MiR-200a Regulates CDK4/6 Inhibitor Effect by Targeting CDK6 in Metastatic Melanoma

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The CDK4/6 pathway is frequently dysregulated in cutaneous melanoma. Recently, CDK4/6 inhibitors have shown promising clinical activity against several cancer types, including melanoma. Here, we show that microRNA-200a decreases CDK6 expression and thus reduces the response of CDK4/6 inhibitor in highly proliferative metastatic melanoma. Down-regulation of microRNA-200a expression in melanoma cells is associated with disease progression and a higher number of lymph node metastases. Furthermore, microRNA-200a expression is epigenetically modulated by both DNA methylation at the promoter region and chromatin accessibility of an upstream genomic region with enhancer activity. Mechanistically, overexpression of miR-200a in metastatic melanoma cells induces cell cycle arrest by targeting CDK6 and decreases the levels of phosphorylated-Rb1 and E2F-downstream targets, diminishing cell proliferation; these effects are recovered by CDK6 overexpression. Conversely, low microRNA-200a expression in metastatic melanoma cells results in higher levels of CDK6 and a more significant response to CDK4/6 inhibitors. We propose that microRNA-200a functions as a “cell cycle brake” that is lost during melanoma progression to metastasis and provides the ability to identify melanomas that are highly proliferative and more prompted to respond to CDK4/6 inhibitors.

INTRODUCTION

Malignant cutaneous melanoma is one of the most aggressive and highly metastatic cancers (Bristow et al., 2013). Despite significant advancements in immunotherapy and targeted therapies, many patients still do not respond to these treatments, and if they do, they often develop resistance (Millet et al., 2016). We have proposed that although primary melanomas (PRMs) are affected by several driver mutations, the metastatic potential is determined by epistatic (Lessard et al., 2015; Marzese et al., 2015a) and epigenomic alterations (Marzese et al., 2014a, 2014b, 2015b; Ono et al., 2015). Among the numerous epistatic mechanisms that control gene expression, microRNAs (miRs) have gained increasing attention in cancer. MiRs possess a regulatory ability to target tumor-related mRNAs, which affects specific signaling pathways and determines drug resistance (Lee et al., 2013; Magee et al., 2015; Rupaimoole et al., 2016). Members of the miR-200 family are major regulators of epithelial to mesenchymal transition, a critical step for metastatic progression observed in different carcinomas; however, because melanomas are not derived from epithelial lineage, they do not experience the classic epithelial to mesenchymal transition changes (Oom et al., 2014; Rupaimoole et al., 2016; Tan et al., 2014). We recently showed that miR-200a down-regulation significantly induces malignant growth, invasion, and metastasis progression in triple-negative breast cancer (Lee et al., 2013) and that loss of miR-200a expression triggers molecular events that induce invasion and tumor metastasis in solid primary carcinomas (Davalos et al., 2012; Wiklund et al., 2011); however, the role of miR-200a in metastatic melanoma remains unexplored. Epigenetic mechanisms, including DNA methylation and histone posttranslational modifications, are critical for the expression of miR-200a in different types of cancers (Attema et al., 2013; Ceppi et al., 2010; Davalos et al., 2012; Lim et al., 2013; Neves et al., 2010; Wiklund et al., 2011); however, little is known about how miR-200a is regulated in melanoma.

CDK6, along with CDK4, plays a key role in controlling cell cycle transition from G1 to S phase by phosphorylating Rb1, which promotes the release of the transcription factor E2Fs and the expression of specific proliferative genes (Weinberg, 1995). The functional and clinical relevance of the pathway in different cancers has fueled the development of improved selective CDK inhibitor compounds, including PD-0332991 (palbociclib) (Asghar et al., 2015; Roskoski, 2016; Xu et al., 2016). Palbociclib is a specific CDK4/6 inhibitor recently approved for treatment of postmenopausal women with metastatic estrogen

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Abbreviations: CGI, CPG island; DOM, distant organ metastasis; LNM, lymph node metastasis; miR, microRNA; miR-ctrl, microRNA-control; p, phosphorylated; PRM, primary melanoma; qRT-PCR, quantitative real-time reverse transcriptase–PCR; TCGA, The Cancer Genome Atlas; UTR, untranslated region; WB, Western blot

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receptor-positive breast cancer in combination with letrozole (Finn et al., 2015) and showed response in cutaneous melanoma (Young et al., 2014).

