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ORIGINAL ARTICLE

# MSX1-Induced Neural Crest-Like Reprogramming Promotes Melanoma Progression

Markus V. Heppt[1,2,9], Joshua X. Wang[1,9], Denitsa M. Hristova[1], Zhi Wei[3], Ling Li[1], Brianna Evans[1], Marilda Beqiri[1], Samir Zaman[1], Jie Zhang[3], Martin Irmler[4], Carola Berking[2], Robert Besch[2], Johannes Beckers[4,5,6], Frank J. Rauscher 3rd[1], Rick A. Sturm[7], David E. Fisher[8], Meenhard Herlyn[1,10] and Mizuho Fukunaga-Kalabis[1,10]

Melanoma cells share many biological properties with neural crest stem cells. Here we show that the homeodomain transcription factor MSX1, which is significantly correlated with melanoma disease progression, reprograms melanocytes and melanoma cells toward 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 nonmigratory state toward 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 show that neural crest-like reprogramming achieved by a single factor is a critical process for melanoma progression.

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## 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 toward a committed melanoblast is made early by expression of the melanocyte-restricted isoform of 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.

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

[1]The Wistar Institute, Philadelphia, Pennsylvania, USA; [2]Department of Dermatology and Allergology, Ludwig-Maximilian University, Munich, Germany; [3]Department of Computer Science, New Jersey Institute of Technology, Newark, New Jersey, USA; [4]Institute of Experimental Genetics, Helmholtz Zentrum München GmbH, Neuherberg, Germany; [5]German Center for Diabetes Research (DZD), Neuherberg, Germany; [6]Technische Universität München, Chair of Experimental Genetics, Freising, Germany; [7]Dermatology Research Centre, The University of Queensland, School of Medicine, Translational Research Institute, Brisbane, Queensland, Australia; and [8]Cutaneous Biology Research Center, Massachusetts General Hospital, Boston, Massachusetts, USA

[9]These authors share first authorship.

[10]These authors share senior authorship.

Correspondence: Mizuho Fukunaga-Kalabis, The Wistar Institute, 3601 Spruce Street Philadelphia, Pennsylvania 19104, USA. E-mail: mkalabis@wistar.org or Meenhard Herlyn, The Wistar Institute, 3601 Spruce Street Philadelphia, Pennsylvania 19104, USA. E-mail: herlynm@wistar.org

*Abbreviations: hESC, human embryonic stem cell; NCSC, neural crest stem cell; shRNA, short hairpin RNA*

