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Transcription Factor/microRNA Axis Blocks Melanoma Invasion Program by miR-211 Targeting NUAK1

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Abstract

Melanoma is one of the deadliest human cancers, responsible for approximately 80% of skin cancer mortalities. The aggressiveness of melanoma is due to its capacity to proliferate and rapidly invade surrounding tissues, leading to metastases. A recent model suggests melanoma progresses by reversibly switching between proliferation and invasion transcriptional signatures. Recent studies show that cancer cells are more sensitive to microRNA (miRNA) perturbation than non-cancer cells; however, the roles miRNAs play in melanoma plasticity remain unexplored. Here, we use gene expression profiles of melanoma and normal melanocytes to characterize the transcription factor-miRNA relationship that modulates the proliferative and invasive programs of melanoma. We identified two sets of miRNAs that likely regulate these programs. Interestingly, one of the miRNAs involved in melanoma invasion is miR-211, a known target of the master regulator microphthalmia-associated transcription factor (MITF). We demonstrate that miR-211 contributes to melanoma adhesion by directly targeting a gene, *NUAK1*. Inhibition of miR-211 increases *NUAK1* expression and decreases melanoma adhesion, whereas upregulation of miR-211 restores adhesion through *NUAK1* repression. This study defines the MITF/miR-211 axis that inhibits the invasive program by blocking adhesion. Furthermore, we have identified NUAK1 as a potential target for the treatment of metastatic melanoma.

Introduction

Clinical and experimental studies have shown that as cancers progress, they sequentially acquire new traits that enable them to disseminate to and proliferate in distant organs. Detachment from the host stroma requires loss of cell-cell and cell-matrix adhesive contacts, enabling the escape of cells from the primary tumor. In addition to surviving in the circulation, such disseminated cells must regain their adherent capacity to enable them to colonize in distant organs (Fidler, 2003).

Cancer types vary greatly in the kinetics of their dissemination. Malignant cutaneous melanoma is characterized by a short relapse phase. It has been proposed that the rapid acquisition of aggressive capabilities by these cells is related to the inherent potential of neural-crest cells to survive migration through different environments and tissues (Gupta *et al.*, 2005). Early acquisition of metastatic potential by and increased treatment resistance of melanomas might also be the consequence of dynamic transitions between transcriptional programs (Pinner *et al.*, 2009; Quintana *et al.*, 2010), comparable to the epithelial-mesenchymal transition (EMT) and its reverse process, MET (mesenchymal-epithelial transition) (Scheel and Weinberg, 2011). A recent model for melanoma progression, the 'state switching' model, suggests that melanoma cells have the potential to switch between invasive and proliferative transcription signatures in response to cellular stress and variations in the tumor microenvironment (Hoek and Goding, 2010). This model of melanoma plasticity can also explain clonal heterogeneity and resistance to treatment. Still, the roles miRNAs play in these transcription signatures are largely unknown.

MiRNAs have unique roles in post-transcriptional regulation of gene expression and are important for maintaining homeostasis in normal cells. Numerous studies have linked dysregulated miRNA expression with tumor growth and progression (Croce, 2009). Interestingly, cancer cells are more sensitive to miRNA perturbations compared with normal cells. This enhanced sensitivity is likely a result of endogenous cellular stress and reciprocal crosstalk with the tumor microenvironment (Inui *et al.*, 2010; Mendell and Olson, 2012). Since a single miRNA can target multiple signaling pathways (Friedman *et al.*, 2009), perturbation of miRNA expression can lead to substantial phenotypic outcomes, particularly in cancer cells.

To investigate the roles miRNAs play in melanoma transcriptional programs, we analyzed microarray gene expression data of melanomas and normal human melanocytes. We applied

the hierarchical clustering algorithm and identified two inversely correlated gene clusters: one was significantly enriched in genes associated with melanoma invasion, and the other was significantly enriched in genes associated with melanoma proliferation (Hoek *et al.*, 2008a; Widmer *et al.*, 2012). Gene Ontology (GO) analysis revealed that these clusters are significantly enriched with genes involved in biological adhesion and melanocyte differentiation processes. We identified two sets of miRNAs, each preferentially regulating one sub-cluster. One of the miRNAs identified as potentially regulating the invasive gene cluster is a lineage-specific miRNA, miR-211. We and others have previously established the involvement of miR-211 in inhibition of melanoma invasion and migration (Boyle *et al.*, 2011; Levy *et al.*, 2010; Mazar *et al.*, 2010). Since the mechanism of cancer cell invasion and dissemination involves loss of adhesive contacts with neighboring cells, we examined the role of miR-211 in mediating melanoma invasion through disruption of cellular adhesion. We identified a miR-211 target, *NUAK1*, and showed that miR-211 inhibits loss of adhesion via direct regulation of this target gene. Moreover, our data suggest that miR-211 is a key player in the MITF-mediated phenotypic plasticity of melanoma.