This study shows a significant and functionally relevant down-regulation of miR-200a in metastatic melanomas. Epigenetic analyses identified a DNA methylation at the promoter region of miR-200b/200a/429 polycistronic unit that was successfully reverted by the use of the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, and an increase in chromatin accessibility at its enhancer element. High-throughput screening identified CDK6 as a potential miR-200a target with functional relevance for metastatic melanoma. Functional analysis confirmed that miR-200a targets CDK6, arrests cell cycle at the G1/S checkpoint, and decreases metastatic melanoma cell proliferation. In agreement with these results, CDK6 overexpression is able to overcome the miR-200a–induced reduction on cell proliferation. Additionally, highly proliferative metastatic melanoma cells with low expression of miR-200a, and hence high CDK6 levels, are more responsive to palbociclib treatment. Conversely, in metastatic melanoma cells with high miR-200a expression and low CDK6 expression, the response to palbociclib is limited to CDK4 inhibition and therefore has a reduced efficiency.

RESULTS AND DISCUSSION
MiR-200a expression decreases during melanoma progression
To evaluate the role of miR-200a in melanoma progression, publicly available miR expression data from The Cancer Genome Atlas (TCGA) cutaneous melanoma database was assessed. We identified significantly lower expression of miR-200a in regional lymph node metastases (LNMs) (Wilcoxon, \( P = 1.1 \times 10^{-19} \)) and distant organ metastases (DOMs) (Wilcoxon, \( P = 1.5 \times 10^{-5} \)) compared with PRMs (Figure 1a and b). Expression of miR-200a in PRMs did not exhibit any correlation with Breslow’s thickness and was not associated with prognostic factors such as tumor size and ulceration (see Supplementary Figure S1a–c online). However, PRMs from patients with two or more LNMs (N2 and N3) presented a significantly lower miR-200a expression compared with PRMs from patients with none or only one lymph node involved (N0 and N1) (t test, \( P = 0.03 \)) (see Supplementary Figure S1d). Associations between miR-200a and common melanoma driver mutations (BRAF and NRAS) were assessed; however, no significant differences in miR-200a expression occurred between wild-type and respective melanoma mutations (see Supplementary Figure S1e and f). miR-200a expression decreased in PRMs that metastasized to regional lymph nodes or distant organs compared with those that did not metastasize \( (P < 0.05) \) (see Supplementary Figure S1g). Overall, miR-200a expression was negatively correlated with American Joint Committee on Cancer stages \( (\text{Kendall's tau} = -0.275, \ P = 0.001) \) (see Supplementary Figure S1h). Finally, overall survival curves showed a trend toward poorer overall survival for patients with PRMs with low miR-200a expression compared with those with high miR-200a expression (see Supplementary Figure S1i). These results suggest a significant association between the loss of miR-200a expression...
and melanoma progression. We further verified this observation in an independent cohort (n = 75) of paraffin-embedded archival tissues using miR-quantitative PCR. In agreement with the in silico analyses, miR-200a expression was significantly down-regulated in metastaticmelanomas (LNMs and DOMs) compared with PRMs (Wilcoxon, \( P = 3.0 \times 10^{-4} \) and \( P = 6.0 \times 10^{-4} \), respectively) (Figure 1c, and see Supplementary Table S1 online) and benign nevi specimens (Wilcoxon, \( P = 0.001 \) and \( P = 0.001 \) respectively) (Figure 1c). MiR-200a expression was also profiled in established early-passaged primary melanocyte and metastatic melanoma lines (n = 32). Consistent with the in silico and paraffin-embedded archival tissue results, LNM- and DOM-derived cell lines presented a significantly lower miR-200a expression (LNM, \( P = 0.008 \) and DOM, \( P = 0.03 \)) than primary melanocytes (Figure 1d and e). Several studies have shown that members of the miR-200 family are deregulated during the metastatic cascade and act as modulators of tumor growth, angiogenesis, epithelial to mesenchymal transition, intra- and extravasation, and tissue colonization through a number of different targets (Humphries and Yang, 2015; Xu et al., 2012). Here, we showed that miR-200a down-regulation is important for the metastatic melanoma cascade; therefore, we focused on the characterization of genes targeted by miR-200a and the mechanisms that control miR-200a expression.

