed tight spheres. Only a small number of cells showed EthD-1 positivity, which was indicative of central necrosis (Figure 2C). These results suggested that MSX1 confers melanocytes stem cell-like properties such as sphere-forming ability and a highly migratory phenotype.

MSX1-melanocytes are able to differentiate to neural crest derivatives

Our observation that ectopic MSX1 enables mature melanocytes to survive under stem cell-selective conditions implied that MSX1-melanocytes are similar to neural crest-derived progenitors. Notably, MSX1-spheres expressed high levels of p75 (nerve growth factor receptor, CD271), a well-reported neural crest marker, at levels similar to NCSC-like cells (Figure 3A). The expression of p75 was not restricted to MSX1-spheres, as MSX1-expressing melanocytes that were grown as an adherent layer displayed intermediate

levels of p75, while control cells did not (Figure 3B). These data suggest that MSX1-cells up-regulate expression of the neural crest marker p75 under hESC conditions, regardless of sphere formation. We therefore investigated whether MSX1-melanocytes could give rise to neural crest derivatives. When cultured in differentiation media, MSX1-melanocytes started to express lineage-specific markers and underwent characteristic morphological changes (Figure 3C). Transdifferentiated smooth muscle cells presented smooth muscle actin filaments in their abundant cytoplasm (Figure 3C, top panels). Upon neuronal differentiation, neurofilament-L was predominantly expressed in the perinuclear cytoplasm, an area that corresponds to axon hillocks in neurons. Some cells even developed very long and subtle dendrites resembling an entire axon (Figure 3C middle panels and Figure S2, white arrow and white dotted line, respectively). Adipogenesis was confirmed by Oil Red O staining (Figure 3C, bottom panels). Approximately 5% (smooth muscle actin filament and neurofilament-L) and 1% (Oil Red O) of cells exhibited these distinctive changes, indicating that this differentiation process was not very efficient. Together, these data demonstrated that ectopic MSX1 dedifferentiates mature melanocytes to a multipotent, neural crest-like state.

Expression of MSX1 correlates with disease progression in melanoma

The experiments in melanocytes suggested that MSX1 induces neural crest-like reprogramming in the melanocytic lineage. The process of dedifferentiation has been implicated in cancer progression. We therefore hypothesized that MSX1 is reactivated in melanoma and contributes to disease progression. Notably,

expression of MSX1 was up-regulated in most melanoma cell lines tested compared to normal melanocytes. WM35 (RGP), WM115 (VGP), and WM858 (metastatic phase) cells displayed lower endogenous levels of MSX1 compared to 451Lu (metastatic phase) and WM1366 (VGP) cells, which exhibited strong expression of MSX1 (Figure 4A). Since a correlation between total levels of MSX1 and disease progression was not obvious from our panel of melanoma cell lines, we used publicly available dataset GSE12391 (Scatolini et al., 2010) to analyze the relationship between expression of MSX1 and clinical stage of melanoma. This dataset consists of 58 nevus, 16 RGP melanoma, 30 VGP melanoma, and 10 melanoma metastasis samples. Within this cohort, expression of MSX1 was significantly correlated with disease progression ($p = 5.15 \times 10^{-6}$) (Figure 4B). Furthermore, out of 7 primary melanomas, 3 showed strong positivity for MSX1 staining (Figure S3). MSX1 was expressed specifically in melanoma cells and not in adjacent stromal cells or in keratinocytes. Both nodular melanoma and superficial spreading melanoma tissues expressed MSX1 regardless of their differences in histological type. To assess the clinical significance of MSX1 in a bigger cohort, we analyzed TCGA melanoma RNA-seq data together with the patient clinical information. Kaplan-Meier analysis showed that patients with higher levels of MSX1 expression had significantly poorer overall survival (Figure 4C). Genetic alteration of MSX1 in patient melanoma samples was observed in only 2% (6 out of 278 cases), suggesting that MSX1 expression is regulated by epigenetic mechanisms in majority of melanoma cases (Figure 4D).