Results

Identification of Two Inversely Correlated Gene Clusters Corresponding to Melanoma Invasive and Proliferative Programs. Hierarchical clustering (Eisen *et al.*, 1998) of normalized and filtered microarray gene expression data of melanomas (N=88) (Lin *et al.*, 2008) enabled identification of three gene 'master clusters' (marked 1 to 3, Figure 1a), three melanoma clusters (horizontal bar, top, Figure 1a), and two sub-clusters of highly correlated genes (numbered 2' and 3', marked green and red, respectively, Figure 1a). To examine further the two sub-clusters, we performed an enrichment analysis of Gene Ontology (GO) annotations within these sub-clusters and found that biological adhesion and melanin/pigmentation processes were the most highly enriched (Table S1). In order to examine whether these sub-clusters play roles in melanoma progression, we computed their enrichment with invasive and proliferative genes (Widmer *et al.*, 2012) using the hypergeometric test (Figure 1b). Interestingly, we found that sub-cluster 3' (marked red, Figure 1b) was enriched with invasive genes (P=2.3x10^{-13}) but had no proliferation genes (P=1.2x10^{-2}). In contrast, sub-cluster 2' (marked green, Figure 1b) was enriched with proliferation genes (P=4.2x10^{-30}) but had no invasive genes (P=1.5x10^{-2}) (Figure 1b and Figure 1c). We note that the datasets used to

deduce the invasive and proliferative signatures (Widmer *et al.*, 2012) contain largely different melanoma expression profiles than those used in this analysis. Our data suggest that the identified sub-clusters represent proliferative and invasive transcription signatures. As a consequence, we refer to sub-cluster 2' as "PROL" for proliferation, and sub-cluster 3' as "INV" for invasion. Next, we performed a differential expression analysis of the "INV" and "PROL" clusters by comparing each of the melanoma clusters (horizontal bar, Figure 1a) with normal melanocytes (Lin *et al.*, 2008). This analysis revealed a reversal of the differential expression pattern between different sub-sets of melanomas (Figure S1). One of the "PROL" genes that is differentially expressed is MITF, the 'master regulator' of the melanocyte lineage (Levy *et al.*, 2006).

Melanoma Transcription Signatures Dictate Distinct Sets of miRNAs. Contrasting results suggest that *MITF* can act as an oncogene in a subset of melanoma samples (Garraway *et al.*, 2005b) or as a suppressor of melanoma invasion and metastasis (Cheli *et al.*, 2011; Levy *et al.*, 2010; Pinner *et al.*, 2009; Shah *et al.*, 2010; Thurber *et al.*, 2011). Studies show that decreased levels of *MITF* are correlated with increased proliferation (Carreira *et al.*, 2006; Garraway *et al.*, 2005a; Garraway *et al.*, 2005b; Hoek *et al.*, 2008a). Whereas high invasiveness is associated with reduced *MITF* levels and increased expression levels of numerous transcription factors, including *ATF2* (Shah *et al.*, 2010), *GLI2* (Alexaki *et al.*, 2010; Javelaud *et al.*, 2011), *DEC1* (Feige *et al.*, 2011), *POU3F2*/*BRN2* (Boyle *et al.*, 2011; Pinner *et al.*, 2009; Thurber *et al.*, 2011), and *TCF4* (Eichhoff *et al.*, 2011). We suggest that another way to interpret the 'state switching' model of melanoma progression (Hoek *et al.*, 2008a) is that it is a result of a double-negative feedback loop (Alon, 2006), which can be induced by transcription factors and/or by miRNAs (Figure 2a).

A single miRNA can potentially affect hundreds of genes, amplifying pathways and cellular programs as well as increasing tumor plasticity (Friedman *et al.*, 2009; Inui *et al.*, 2010; Mendell and Olson, 2012). In order to examine the roles miRNAs play with respect to melanoma's transcription signatures, we matched gene expression profiles of melanoma samples (Lin *et al.*, 2008) with miRNA expression levels (see Supplementary information). It has been shown that paired miRNA-mRNA expression profiles can be used to improve target prediction (Huang *et al.*, 2007; Nunez-Iglesias *et al.*, 2010). To improve the predictions of biologically relevant miRNA-target relations, we calculated Pearson's correlations between miRNAs and genes belonging to

the “INV” and “PROL” clusters (Figure 2). Our analysis identified two distinct sets of miRNAs that may play important roles in regulating these sub-clusters (Table S2). We recently generated a literature-curated list of miRNAs that exhibit dysregulated expression in melanoma (Bell and Levy, 2011). Intriguingly we found that many of the miRNAs potentially regulating the "INV" or "PROL" clusters appear in our literature-based list. For example, copy number lost was shown for miR-302b and miR-302c (Zhang *et al.*, 2006), miR-125b and miR-30e-3p are dysregulated (Leidinger *et al.*, 2010), miR-214, miR-218 and miR-31 were found to be significantly associated with acral melanoma (Chan *et al.*, 2011), and circulating miR-221 is a marker of metastatic melanoma (Kanemaru *et al.*, 2011). One of the miRNAs we identified was miR-211 (Figure 2d), which is frequently dysregulated in melanomas (Bell and Levy, 2011), is a direct target of MITF, and has been established as an inhibitor of melanoma migration and invasion (Boyle *et al.*, 2011; Levy *et al.*, 2010; Mazar *et al.*, 2010). For this reason, we continued our analysis by focusing on miR-211 and examined its potential targets within the “INV” cluster, corresponding to the invasive phenotype.