Epigenetic regulation of miR-200a expression in melanoma progression

Given the significant down-regulation of miR-200a observed during melanoma progression, we investigated the potential influence of epigenomic transcriptional regulation in the miR-200b/200a/429 polycistronic unit associated with DNA methylation and histone modifications. Initially, we assessed the DNA methylation level of 58 CpG sites (chromosome 1: 1,089,668–1,107,439) with a putative role in miR-200b/200a/429 polycistronic unit regulation in a large cohort of melanoma cell lines. This segment of the genome includes the promoter region, two upstream CpG islands (CGIs), and its context of CpG shores and CpG shelves (Figure 2a). We observed a large variance in DNA methylation level of CpG sites located in the promoter CGI, CpG shore, and CpG shelf located in the miR-200b/200a/429 cluster (Figure 2b). Next, based on the mean value of miR-200a expression, melanoma lines were grouped into “low” and “high” miR-200a, after which average DNA methylations of the 58 CpG sites were compared (Figure 2b). The analysis showed significantly higher DNA methylation level at CpG sites located both in...
the promoter and in the body of the polycistronic unit of low miR-200a—expressing melanoma cells (Figure 2b). Furthermore, examination of the correlation between miR-200a expression and the DNA methylation level for each of the 58 CpG sites showed a significant negative correlation with CpG sites located in the promoter CGI (Figure 2c). Supporting the functional role of DNA methylation on miR-200a expression, the treatment of four metastatic melanoma lines with the DNA methyltransferases inhibitor 5-aza-2′-deoxycytidine significantly increased the expression of miR-200a by 3- to 8-fold (see Supplementary Figure S1j).

A recent breast cancer study has reported that in addition to DNA methylation, chromatin accessibility determined by histone modifications in an upstream enhancer element stimulates the transcription of the miR-200b/200a/429 cluster (Attema et al., 2013). Data obtained from the chromatin state segmentation by hidden Markov model from the ENCODE project (Rosenbloom et al., 2013) supported the existence of an active enhancer element on this region in several cell types besides breast cancer cells (Figure 2d). To test this hypothesis in melanoma, we evaluated chromatin accessibility maps for one melanocyte (Melano; GSM816631) and two metastatic melanoma cell lines (MEL 2183; GSM1008599 and COLO 829; GSM1008571). We identified a progressive decrease in chromatin accessibility of the genomic region overlapping the upstream enhancer element of miR-200a (Figure 2e), which is in agreement with a decrease in miR-200a expression through metastatic progression. Consistent with other cancers reported in agreement with a decrease in miR-200a expression through metastatic progression, we evaluated whether miR-200a also regulates CDK4 expression (Bracken et al., 2008; Gregory et al., 2008). The miR-200a target in metastatic melanomas. Thus, because of its key regulatory role on G1/S checkpoint and its newly defined therapeutic importance for human solid tumors (Cristofanilli et al., 2016; Turner et al., 2015), we focused our study on the influence of miR-200a on CDK6 expression.

**MiR-200a down-regulates CDK6 expression**

To better understand the regulatory mechanism of miR-200a on CDK6, metastatic melanoma cell lines (DP-0574, HM-0525, and WP-0614) with low miR-200a and concurrently CDK6 expressions (Figure 1e, and see Supplementary Figure S2a online) were transfected with miR-200a or miR-ctrl precursors (Figure 3c). miR-200a significantly reduced CDK6 expression, as confirmed by quantitative real-time reverse transcriptase—qRT-PCR (Figure 3d), Western blot (WB) (Figure 3d and e), and indirect immunofluorescence (see Supplementary Figure S3 online). Consistent with these findings, HM-0525, which exhibited greater CDK6 expression (see Supplementary Figure S2a), required higher miR-200a precursor concentrations to generate a comparative CDK6 down-regulation to DP-0574 and WP-0614 (see Supplementary Figure S2b). Additionally, indicating a higher dependency, down-regulation of CDK6 on HM-525 resulted in a more drastic reduction in cell proliferation.