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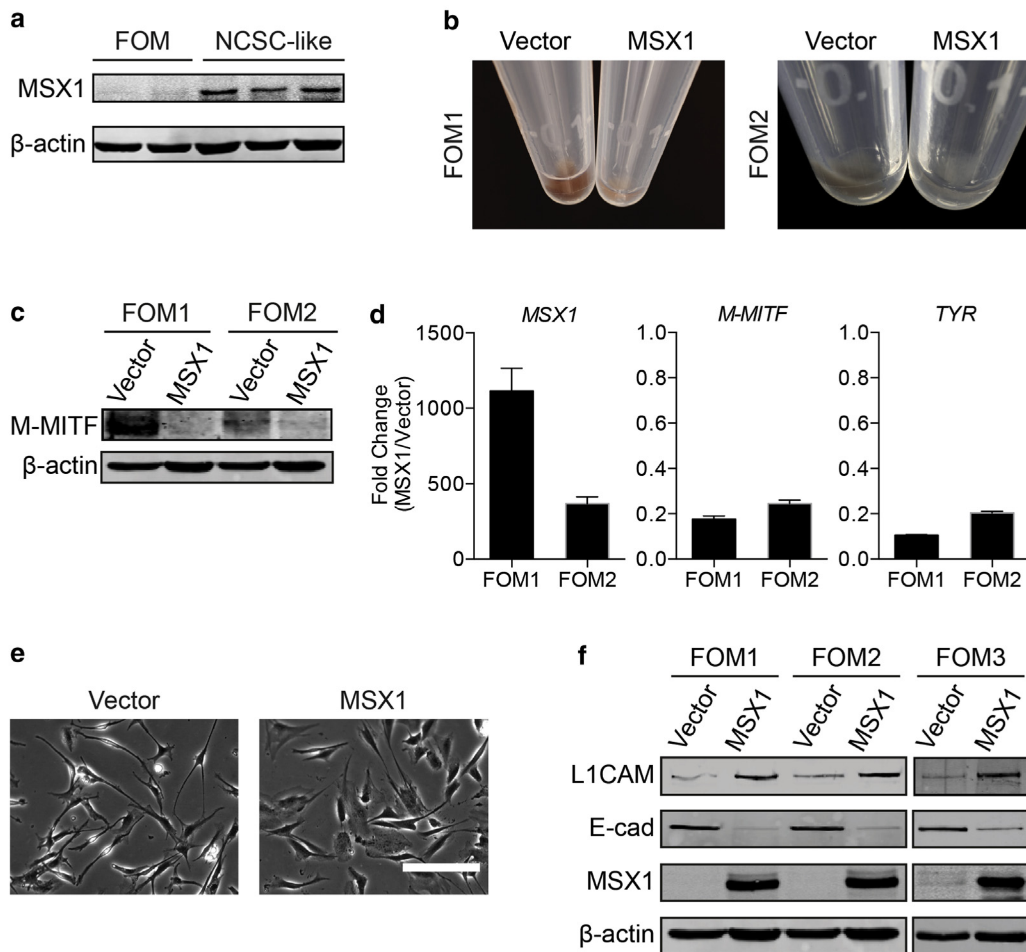


**Figure 1. Expression of MSX1 attenuates pigmentation and the expression of melanocytic markers in melanocytes.** (**a**) Immunoblot analysis of MSX1 in two foreskin-derived melanocytes (FOMs) and three foreskin-derived neural crest stem cell-like (NCSC-like) cells. (**b**) Macroscopic images of cell pellets from control vector and MSX1 vector-transduced melanocytes. (**c**) Immunoblot analysis of M-MITF in control vector and MSX1 vector-transduced foreskin-derived melanocytes. (**d**) Quantitative gene expression analysis of MSX1, MITF, and TYR in control vector and MSX1 vector-transduced melanocytes from two different donors. Error bars indicate one standard deviation of technical replicates. Sequences of the primers used are in Supplementary Table S1 online. (**e**) Phase images of control vector and MSX1 vector-transduced foreskin-derived melanocytes. (**f**) Immunoblot analysis of L1CAM, E-cad, and MSX1 in control vector and MSX1 vector-transduced melanocytes from three different donors. The left side images for FOM1 and FOM2 samples were derived from the same membrane used in **c**.

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, and silencing MSX1 by short hairpin RNA (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 used 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