Prediction of miR-211 Involvement in Melanoma Adhesion. In order to seek potential targets of miR-211 that may be involved in mediating the invasive program, we examined which of the miR-211 predicted targets were significantly inversely correlated with miR-211 (P<0.05, FDR corrected). We selected those that fulfill these requirements and belong to the “INV” cluster, identifying five genes: *BDNF, EFEMP2*, *FBN2*, *NUAK1*, and *TRAM2* (Figure 3a). One of the cancer-related (Hanahan and Weinberg, 2011) GO annotations pertaining to this group was ‘cell adhesion’. Since disruption of adhesive contacts contributes to cancer cell motility and invasion (Fidler, 2003), we proceeded to examine the role of miR-211 in modulation of cellular adhesion.

MiR-211 Modulates Adhesion Ability of Melanomas. We previously described two types of melanoma cell lines differing in their invasive potential and miR-211 expression levels (Levy *et al.*, 2010). We observed an *in vitro* correlation between invasive potential and adhesion. Cell lines with high miR-211 levels formed dense and confluent two-dimensional monolayers that adhered to the culture plates. Whereas, cell lines with lower miR-211 levels were less adhesive (Figure 3b). To test the effects of miR-211 on melanoma adhesion, melanoma cells with high (WM3682) or low (WM1716) miR-211 levels were transfected either with miR-211-specific Anti-miRs (Krutzfeldt *et al.*, 2005) or with miR-211 mimics. Next, cells were seeded, and the amount of adherent cells was calculated as a function of time. Inhibition of miR-211 reduced adhesion

compared with a scrambled control (Figure 3b, left panel), whereas transfection of a miR-211 mimic increased adhesion (Figure 3b, right panel). These data (and Figure S2) indicate that reduction in miR-211 levels diminishes melanoma adhesiveness.

MiR-211 Modulates *NUAK1* Levels. miRNAs typically repress protein-coding genes by binding to their 3' untranslated regions (UTR) (Bartel, 2009). We measured the mRNA levels of potential miR-211 targets after modulating miR-211 levels (Figure S3a). One the potential targets that was most affected by miR-211 was *NUAK1*. *NUAK1* (also named *ARK5*) is an AMP-activated protein kinase-related kinase directly phosphorylated by LKB1 downstream of the AKT pathway (Shaw *et al.*, 2004; Suzuki *et al.*, 2004). It has three binding sites for miR-211 in its 3'UTR (Figure 4a). Functional studies reveal its involvement in induction of senescence (Humbert *et al.*, 2010), regulation of proliferation through p53 phosphorylation (Hou *et al.*, 2011), promotion of cell survival (Suzuki *et al.*, 2003), invasion, and metastasis (Chang *et al.*, 2012; Suzuki *et al.*, 2004), and loss of cellular adhesion (Zagorska *et al.*, 2010). Although *NUAK1* is overexpressed in several cancers (Chang *et al.*, 2012), little is known about its role in melanoma.

In order to understand the roles of MITF, NUAK1, and miR-211 in melanoma progression, we performed an expression analysis of normal melanocytes and melanoma tumor samples at different phases of the disease (Smith *et al.*, 2005). The expression levels of MITF were assumed to be indicative of miR-211 expression, since miR-211 is directly targeted by MITF and is highly correlated with MITF (see Supplementary information). We found that MITF and NUAK1 are inversely correlated (*rho*=-0.55) and exhibit differential expression across different phases of melanoma progression (Figure S4a). Next, we used an *in situ* approach to examine the correlation between the expression of *NUAK1* and miR-211 using 100 tumor samples at various stages of melanomagenesis (Figure 4b, Figure S4b and Table S3). *In situ* hybridization was used to detect miR-211 expression, and immunohistochemistry was used to detect NUAK1 expression. Double-blind analysis of miR-211 and NUAK1 signal intensities across the tissue samples (Table S3, Figure S4b) revealed an inverse correlation (P=3.0x10^{-3}), suggesting an inhibitory relationship.

To determine whether NUAK1 is biologically regulated by miR-211, we modulated miR-211 levels in melanomas with high (WM3682, WM3526, and 451LU) or low (WM1745 and WM1716) miR-211 levels and examined the effects on NUAK1 protein (Figure 4c) and mRNA levels (Figure S4c). Transfection of miR-211 mimic led to a 2- to 4-fold decrease in NUAK1 protein levels in all cell lines, indicating that miR-211 can modulate NUAK1 levels. Transfection of

WM3526, WM3682, and 451LU with miR-211-specific Anti-miRs led to a robust reduction in miR-211 levels, yet only a modest increase in NUAK1 protein levels. These findings indicate that additional factors and mechanisms may be involved in regulating NUAK1 protein levels. Nonetheless, we found that miR-211 expression had a strong effect on cellular NUAK1 levels.

To determine whether *NUAK1* is a direct target of miR-211, we transfected luciferase-expressing constructs containing *NUAK1* 3'UTR with the wild-type or seed mutant predicted miR-211 binding-site into HeLa cells (which lack endogenous miR-211) with or without a miR-211 mimic. Our data demonstrate that *NUAK1* is a direct target of miR-211 (Figure 4d). Finally, we examined whether miR-211 can target *NUAK1* in the endogenous context of the MITF signaling pathway. Cells with high (WM3682 and 451LU) or low (WM1716 and WM3314) endogenous MITF levels were transfected with an MITF-specific siRNA or a cDNA construct overexpressing MITF, respectively (Figure 4e). MITF knockdown decreased miR-211 levels and increased *NUAK1* levels. MITF overexpression increased miR-211 levels and decreased *NUAK1* levels. Our data suggest that *NUAK1* is a downstream target of MITF via miR-211.