As shown in Figure 2e, CDK6 3′-UTR possesses miR-recognition elements for miR-200a that span over 10 kilobase pairs and spread in two regions; thus, we introduced them into two different luciferase gene reporter constructs: 3′-UTR-1 (419-2,041 base pairs) and 3′-UTR-2 (7,471-8,729 base pairs) (Figure 3e). Then, cells were co-transfected with miR-200a and 3′-UTR1 or 3′-UTR2 constructs, and luciferase activity was measured. MiR-200a significantly decreased the luciferase activity by targeting 3′-UTR-2 but not 3′-UTR-1 (Figure 3f), thus confirming that the regions between 7,471 and 8,729 of CDK6 3′-UTR is a target of miR-200a. Finally, changes in expression of ZEB1, a known miR-200a target, were assessed as readout of miR-200a regulatory effect (Bracken et al., 2008; Gregory et al., 2008). The miR-200a—induced down-regulation of ZEB1 was confirmed by qRT-PCR (see Supplementary Figure S2c) and WB (see Supplementary Figure S2d and e) in ZEB1-expressing melanoma cells (see Supplementary Figure S2a). Because both CDK6 and its functional homolog CDK4 act as key promoters of G1/S checkpoint (Asghar et al., 2015), we evaluated whether miR-200a also regulates CDK4 expression in melanoma cells. Our results showed that CDK4 levels were not affected by miR-200a in melanoma cell lines, as shown by qRT-PCR (see Supplementary Figure S2f) and WB (see Supplementary Figure S2g and h). These findings indicate that miR-200a is an epigenetic regulator of the cell cycle checkpoint G1/S through the modulation of CDK6 expression.
MiR-200a–induced CDK6 down-regulation arrests cell cycle through p-Rb1 reduction

Cyclin D-CDK4/6 dimers phosphorylate Rb1, resulting in the release of E2F transcription factors, which induce the expression of many key proliferative genes that promote G1 to S phase transition (Du and Pogoriler, 2006). To investigate the functional effect of miR-200a–induced CDK6 down-regulation, changes in Rb1 phosphorylation (p-Rb1) were evaluated. Among the 16 known phosphorylation sites on the Rb1 protein, at least four of these serine residues (Ser780, Ser795, Ser807, and Ser811) are known to be specifically phosphorylated by cyclin D-CDK4/6 dimers (Takaki et al., 2005). We assessed these four specific serine residues by WB. In line with CDK6 down-regulation, levels of p-Rb1 significantly decreased on Ser780, Ser795, and Ser807/811 after miR-200a overexpression (Figure 4a). As with its subcellular localization and function, Rb1 and p-Rb1 were localized to the melanoma cells’ nuclei. Additionally, p-Rb1 levels were evaluated by counting the number of melanoma...
cells showing positive p-Rb1 Ser795 staining by indirect immunofluorescence. MiR-200a–induced CDK6 down-regulation significantly decreased p-Rb1 Ser795 levels (t test, \( P < 0.05 \)) (Figure 4b). Flow cytometry analysis showed that miR-200a–induced reduction of p-Rb1 levels was accompanied by a significant increase in cells arrested in G0/G1 phases (Figure 4c and d). In line with the functional effects of p-Rb1 level reduction, we observed that although E2F1 expression was not affected (t test, NS \( P > 0.05 \)) (Figure 4e and f), expression of Ki-67 (t test, \( P < 0.05 \)) (Figure 4g, and see Supplementary Figure S6 online), and CDK2 (t test, \( P < 0.05 \)) (see Supplementary Figure S2i), two representative E2F1 downstream proliferative targets, decreased significantly. These data show that miR-200a–induced CDK6 down-regulation leads to a reduction in p-Rb1 levels. Consequently, hypophosphorylated Rb1 binds and represses E2F1 transcriptional activity and therefore blocks its signaling pathway, reflected in a reduction of E2F1 downstream targets. This mechanistic role of miR-200a leads to an arrest of metastatic melanoma cells in G0/G1 phases. Thus, miR-200a has an important role in cell cycle G1/S checkpoint regulation by targeting CDK6 and its downstream pathway on cutaneous metastatic melanoma.