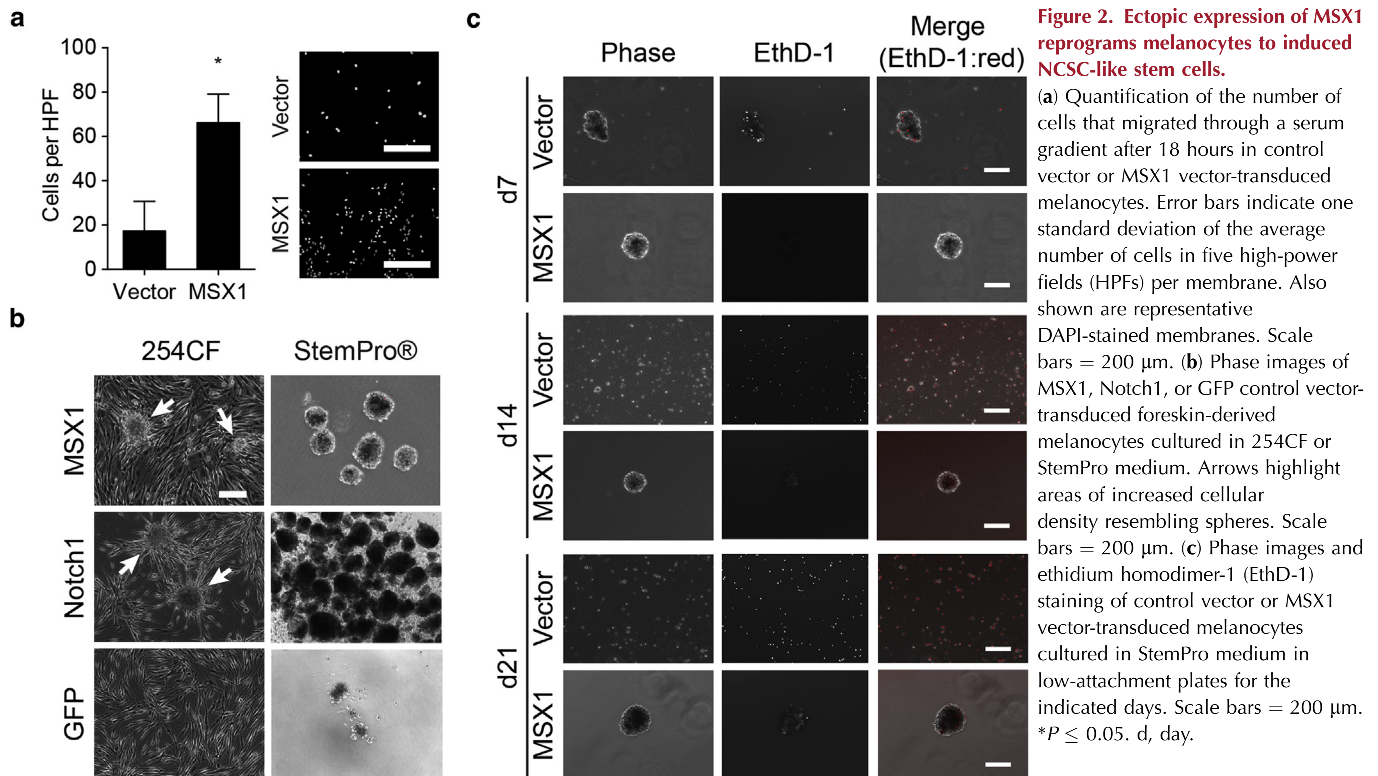


**Figure 2. Ectopic expression of MSX1 reprograms melanocytes to induced NCSC-like stem cells.**
(**a**) Quantification of the number of cells that migrated through a serum gradient after 18 hours in control vector or MSX1 vector-transduced melanocytes. Error bars indicate one standard deviation of the average number of cells in five high-power fields (HPFs) per membrane. Also shown are representative DAPI-stained membranes. Scale bars = 200 μm. (**b**) Phase images of MSX1, Notch1, or GFP control vector-transduced foreskin-derived melanocytes cultured in 254CF or StemPro medium. Arrows highlight areas of increased cellular density resembling spheres. Scale bars = 200 μm. (**c**) Phase images and ethidium homodimer-1 (EthD-1) staining of control vector or MSX1 vector-transduced melanocytes cultured in StemPro medium in low-attachment plates for the indicated days. Scale bars = 200 μm. *$P \leq 0.05$. d, day.

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 2−3 weeks of stable expression, we detected a dramatic decrease in pigmentation in MSX1-melanocytes (Figure 1b). Most melanocytes (5/7 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 posttranslational 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 with 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 (see Supplementary Figure S1 online). 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, because the expression of E-cadherin was strongly reduced in MSX1-melanocytes (Figure 1f). Ectopic expression of MSX1 in melanocytes consistently increased the expression of 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 toward 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 with control vector-infected melanocytes (Figure 2a). When cultured in melanocyte-specific medium (254CF, Thermo Fisher Scientific, Waltham, MA), 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 2b, middle left panel) (Li et al., 2010). The