Mir-211 Modulates Melanoma Cellular Adhesion by Directly Targeting *NUAK1*. To date, no established role of NUAK1 in melanomagenesis has been described. We show that miR-211 perturbation affects melanoma adhesion (Figure 3). GO annotations of miR-211 targets (Figure 3a) reveal their involvement in cell adhesion. The extent of melanoma adhesion following modulation of miR-211 or *NUAK1* levels (Figure 5 and Figure S5) and treatment with EDTA-containing buffer was evaluated. We modulated miR-211 and NUAK1 levels in EDTA-treated melanoma cell lines and measured relative change in cell area using real-time video microscopy (Figure 5a, Figure S5a and Supplementary movies). Cell area shrinkage was attributed to loss of adhesive contacts with the surface. Melanoma cells were transfected with NUAK1 cDNA, NUAK1 siRNA, miR-211-specific Anti-miR, miR-211 mimic, or appropriate controls (*Renilla* luciferase cDNA, control siRNA, scrambled Anti-miR or scrambled miRNA), followed by EDTA treatment. Significant changes in cell area were observed (Figure 5a). *NUAK1* overexpression and miR-211 downregulation resulted in significant cell area shrinkage (left panel, WM3682). Knockdown of *NUAK1* and miR-211 overexpression resulted in a significant suppression of cell area shrinkage (right panel, WM3314). Our data show that downregulation of *NUAK1* enhances adhesiveness, while *NUAK1* overexpression reduces cell adhesion, indicating disruption of adhesive contacts (Figure 5a, Figure S5a and Supplementary movies).

In order to confirm a direct link between MITF, miR-211, and NUAK1 in regulating melanoma adhesion, we performed a number of rescue experiments (Figure 5c and Figure 5d). In WM3682 melanoma cell lines, MITF knockdown decreased the number of adherent cells, which was reversed upon overexpression of miR-211 (Figure 5c). These experiments demonstrate that the modification of melanoma adhesion by MITF is dependent on miR-211 regulation. We then verified that the effect of miR-211 on adhesion was mainly due to the regulation of NUAK1. Towards this aim, miR-211 was inhibited using an Anti-miR in WM3682 melanoma cells. Under these conditions, adhesion was decreased significantly compared with control, which was rescued upon NUAK1 knockdown (Figure 5d). These experiments demonstrate that the modification of melanoma adhesion by miR-211 is dependent on NUAK1 regulation.

To study further the effect of NUAK1 and miR-211 on melanoma adhesion, we examined the response of the actomyosin cytoskeleton during detachment. Three days after modulation of miR-211 or NUAK1 levels, melanoma cells treated with EDTA showed morphological changes from an elongated to a rounded structure. Loss of stress fibers and the formation of an actomyosin “contractile ring” at the cell periphery containing polymerized actin were also observed (Figure 5b). Reduction in NUAK1 levels suppressed formation of the rounded cells. Increased NUAK1 levels induced formation of round cells, indicating decreased adhesiveness. MiR-211 overexpression increased cell adhesion, indicated by an increase in Phalloidin-stained F-actin stress fibers. We and others previously established the role of miR-211 in inhibition of melanoma migration and invasion (Boyle *et al.*, 2011; Levy *et al.*, 2010; Mazar *et al.*, 2010). Here, we provide evidence that miR-211 regulates melanoma adhesion by directly targeting *NUAK1*. We also show that melanoma invasion is inhibited upon reduction of *NUAK1* levels (Figure S5c and Figure S5d). Our study thus places miR-211 as a central regulator of melanoma invasion.

BX795, is a potent protein kinase inhibitor that has been shown to inhibit the PDK1/Akt pathway (Feldman *et al.*, 2005), TBK1 and IKKepsilon (Clark *et al.*, 2009), and the LKB1/NUAK1 pathway (Zagorska *et al.*, 2010). Consistent with the inhibitory effect of BX795 on NUAK1, we treated melanoma cells with low miR-211 levels (WM1716 and WM3314) with BX795. This treatment led to visible changes in the cell morphology from spindle-shaped cells to rounded cells with many plasma membrane protrusions (Figure S6a and Figure S6b). Similar to the effect of miR-211 or siNUAK1, BX795 increases cell adhesion (Figure S6b) and reduces cell invasion (Figure S6c and Figure S6d). BX795 and its analogs have previously been shown to

have anti-cancer effects (Feldman *et al.*, 2005). Our results support these findings by showing that in melanoma cells, BX795 significantly reduces loss of cell adhesion and invasion ($P<0.05$).