**MiR-200a expression blocks melanoma cell proliferation**

To gain more insight into the functional role of miR-200a expression in melanoma, we evaluated whether the arrest in cell cycle and the reduction in p-Rb1 levels cause a decrease in melanoma cell proliferation and colony formation. As expected, our results showed a significant reduction in cell proliferation and colony formation of melanoma cells transfected with miR-200a (see Supplementary Figure S7a–f online). Additionally, melanoma cells overexpressing miR-200a showed lower cell aggregation capability and lower potential to form spheroids after 9 days in three-dimensional spheroid formation assays (see Supplementary Figure S7g–j). In concordance with reduced CDK6 expression, these results suggest that miR-200a overexpression decreases metastatic melanoma cell proliferation and tumor colony formation ability. Therefore, we inferred that the opposite occurs in metastatic melanoma where miR-200a down-regulation increases CDK6 levels and promotes cell growth.

**High CDK6 expression levels overcome miR-200a effect in melanoma**

We assessed CDK6 levels in primary and metastatic melanoma tumors using the TCGA database. As shown in Supplementary Figure S8a online, CDK6 expression is significantly higher in metastatic melanoma than in primary melanoma specimens. We verified these results using our gene expression database for melanocyte (n = 3), LNM (n = 37), and DOM (n = 11) cell lines, available at the John Wayne Cancer Institute (Santa Monica, CA). DOM cell lines showed a higher expression of CDK6 compared with melanocytes (see Supplementary Figure S8b). We further validated these results by qRT-PCR in the melanoma cell lines (n = 29) (see Supplementary Figure S8c). Then, we analyzed the...
correlation between CDK6 and miR-200a expression levels, using a cohort of TCGA patients \( (n = 125) \). We observed a significant negative correlation between miR-200a expression and CDK6 expression \( (\text{see Supplementary Figure S8d}) \), which was more significant when comparing patients with low \( (n = 31) \) and high \( (n = 31) \) miR-200a expression \( (\text{see Supplementary Figure S8e}) \).

Then, we characterized other metastatic melanoma cell lines to test for cells naturally expressing high CDK6 levels. M-204 and LP-0024 cell lines were selected to determine whether miR-200a overexpression affects cell proliferation when CDK6 levels are high \( (\text{see Supplementary Figure S8f}) \). The results showed that CDK6 levels were not significantly affected by miR-200a overexpression as shown by WB \( (\text{see Supplementary Figure S8g}) \). We also analyzed cell proliferation after miR-200a transfection, and the results showed no differences in cell proliferation \( (\text{see Supplementary Figure S8h}) \) or colony formation \( (\text{see Supplementary Figure S8i}) \) when cells had high CDK6 levels. However, higher miR-200a doses showed significant changes in LP-0024 and HM-0525 cell proliferation \( (\text{see Supplementary Figure S9h and i}) \), indicating that CDK6 regulation is critical; our results consistently show that high CDK6 expression overrides the inhibition of cell proliferation induced by miR-200a overexpression.

To further understand this mechanism, we established two clones overexpressing CDK6 (WP-C2 and WP-C3) and one clone with low expression of exogenous CDK6 (WP-C1) using the WP-0614 cell line \( (\text{see Supplementary Figure S9a and b}) \). Exogenous overexpressed CDK6 did not contain a 3’-UTR region \( (\text{see Supplementary Figure S9c}) \), and hence is not targeted by miR-200a. Therefore, we evaluated whether stable CDK6 overexpression can rescue the effects induced by miR-200a. Our results showed that p-Rb1 level did not significantly change after miR-200a overexpression in WP-C3 but was reduced in control WP-C1 clone. We also observed decreased endogenous CDK6 levels \( (\text{see Supplementary Figure S9e}) \) in both WP-C1 and WP-C3 that can be targeted by miR-200a. Using the same strategy, the decrease in proliferation observed in WP-0614 cells was rescued by CDK6 overexpression in WP-C3 but not in WP-C1 clones \( (\text{see Supplementary Figure S9f}) \). Our data strongly support that miR-200a has an effect on metastatic melanoma growth that can be rescued by CDK6 overexpression.