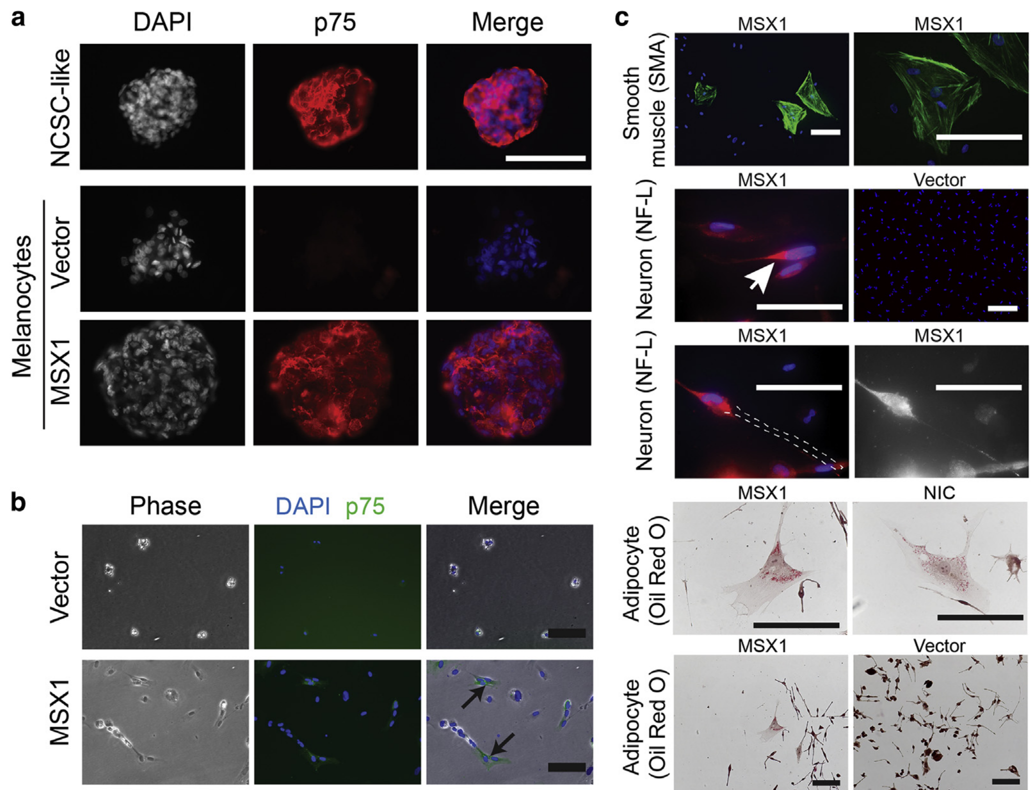


**Figure 3. MSX1 melanocytes are able to differentiate to neural crest derivatives.** (**a**) Immunofluorescence staining for p75 in dermis-derived NCSC-like stem cells, control vector-transduced melanocytes, and MSX1 vector-transduced melanocytes. Scale bars = 100 μm. (**b**) Phase images and immunofluorescence staining for p75 in control vector or MSX1 vector-transduced melanocytes cultured as adherent cells under stem cell conditions (StemPro). Black arrows indicate p75 cytoplasmic localization. Scale bars = 100 μm. (**c**) Immunofluorescence staining for smooth muscle actin (green) and neurofilament-L (red) and Oil Red O staining for lipid droplets in MSX1, Notch1 (NIC), and control vector-transduced melanocytes. Cells were differentiated in each differentiating media for 2 weeks. Nuclei are stained with DAPI. Scale bars = 100 μm. FOM, foreskin melanocyte; NCSC, neural crest stem cell.

formation of spheres was more evident under hESC growth conditions (StemPro hESC SFM, Thermo Fisher Scientific). 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 a 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, whereas 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. 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, because MSX1-expressing melanocytes that were grown as an adherent layer displayed intermediate levels of p75, whereas 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 Supplementary Figure S2 online, 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 showed 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. Expression of MSX1 was up-regulated in most