Discussion

In addition to the 'clonal evolution' (Nowell, 1976) and 'cancer stem cell' models of cancer propagation (Al-Hajj *et al.*, 2003; Clarke *et al.*, 2006; Dalerba *et al.*, 2007), the 'phenotypic plasticity' model (also named the 'interconversion model') has been recently proposed based on observations of cancer cell plasticity (Quintana *et al.*, 2010). This model underscores the capacity of cancer clones to undergo reversible changes giving rise to tumor heterogeneity (Bell and Levy, 2011; Gupta *et al.*, 2011; Hoek and Goding, 2010; Scheel and Weinberg, 2011). A similar model, the 'state switching' model, describes melanoma progression via two transcriptional signatures corresponding to the proliferative or invasive potential (Hoek *et al.*, 2008a; Hoek and Goding, 2010). This model can be explained by a positive feedback loop (*e.g.*, double-negative feedback), in which activation of one program suppresses the other (Alon, 2006). Suppression can be achieved via transcription factors and/or miRNAs (Figure 2a). Still, little is known about the roles that miRNAs play in melanoma plasticity or about their cooperation with the transcription factors in co-regulating these transitions. Using microarray gene expression analysis of melanoma and normal melanocytes, we identified two inversely correlated gene clusters, which correspond to two transcription signatures of melanoma invasion and proliferation (Figure 1). Subsequently, we identified distinct sets of miRNAs that can preferentially mediate each of these signatures (Figure 2d). Our data provide additional understanding of these phenotypic states and suggest that melanoma invasion is mediated by miRNAs as well.

We and others have previously shown that miR-211 and MITF are together important players in melanoma invasion and migration (Boyle *et al.*, 2011; Levy *et al.*, 2010; Mazar *et al.*, 2010). As discussed above, the transition between an invasive and proliferative state involves the reduction or increase of MITF expression, respectively. Bioinformatic exploration of the networks of miRNAs and their targets reveal that miRNAs commonly cooperate with transcription factors in regulating large sets of target genes (Shalgi *et al.*, 2007). Our data suggest that miR-211 is a key player in the MITF-mediated inhibition of melanoma invasion (Figure 3, Figure 4e, Figure S2, and Figure S4). Our data further show that miR-211-mediated suppression of *NUAK1* increases melanoma adhesion and reduces invasion (Figure 5, Figure

S2, Figure S3, Figure S4, Figure S5, and Supplementary movies), whereas miR-211 depletion promotes morphological changes and loss of adhesion as a result of de-repression of *NUAK1*. Additionally, we show that a protein kinase inhibitor of NUAK1, BX795 (Feldman *et al.*, 2005) leads to similar morphological effects and inhibits loss of melanoma adhesion and invasion. We demonstrate that MITF and miR-211 work together to inhibit these programs. MITF regulates many cellular programs in melanoma, including replication, genomic stability, mitosis, and senescence (Hoek *et al.*, 2008b; Strub *et al.*, 2011). It would be interesting to explore additional programs that MITF orchestrates together with the miRNAs that it directly regulates.

MiR-211 inhibits melanoma invasion by targeting multiple genes, such as *KCNMA1*, *TGFB2R, NFAT5,* and *POU3F2*/BRN2 (Boyle *et al.*, 2011; Levy *et al.*, 2010; Mazar *et al.*, 2010; Zbytek *et al.*, 2012). Here, we show that miR-211 regulates melanoma adhesion. Clearly, a number of genes are expected to contribute to this phenotype. Indeed, a number of predicted direct targets were identified. In this study, we focused on one gene, *NUAK1*, which is at least partially regulated by miR-211 and whose levels are reduced with miR-211 overexpression. We showed that *NUAK1* is a direct target of miR-211 and that NUAK1 is involved in mediating the effect of reduced miR-211 levels, since *NUAK1* knockdown phenocopies transfection of the miR-211 mimic. Still, many other genes directly or indirectly targeted by miR-211 may affect melanoma invasiveness and adhesion. Additionally, there may be other factors affecting *NUAK1* expression in cancers that are independent of miR-211. For example, the MAF family leads to upregulation of *NUAK1* in malignant multiple myeloma and colorectal cancers (Kusakai *et al.*, 2004; Suzuki *et al.*, 2005), while BRAF negatively regulates LKB1 phosphorylation of NUAK1, promoting melanoma proliferation (Zheng *et al.*, 2009).

Recent studies have shown that cancer cells are more sensitive than non-tumor cells to miRNA regulation (Inui *et al.*, 2010; Mendell and Olson, 2012). In addition to their long half-lives, one of the major advantages of using miRNAs as a strategy for anti-cancer therapy is that a single miRNA can target multiple pathways and even revert diseased phenotypes. Therefore, therapeutic manipulation of a single miRNA offers promise for drug-resistant therapies or for enhancing responsiveness to standard cancer strategies (Kasinski and Slack, 2011). miR-211 is an intronic miRNA, hosted within a protein coding gene, melastatin (Levy *et al.*, 2010). Similar to its host gene, miR-211 demonstrates highly restricted expression and is detected primarily in the melanocyte lineage. Our data demonstrates that high expression levels of miR-211 correlate with decreased invasiveness and agrees with previously published mRNA expression profiles (Hoek *et al.*, 2008a; Levy *et al.*, 2010). In this respect, low miR-211 expression could be a

marker for invasive melanomas; moreover, miRNA expression profiles may be used as a molecular taxonomy of cancers (Lu *et al.*, 2005) that can serve to identify cancer origin (Rosenfeld *et al.*, 2008). The absence of miR-211 expression in known cases of melanoma might signify the presence of invasive melanoma cells. Our data suggest that miR-211 may be useful for differential diagnosis and detection of particular melanoma variants with metastatic potential. Given its crucial roles in melanoma adhesion and invasion, miR-211 may be an excellent therapeutic target.