MSX1 induces phenotype switching and promotes migration of melanoma cells

Since the process of dedifferentiation has been implicated in cancer progression, we investigated whether MSX1 contributes to an aggressive phenotype in melanoma. We performed gain of function-experiments in two melanoma cell lines that exhibited low intrinsic levels of MSX1: radial growth phase (RGP) WM35 cells and metastasis-derived WM3451 cells (Figures 5). The effects of MSX1 on cell morphology of WM35 cells were not evident when cells were cultured in Mel2% (Figure S4A, left panels). However, upon exposure to hESC media, control cells formed large clusters, the typical growth pattern of WM35 cells, whereas MSX1-WM35 cells were finely dispersed and grew in multiple small colonies (Figure S4A, right panels). WM3451 control cells retained their bipolar spindle-shape, while MSX1-WM3451 cells were polygonal and flattened (Figure S4B). MSX1 increased the migration of WM35, a cell line that has little migratory and invasive potential *per se* (Figure 5A). In WM3451 cells, expression of MSX1 led to a 7-fold increase in migration (Figure 5B). Importantly, ectopic MSX1 did not alter cell growth and proliferation in any of the cells (Figures 5C). Switching between a MITF-high differentiated phenotype and a Wnt5a-high invasive phenotype is implicated in melanoma (Eichhoff et al., 2011, Hoek et al., 2008). Similarly, a previous report suggested that a transcriptional switch from ZEB2 to ZEB1 and loss of E-cadherin is critical to melanoma initiation and progression (Caramel et al., 2013). MSX1 overexpression in WM35 cells increased *WNT5A* and *ZEB1*, and down-regulated *MITF*, *ZEB2,* and E-cadherin (Figures 5D and 5E). This suggests that MSX1 promotes a dedifferentiated and invasive phenotype in melanoma cells. In order to identify other downstream molecules responsible for MSX1-induced phenotypes,

we tested whether the MSX1-induced migration could be rescued by overexpression of MITF or depletion of ZEB1. ZEB1 knockdown, but not MITF overexpression, significantly reduced the MSX1-induced migratory phenotype in WM35 (Figure 5F and 5G, S5). These results suggested not MITF down-regulation, but ZEB1 up-regulation was responsible for the MSX1-induced migratory phenotype in melanoma cells.

Depletion of MSX1 decreases melanoma migration and liver metastasis

We then performed loss-of-function experiments in melanoma cells using shRNAs specific to MSX1. Silencing of endogenous MSX1 impaired cell migration in both clones compared to control vector-infected cells (Figure 6A), while it did not alter cell proliferation (Figure 6B). To evaluate the functional significance of MSX1 in melanoma tumorigenicity *in vivo,* we injected 451Lu cells into immunodeficient NOD-*scid* IL2Rgamma^{null} (NSG) mice intravenously and evaluated the extent of metastases. 451Lu cells tended to colonize the liver after tail vain injection. MSX1 knockdown in 451Lu cells significantly decreased metastatic tumor volume in the liver at 43 days after the injection (Figure 6C). Although one of the shRNAs (shRNA MSX1_3) showed a trend towards decreased lung lesions compared with control cells, the other shRNA (shRNA MSX1_5) did not decrease the area of metastasis, suggesting MSX1 knockdown does not influence lung colonization in 451Lu cell line in this context (Figure S6A). When 451Lu cells were subcutaneously injected into NSG mice, we observed no difference in tumor growth (Figure S6B) between MSX1 knockdown-infected cells and control cells, suggesting that the decreased metastatic colonization in MSX1 knockdown cells is not due to the disturbance of

anchorage-dependent growth. Taken together, our data suggest that MSX1 plays a critical role in promoting migration and metastasis of melanoma cells, especially in the liver microenvironment.

Discussion

In this study, we investigated the function of MSX1 in the melanocytic lineage. Both in melanocytes and melanoma cells, expression of MSX1 decreased pigmentation and expression of melanocytic markers, and the cells behaved in a manner similar to a neural crest precursor-like population. Our previous study had determined that active Notch1 is also capable of reprogramming melanocytes to the neural crest-like state. Little is known about the interactions between these two molecules; however, MSX1 was one of the highly expressed genes in Notch-reprogrammed NCSC-like cells compared to control melanocytes (Zabierowski et al., 2011). Interestingly, in neuroblastoma cells, expression of MSX1 quickly and strongly up-regulates the Notch pathway genes delta-like 1 homolog, NOTCH3, and hairy and enhancer-of-split related with YRPW motif protein 1 (HEY1) (Revet et al., 2008). Our preliminary data suggest that hairy and enhancer of split-1 (HES1), a known target of the Notch pathway, was among the genes which were most up-regulated in melanocytes shortly after the induction of MSX1 (data not shown). *Hes1* is expressed at the neuroepithelial stage during embryonic development when *Notch* and *Delta* are not expressed (Kageyama et al., 2005) indicating that, in addition to Notch, other factors may directly regulate Hes1 expression. Notably, HES genes play a role in the

maintenance of neural stem cells (Kageyama et al., 2005). It remains to be elucidated whether HES1 is a mediator in the reprogramming process by MSX1 and Notch1.