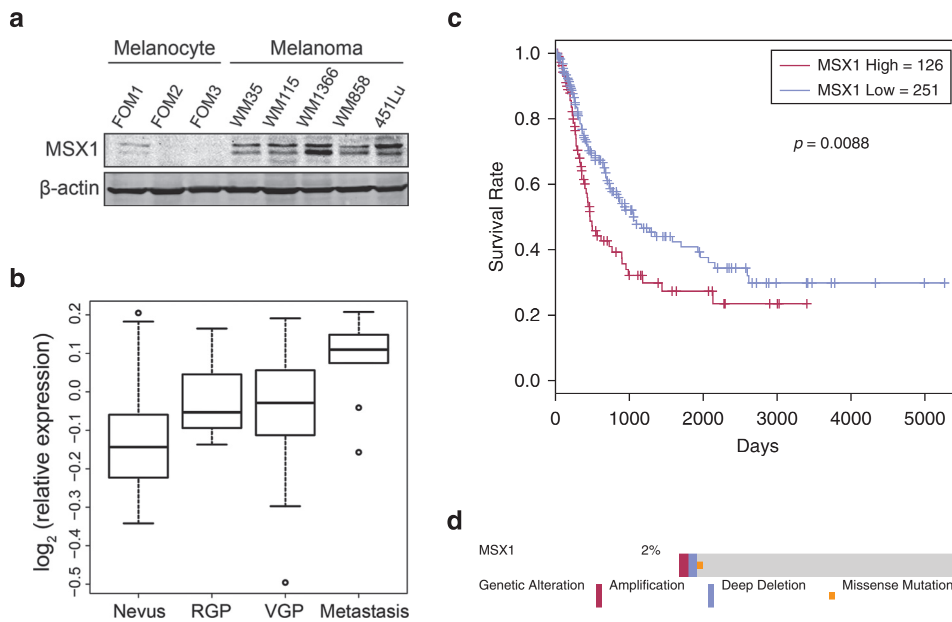


**Figure 4. Expression of MSX1 correlates with disease progression in melanoma.** (**a**) Immunoblot analysis of MSX1 in foreskin-derived melanocytes (FOMs), WM35, WM115, WM1366, WM858, and 451Lu melanoma cells. (**b**) Box-and-whisker plots for MSX1 expression levels in dataset GSE12391 (probe 41313) after grouping the samples by nevus, radial growth phase (RGP), vertical growth phase (VGP), and metastasis. (**c**) Kaplan-Meier survival curve for overall survival of patients with low and high expression of MSX1. The curves indicate a statistically significant reduction in overall survival with higher MSX1 mRNA expression. (**d**) Genetic alteration of MSX1 in patient melanoma samples. TCGA melanoma data was analyzed using cBioPortal (Gao et al., 2013) (http://cbioportal.org).

melanoma cell lines tested compared with normal melanocytes. WM35 (radial growth phase), WM115 (vertical growth phase), and WM858 (metastatic phase) cells displayed lower endogenous levels of MSX1 compared with 451Lu (metastatic phase) and WM1366 (vertical growth phase) cells, which exhibited strong expression of MSX1 (Figure 4a). Because 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 radial growth phase melanoma, 30 vertical growth phase 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 seven primary melanomas, three showed strong positivity for MSX1 staining (see Supplementary Figure S3 online). 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 The Cancer Genome Atlas melanoma RNA sequencing 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% (6/278 samples), suggesting that MSX1 expression is regulated by epigenetic mechanisms in most melanoma patients (Figure 4d).

### MSX1 induces phenotype switching and promotes migration of melanoma cells

Because 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 WM35 cells and metastasis-derived WM3451 cells (Figure 5). The effects of MSX1 on cell morphology of WM35 cells were not evident when cells were cultured in Mel2% (see Supplementary Figure S4a online, 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 (see Supplementary Figure S4a, right panels). WM3451 control cells retained their bipolar spindle-shape, whereas MSX1-WM3451 cells were polygonal and flattened (see Supplementary 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). Ectopic MSX1 did not alter cell growth and proliferation in any of the cells (Figures 5c). Switching between an 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 (Figure 5d, 5e). This suggests that MSX1 promotes a dedifferentiated and invasive phenotype in melanoma cells. 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, 5g, and Supplementary Figure S5 online). These results suggested that not MITF down-regulation but ZEB1