Materials and Methods

Microarray Expression Data

In silico analyses were performed using published melanoma and normal melanocyte microarray expression profiles (Lin *et al.*, 2008). MiRNA expression was derived for eleven melanoma cell lines and matched to samples within the gene expression data. Gene expression profiles of different phases of melanoma progression were downloaded from GEO database (GSE4587) (Smith *et al.*, 2005). Detailed methods are described in Supplementary information.

Statistical Analysis

Statistical calculations of differential expression and microscopy real-time analyses were performed using two-tailed Student's t-test. The hypergeometric test was used to perform enrichment analyses.

Cell Culture, Invasion, Migration and Adhesion Assays, and Real-Time Microscopy Analysis

Melanoma cells were selected and subjected to matrigel invasion assay (BD Biosciences) as described (Levy *et al.*, 2010). Cell attachment and detachment analyses were based on the measurement of adherent cells at the indicated time points, normalized to t=0. Detailed methods are described in Supplementary information.

In Situ Hybridization

Tissue microarray slides including 100 specimens (US Biomax) were deparaffinized using EZ-prep solution (Ventana Medical Systems), fixed with 10% PFA (37°C for 20 min), digested with proteinase K (20 µg/ml; 37°C for 10 min; Roche), then denatured (70°C for 10 min). Next, the probe was added to the slides diluted in RiboHybe Reagent (Ventana) and allowed to hybridize at 53°C for 6h. Slides were washed twice with 2X SSC at 73°C for 6 min followed by the use of Ventana bluemap kit according to manufacturer's instructions. After development, slides were counterstained with nuclear fast red solution. Probes were DIG-labeled locked nucleic acid (LNA)-based oligonucleotides specific for miR-211 (Exiqon). Signal intensity in tissue samples were divided into three intensity categories: low (0 and 1), medium (2) and high (3) (Table S3).

Expression of miR-211 and NUAK1 is significantly inversely correlated (Wilcoxon paired two-sided signed rank test P=3.0x10^{-3}).

Oligonucleotide Transfection

MiRNAs (miR-211 mimic, Anti-miR-211, and control scrambled-miRNA) oligonucleotides (Ambion) or siRNAs (si-NUAK1 and control scrambled-siRNA) were transfected using HiPerFect (Qiagen) according to manufacturer's instructions. Cells were transfected twice with 20 pmol miRNA mimic or 200 pmol Anti-miR per well (0.5 x 10^6 cells) at 24 h intervals. Cells were used for the experiments 48h after second transfection.

RNA Purification and qRT-PCR

48h post transfection, total RNA was harvested using Trizol reagent (Invitrogen) according to manufacturer's instructions. qRT-PCR results were normalize to actin. Results are the average of three independent experiments. Table S4 lists the primer sequences used. Additional detailed methods are described in Supplementary information.

Gel Electrophoresis and Immunoblotting

48h post transfection, total protein of melanoma cells were harvested and analyzed by Western blot as detailed in Supplementary information.

Luciferase Assay

Cells were first transfected with miRNA as described, followed by transfection with appropriate plasmids. 48h later, the cells were subjected to luciferase assay (Promega). *Renilla* luciferase levels were used for normalization.

Plasmids

Luciferase-NUAK1 3'UTR were cloned into pcDNA 3.1 containing FireFly luciferase (Addgene plasmid 1265). Six CXCR4 sites were cut out with Xho1 and EcoRV and replaced by *NUAK1* 3'UTR.

The authors declare no conflict of interest.

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Figure Legends

Figure 1.

Gene Clustering Identifies Melanoma Transcription Signatures. *(a)* Hierarchical clustering of gene expression data of 88 melanomas (published in (Lin *et al.*, 2008)), enabled identification of gene clusters (rows, numbered 1 to 3), melanoma clusters (columns, horizontal bars), and closely related sub-clusters (marked 2' and 3', green and red, respectively). *(b)* Relative enrichment of invasion and proliferation transcription signatures genes. Sub-clusters 2' and 3' (henceforth "PROL" and "INV" clusters, respectively) were tested for enrichment with invasive and proliferative genes (Widmer *et al.*, 2012), and compared to background gene-set. *(c)* Venn diagram of overlaps of invasive genes (yellow) with "INV" (red), and proliferative genes (cyan) with "PROL" (green). No overlap exists between invasion genes and "PROL" nor between proliferation genes and "INV".

Figure 2.

MiRNA Involvement in Melanoma Transcription Signatures. *(a)* The 'state switching' model of melanoma progression can be described as a double-negative feedback loop, where transcription factors/miRNAs can activate one state while suppressing another. *(b)* A flow chart of the process of identification of miRNAs likely to be involved in the regulation of "INV" and "PROL" clusters (see Supplementary information). *(c)* Paired mRNA-miRNA expression profiles were used to derive miRNAs' mean correlations with "PROL" (x-axis) and "INV" (y-axis) clusters; miRNAs exhibiting significant mean correlation are marked with circles (P<0.025). *(d)* Of the miRNAs selected in (c) we identified those with predicted targets that belong to "INV" or "PROL" clusters and that show significant inverse correlation with the miRNA expression (*rho*<-0.4, *P*<0.05, FDR corrected) (Table S2).