**MiR-200a—induced cell growth arrest decreases the effect of palbociclib**

Considering the regulatory effects of miR-200a on CDK6 expression, the potential interactions with palbociclib, a CDK4/6 inhibitor recently approved by the US Food and Drug Administration, were evaluated \( (\text{Beaver et al., 2015; Turner et al., 2015}) \). Based on previous reports, the minimal inhibitory doses of palbociclib in melanoma vary greatly \( (\text{Young et al., 2014}) \). To identify the half maximal inhibitory concentration, we evaluated different concentrations \( (1–5,000 \text{ nmol/L}) \) for 72 hrs. Cell viability assays established 100 nmol/L as the half maximal inhibitory concentration for palbociclib in metastatic melanoma cells \( (\text{see supplementary Figure S10a online}) \). Then, the effect of palbociclib on the CDK4/6 signaling pathway was examined by analyzing Rb1 phosphorylation status and three-dimensional spheroid formation assays on melanoma cell lines. Palbociclib treatment decreased Rb1 and p-Rb1 levels, as shown by WB analysis \( (\text{see Supplementary Figure S10b–d}) \). In addition, a three-dimensional spheroid formation assay showed a significant reduction in cell growth \( (\text{see Supplementary Figure S10e–j}) \).

Our results further support that metastatic melanoma cell lines are sensitive to palbociclib treatment, as recently shown \( (\text{Young et al., 2014}) \). Because both miR-200a and palbociclib significantly reduce p-Rb1 by targeting CDK6 expression levels or CDK6 kinase activity, respectively, we hypothesized that miR-200a expression may affect the efficacy of palbociclib treatment in melanoma cells. To identify potential interactions between miR-200a expression and palbociclib, melanoma cells transfected with miR-200a or miR-ctrl precursors were treated with palbociclib. As expected, the combination of miR-200a and palbociclib significantly reduced CDK6 but not CDK4 expression. However, because palbociclib inhibits both CDK4 and CDK6, the p-Rb1 levels were more significantly reduced with the combination of palbociclib and miR-200a than with the miR-200a alone \( (\text{Figure 5a}) \). Flow cytometry analysis of cells transfected with miR-ctrl indicated that palbociclib significantly increased the percentage of melanoma cells arrested in G0/G1 phases in a dose-dependent manner \( (\text{Figure 5b}) \).

The percentage of melanoma cells arrested in G0/G1 after miR-200a transfection was similar to that obtained with the highest doses of palbociclib \( (\text{Figure 5b}) \). Nevertheless, we observed that palbociclib treatment of miR-200a—overexpressing cells significantly increased the percentage of melanoma cell population arrested in G0/G1 \( (\text{Figure 5b}) \); however, this effect was mainly due to miR-200a overexpression. Supporting this interaction, we found that cells overexpressing miR-200a showed lower half maximal inhibitory concentration for palbociclib \( (\text{see Supplementary Figure S11a online}) \). Concordantly, we showed a striking decrease in colony formation of miR-200a—transfected melanoma cells treated with palbociclib \( (\text{see Supplementary Figure S11b–d}) \). Overall, these results suggest that metastatic melanoma cells with low miR-200a and high CDK6 expression are more responsive to palbociclib treatment compared with cells with high miR-200a and low CDK6 expression. Nevertheless, miR-200a—overexpressing cells still showed response when combining with palbociclib, likely because of CDK4 inhibition.

Finally, we searched for other miRs that have been reported to target CDK6 mRNA. We found 35 signature mature sequences known to affect CDK6 expression \( (\text{see Supplementary Table S3 online}) \). We reviewed the TCGA database to determine changes in miR expression during melanoma progression. We found that most of the miRs where not altered during melanoma progression or showed insignificant low expression \( (\text{see Supplementary Figure S12d–f online}) \). Only three miRs were decreased in expression during melanoma progression: miR-203a, miR-452, and miR-506 \( (\text{see Supplementary Figure S12a–c}) \).