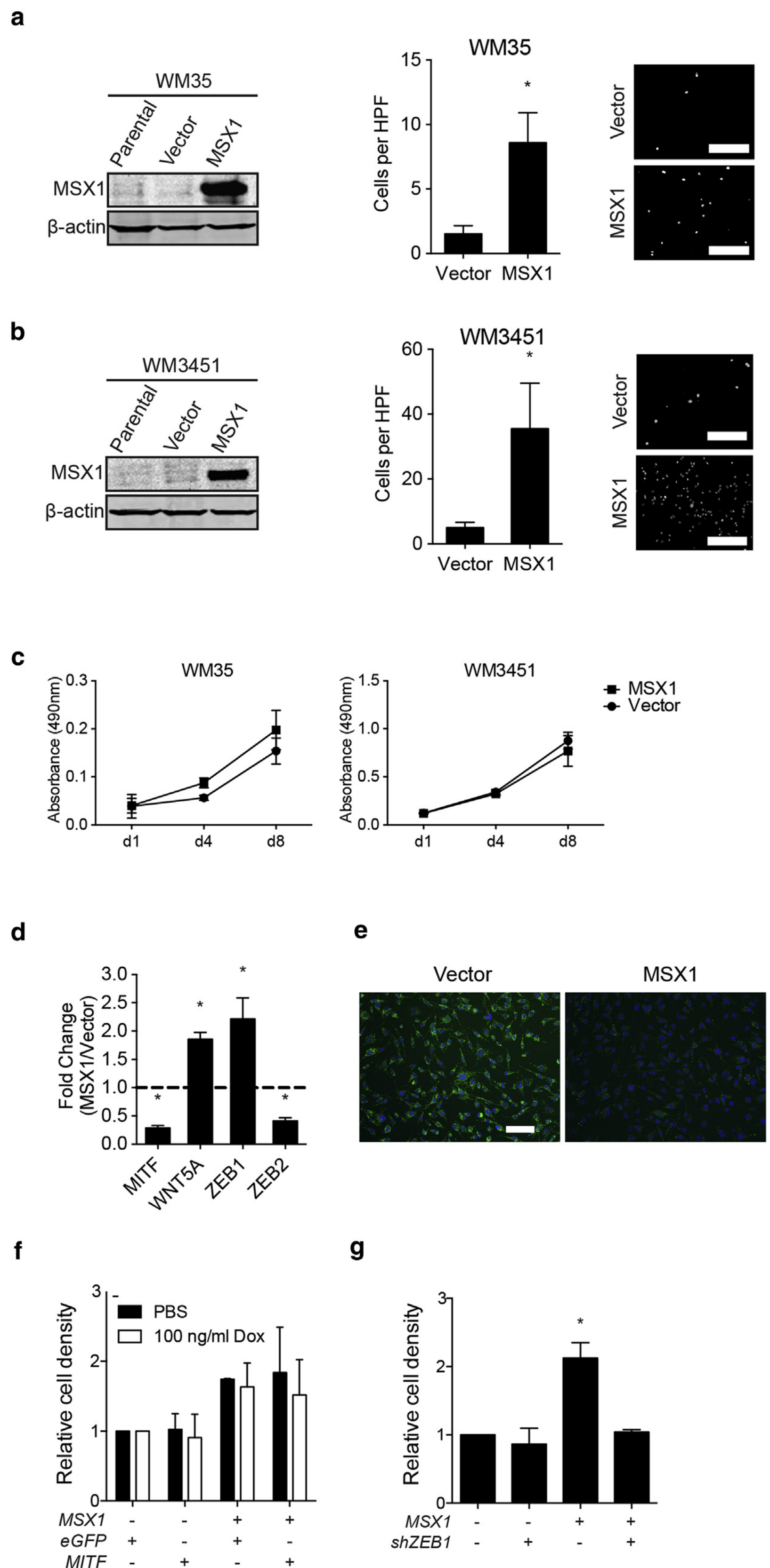


**Figure 5. MSX1 induces phenotype switching and promotes migration of melanoma cells.** (**a, b**) Immunoblot analysis (left) and quantification of cells migrated through a serum gradient after 6 hours (middle) in (**a**) WM35 and (**b**) WM3451 melanoma cells. Representative DAPI-stained membranes (right). Scale bars = 200 μm. (**c**) MTS assay for control vector or MSX1 vector-transduced WM35 and WM3451 cells. (**d**) Quantitative gene expression analysis in control vector or MSX1 vector-transduced WM35 cells. Sequences of the primers used are in Supplementary Table S1. (**e**) Immunofluorescence staining for E-cadherin (green) in WM35. Nuclei are stained with DAPI. Scale bar = 100 μm. (**f, g**) Quantification of cells migrated through a serum gradient in WM35 cells (**f**) after eGFP or MITF induction or (**g**) shRNAs targeting ZEB1. Error bars indicate one standard deviation of replicates. n = 3–4. *$P \leq 0.05$. d, day; HPF, high-power field; PBS, phosphate buffered saline; shRNA, short hairpin RNA.