Figure 3.

Prediction of miR-211 Involvement in Melanoma Adhesion. *(a)* A Venn diagram depicting process of predicting miR-211 involvement in adhesion. We derived a list of five potential targets from the overlap of (*i*) miR-211 predicted targets (http://www.targetscan.org), (*ii*) the "INV" cluster, and (*iii*) genes significantly inversely correlated with miR-211 calculated from the paired miRNA-mRNA expression profiles (Figure 2c). GO annotations (Ashburner *et al.*, 2000)

of these targets reveal 'cell adhesion' as one of the cancer promoting phenotypes (Hanahan and Weinberg, 2011). *(b)* miR-211 mediates melanoma adhesion capacity. Melanoma cells with low miR-211 expression (WM1716) or with high miR-211 levels (WM3682) were transfected as indicated. 48h post transfection, cells were subjected to cell detachment analysis. Graphs represent mean +/- SD of three replicates of adhering cells over time.

Figure 4.

MiR-211 Directly Targets NUAK1. *(a)* Predicted binding-sites for miR-211 in *NUAK1* 3'UTR. Numbers indicate positions on *NUAK1* mRNA (TargetScan). *(b)* H&E staining of malignant melanomas (left panels), immunostaining of NUAK1 (red dots), and *in situ* hybridization of miR-211 (blue dots) (Table S3 and Figure S4). *(c)* Melanomas with high (WM3526, WM3682, and 451LU) or low (WM1745 and WM1716) MITF and miR-211 levels were transfected as indicated. Western blotting illustrates NUAK1 protein levels following transfection. Tubulin is provided as a loading control. *(d)* Luciferase constructs containing wild-type (WT) or miR-211 binding-site mutant (mut) 3'UTRs of *NUAK1* were transfected with miR-211 mimic or control (mean +/- SD). *(e) MITF*, miR-211, and *NUAK1* expression in melanomas upon MITF perturbation, as indicated. MiR-211 was normalized to RNU48 and mRNA was normalized to actin; (mean +/- SD).

Figure 5.

MiR-211 Modulates Melanoma Cellular Adhesion via NUAK1. *(a)* WM3682 and WM3314 melanomas were transfected as indicated followed by EDTA treatment, recorded with real-time video microscopy for 1 min (see Supplementary movies). Two movies were analyzed in each assay. Graphs quantify relative cell area changes (mean +/- SD). *(b)* WM3682 and WM3314 melanomas were grown on glass cover slips and transfected as indicated. 48h post transfection, cells were fixed immediately after aspiration of the media and PBS wash or after incubation with 1mM EDTA buffer for 1 min. Cells were stained with Phalloidin (red) and DAPI (blue). Three sets of cells were analyzed, and representative images are shown. Scale bar=10μm. *(c)* and *(d)* represent results of rescue experiments of melanoma cells transfected as indicated and subjected to adhesion assay.

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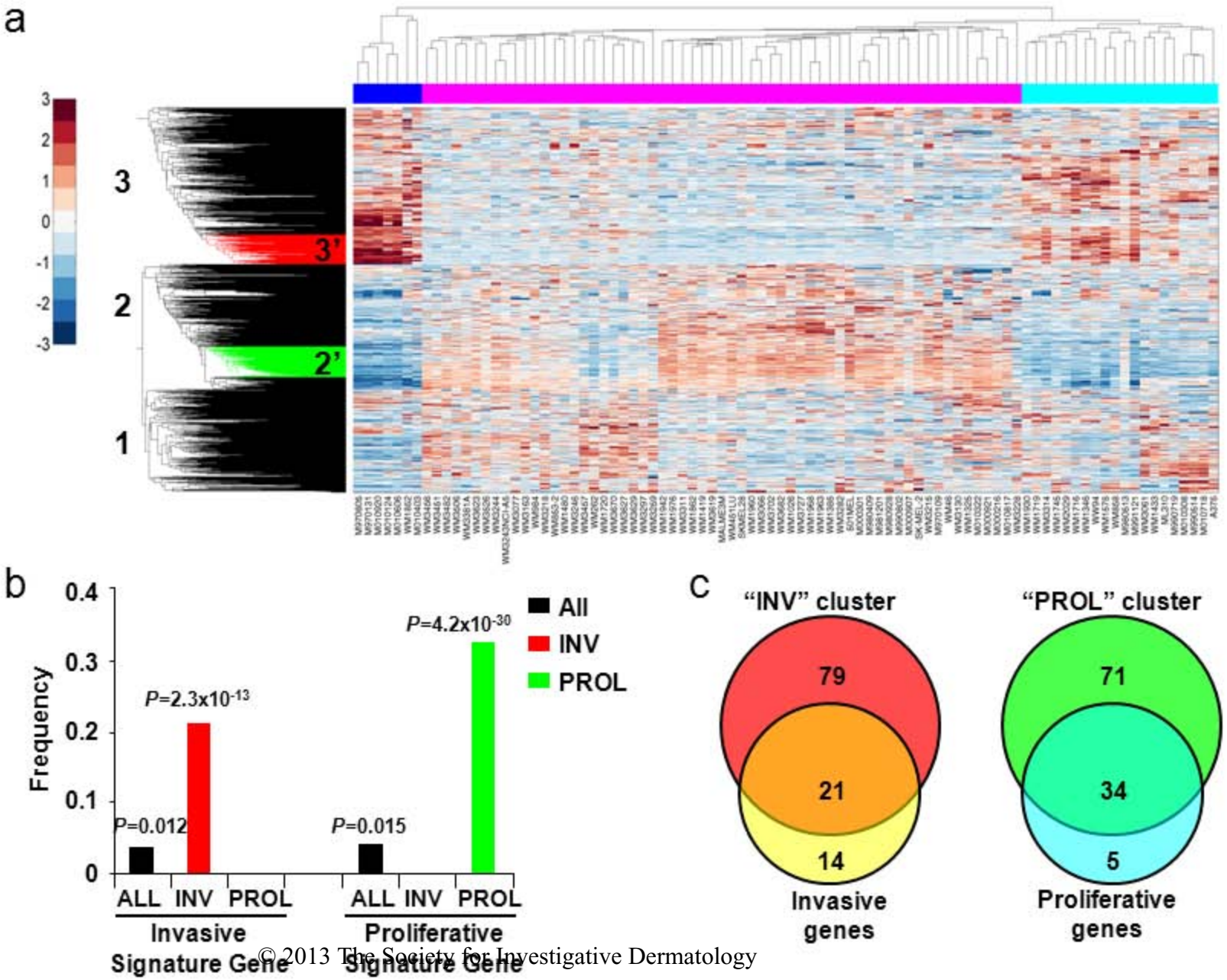