CDK inhibitors targeting CDK4/6 are among the most promising therapeutic agents for cancer treatment, mainly because of their cytostatic effects on tumor growth. Palbociclib has proven to be highly effective on melanoma cells,
where CDK4 amplification and CDKN2A deletion are frequently observed (Hamilton and Infante, 2016; Sheppard and McArthur, 2013; Young et al., 2014); however, little attention has been paid to CDK6, the posttranscriptional mechanisms that control its expression, and how that affects its response to palbociclib. Our findings showed a regulatory effect of miR-200a on CDK6 expression, which is frequently lost in metastatic melanomas because of DNA methylation and chromatin accessibility (Figure 2). This novel mechanism affecting CDK6 expression has a functional role in limiting the response to palbociclib's effect on CDK4 (Figure 5c). Conversely, we identified that the expression of CDK6 is significantly higher in low miR-200a expressing metastatic melanomas (see Supplementary Figure S8e) and, therefore, they have a better response to palbociclib (see Supplementary Figure S11a). Because lower miR-200a expression is associated with melanoma progression, we proposed that CDK6 inhibition would have a greater efficacy in patients with advanced cutaneous melanoma than in those with early stage disease. Low miR-200a expression has also been identified in epithelial origin tumor types (bladder and breast cancer [Davalos et al., 2012; Wiklund et al., 2011]), where DNA methylation is one of the major mechanisms controlling miR-200a expression; however, whether miR-200a is able to determine response to palbociclib by modulating cell cycle in those tumors is still unknown. Further studies are needed to determine how miR expression influences the response to palbociclib and other CDK4/6 inhibitors.

**MATERIALS AND METHODS**

**Melanoma patients**
Seventy-five patients treated at Providence Saint John’s Health Center were included in this study (see Supplementary Table S1). Paraffin-embedded archival tissue specimens were obtained from
benign nevi specimens (n = 10), PRMs (n = 7), LNM s (n = 33), and DOMs (n = 25). All samples and clinical information were included in the study under protocols approved by the Providence Saint John’s Health Center/John Wayne Cancer Institute Institutional and Western institutional review boards. Written, informed consent was obtained from all subjects, and the experiments were performed according to the principles set out in the World Medical Association Declaration of Helsinki and the National Institutes of Health Belmont Report. Tissue specimens were coded according to Health Insurance Portability and Accountability Act recommendations.

**RNA sequencing analysis**

Three metastatic melanoma lines (DP-0574, WP-0614, and BD-0548) were transfected with either 10 nmol/L of miR-200a or miR-ctrl precursors. RNA extraction was performed with TRI Reagent (Sigma-Aldrich, St. Louis, MI). The quality of RNA samples was estimated by RNA integrity number using Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). High quality RNA (RNA integrity number ≥ 8.0) was used to create mRNA libraries using the Illumina (San Diego, CA) TruSeq RNA Sample Preparation Kit v2. The mRNA libraries were then sequenced on the Illumina HiSeq 2500 rapid mode using TruSeq SBS v3–HS 200 cycle kit (Illumina) according to standard procedures, generating minimally 35 million 100-base pair paired-end reads per sample. Base calling and demultiplexing were performed using CASAVA v1.8 (Illumina), alignment was performed using TopHat 2.0.10 (Kim et al., 2013), and expression values were generated using Cufflinks, as previously described (Roberts et al., 2011). The z-score of RNA expression values for each treatment were calculated, and data were analyzed using Multiple Experiment Viewer, version 4.7.1 software (Saeed et al., 2006). Gene expression data are available at Gene Expression Omnibus: GSE83512 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83512).

Further details for drugs, reagents, and supplies; cell culture conditions; qRT-PCR; luciferase reporter assay; colony formation and three-dimensional spheroid culture assays; cell cycle analysis; DNA demethylation assays; WB; indirect immunofluorescence; and biostatistical analysis are available in the **Supplementary Materials and Methods** online.

**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 http://dx.doi.org/10.1016/j.jid.2017.03.039.

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