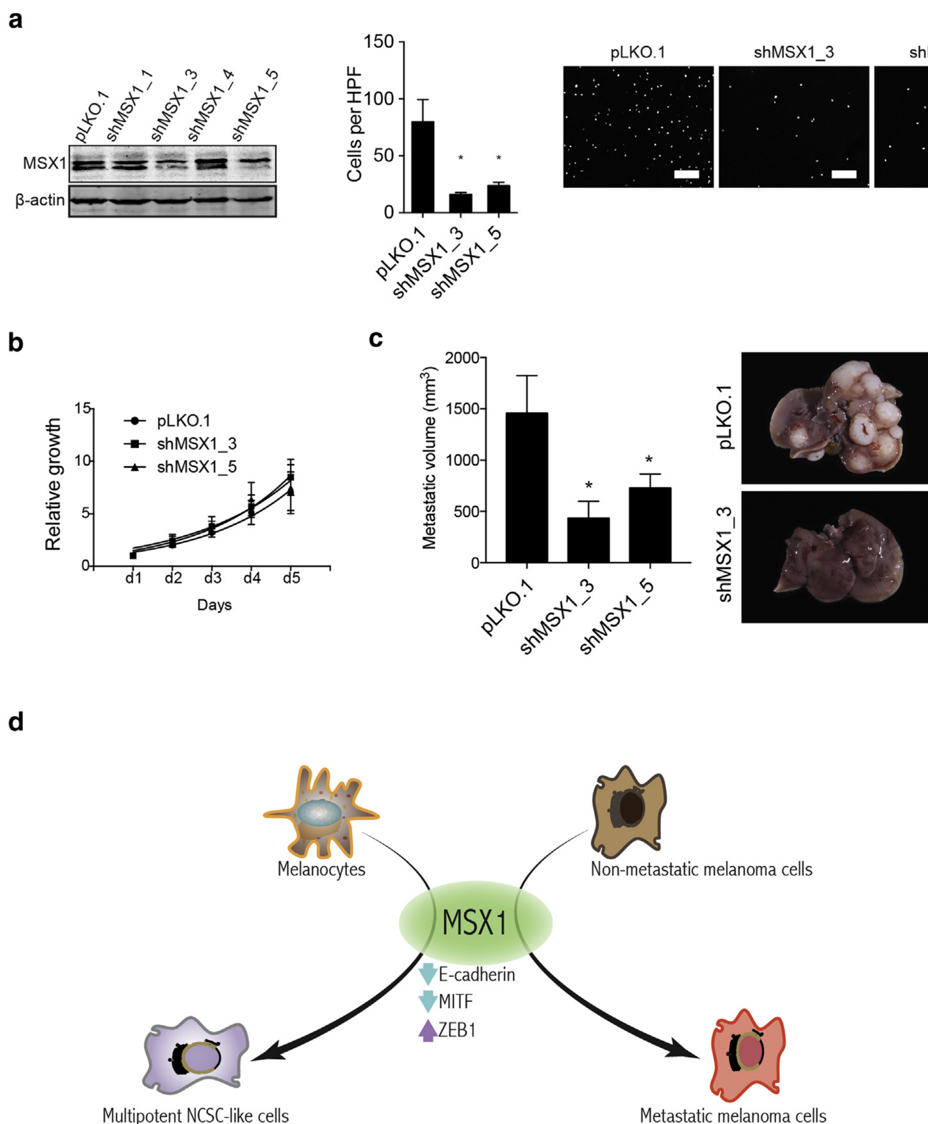


**Figure 6. Depletion of MSX1 decreases melanoma migration and liver metastasis.** (**a**) Immunoblot analysis of MSX1 in control vector or shRNAs targeting MSX1-transduced 451Lu cells (left). Quantification of cells migrated through a serum gradient after 6 hours in 451Lu cells (middle). Error bars indicate one standard deviation (n = 3). Representative DAPI-stained membranes (right). Scale bars = 500 μm. (**b**) MTS assay for control vector or shRNAs targeting MSX1-transduced 451Lu cells. Error bars indicate one standard deviation of biological replicates (n = 3). (**c**) Quantification of the metastasis volume after tail vein injection with 451Lu (left). Error bars indicate 1 standard error (n = 19–20). Macroscopic liver images harvested from representative mice of each group (right). (**d**) Overview schema of MSX1 expression in the melanocytic lineage. $^{*}P \leq 0.05$. HPF, high-power field; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; sh, short hairpin.

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 with control vector-infected cells (Figure 6a), and 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$^{null}$ mice intravenously and evaluated the extent of metastases. 451Lu cells tended to colonize the liver after tail vein 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 toward decreased lung lesions compared with control cells, the other shRNA (shRNA MSX1_5) did not decrease the area of metastasis, suggesting that MSX1 knockdown does not influence lung colonization in 451Lu cell line in this context (see Supplementary Figure S6a online). When 451Lu cells were subcutaneously injected into NOD-*scid* IL2Rgamma$^{null}$ mice, we observed no difference in tumor growth (see Supplementary 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 with control melanocytes (Zabierowski et al., 2011). In neuroblastoma cells, expression of MSX1 quickly and strongly upregulates the Notch pathway genes delta-like 1 homolog (*DLL1*), *NOTCH3,* and *HEY1* (Revet et al., 2008). Our preliminary data suggest that *HES1,* a known target of the Notch pathway, was among the genes that were most upregulated 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 indicate that cancer is composed of heterogeneous cell