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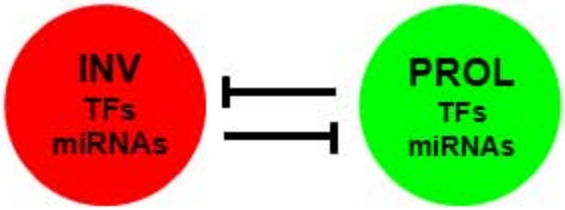

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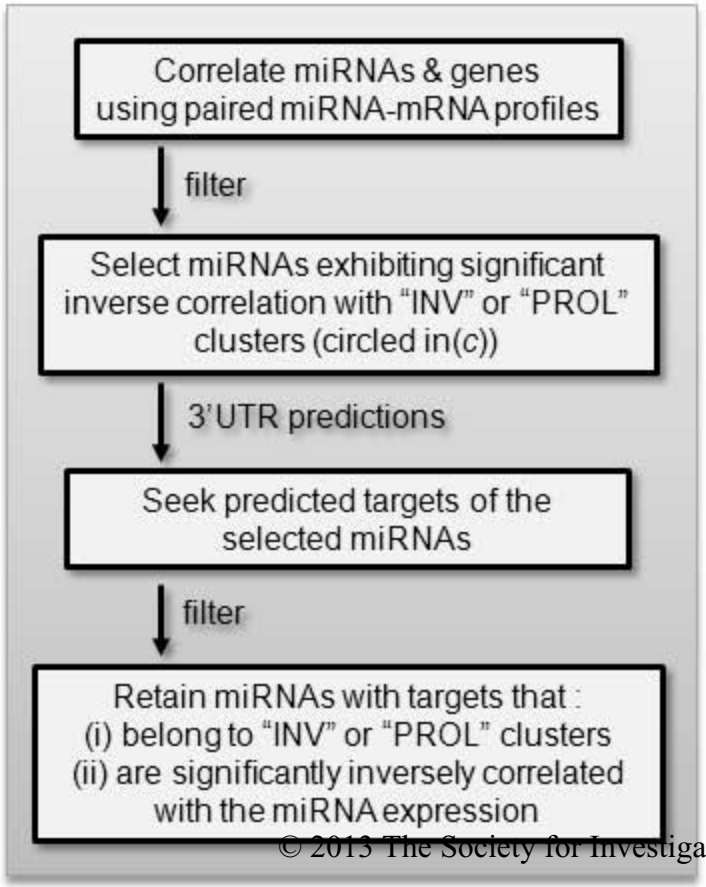

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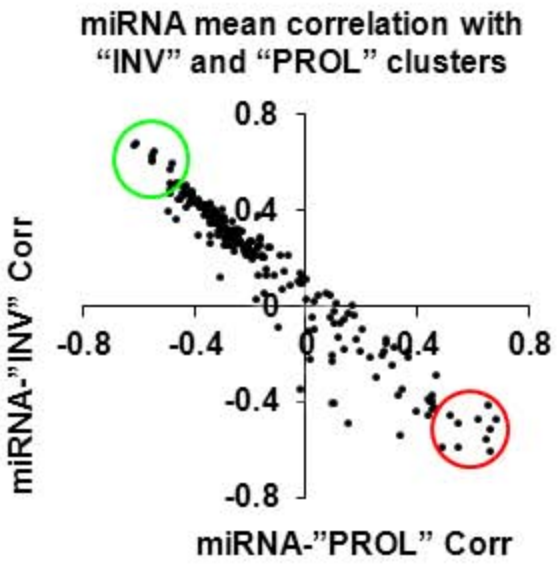

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