Many studies support that cancer is composed of heterogeneous cell populations, and a small subpopulation of stem-like cells play a key role in tumor maintenance and progression. However, more recent reports, including ours, suggest that the phenotypes of subpopulations are not static, but rather dynamic, and a stem-like subpopulation likely continually arises and disappears (Roesch et al., 2010, Vermeulen et al., 2010). Hoek and Goding framed the ‘‘phenotype-switching model’’ of melanoma (Hoek and Goding, 2010) in which melanomas contain M-MITF-high ‘‘proliferative’’ and M-MITF-low ‘‘invasive, stem-like’’ subpopulations (Hoek et al., 2008, Selzer et al., 2002, Wellbrock and Marais, 2005). These phenotypes are only temporarily distinct and each subpopulation has the potential to adopt the other phenotype. In our present study, we identified a novel mechanism for phenotype-switching by demonstrating that MSX1 acts as a master regulator, switching melanomas from an E-cadherin-high, M-MITF-high, ZEB1-low, differentiated state to an E-cadherin-low, M-MITF-low, ZEB1-high undifferentiated state (Figure 6D). Our data suggests that the MSX1 regulatory network plays a critical role in the dynamic phenotypes of human melanomas and that blocking MSX1-driven reprogramming is sufficient to inhibit subsequent metastasis formation primarily in the liver. Precise mechanisms in MSX1-dependent liver metastasis still remain to be elucidated, however, several studies reported that high expression of ZEB1 and ZEB-induced mesenchymal phenotype is correlated with liver

metastasis in several cancer types such as uveal melanoma and colorectal cancer (Chen et al., 2017, Spaderna et al., 2008, Zhang et al., 2013). Thus, it is plausible that the increased liver colonization is regulated by the MSX1- ZEB1 axis. Furthermore, it is noteworthy that the hepatic microenvironment provides a favorable milieu for mesenchymal stem cells, which likely play a role in the promotion of liver regeneration (Liu et al., 2015, Wang et al., 2016).

In summary, we discovered a novel function for MSX1 in the melanocytic lineage and identified it as a new reprogramming molecule that can convert mature melanocytes into a multipotent, uncommitted state. In melanoma, MSX1 induces a phenotypic switch from a non-migratory state towards a migratory, metastatic state. These data imply that reactivation of signaling pathways involved in neural crest-like reprogramming is relevant to the aggressiveness of melanomas. Further elucidation of the dedifferentiation process in the progression of melanoma will help us discover novel approaches for melanoma therapy.

Materials & Methods

All detailed information on experimental procedures and reagents is provided in the Supplemental Experimental Procedures.

Cell culture

Human melanoma cell lines were isolated at the Wistar Institute and cultured in Mel2% medium as previously described (Fang et al., 2005). All cell lines were periodically authenticated by DNA finger printing using the AmpFISTR Identifier microsatellite kit (Life Technologies) and tested for mycoplasma by Mycoalert Assay (Lonza). Human primary melanocytes were isolated from the epidermis of neonatal foreskins and were maintained in 254CF media (Life Technologies). NCSC-like cells were isolated from the dermis of human neonatal foreskins as described elsewhere (Li et al., 2010) and were cultured as spheres in human embryonic stem cell culture medium (StemPro® hESC SFM, Life Technologies) without bFGF**.** Intracellular Notch1 (NIC)-expressing and green fluorescent protein (GFP)-expressing control melanocytes were generated as described (Pinnix et al., 2009). HEK 293T cells used for producing lentiviral particles were cultured in DMEM (Life Technologies) supplemented with 10% FBS.

Statistics

All experiments were done in replicate samples and were repeated at least two or three times for validation. Two-sample t-tests with equal or unequal variances, or one-way ANOVA with post hoc tests were used for data analysis. All statistical tests were performed under a two-sided hypothesis with a *p*-value of less than or equal to 0.05 to reject the null hypothesis.

Conflict of Interest

The authors declare no competing financial interests with this paper.

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