populations and that a small subpopulation of stem-like cells plays 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 (2010) framed the *phenotype-switching model* of melanoma 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 this study, we identified a mechanism for phenotype-switching by showing 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 suggest 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, 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 function for MSX1 in the melanocytic lineage and identified it as a reprogramming molecule that can convert mature melanocytes into a multipotent, uncommitted state. In melanoma, MSX1 induces a phenotypic switch from a nonmigratory state toward 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 effective approaches for melanoma therapy.

## MATERIALS AND METHODS

All detailed information on experimental procedures and reagents is provided in the Supplementary Materials online.

### 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 (Thermo Fisher Scientific) and tested for mycoplasma by Mycoalert Assay (Lonza, Basel, Switzerland). Human foreskin specimens were obtained from the Cooperative Human Tissue Network (http://www.chtn.nci.nih.gov). Written informed consent was obtained when collecting tissues from patients according to the policies and procedures of the Cooperative Human Tissue Network complying with federal human subjects regulations. The Wistar institutional review board approved foreskin sample collection from the Cooperative Human Tissue Network. Human primary melanocytes were isolated from the epidermis of neonatal foreskins and were maintained in 254CF media (Thermo Fisher Scientific). 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, Thermo Fisher Scientific) without basic fibroblast growth factor. NIC-expressing and GFP-expressing control melanocytes were generated as described (Pinnix et al., 2009). HEK 293T cells used for producing lentiviral particles were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum.

### 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 analysis of variance 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 state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.05.038.

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