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TITLE PAGE

MiRNA directed cancer therapies: Implications in melanoma intervention

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Running Title: miRNAs in melanoma and melanoma therapies

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Abbreviations: (**miRNAs/miRs**) MicroRNAs; (**Ago**) Argonaute proteins; (**RISC**) RNA-induced silencing complex; 30 (**UTR**) 30- untranslated region; **TRPM1** (Transcriptional regulation of the melanoma prognostic marker melastatin); (**RUNX2**) Runt-related transcription factor 2; (**IGF2R**) insulin-like growth factor 2 receptor; (**TGFBR2**) TGF-beta receptor 2; (**NFAT5**) and nuclear factor of activated T cells 5; (**bFGF**) basic fibroblast growth factor; (**BMP-4**) bone morphogenetic protein 4; (**CDKs**) cyclin-dependent kinases; (**CTLs**) cytotoxic T lymphocytes; (**NKG2D**) natural killer cell immunoreceptor; (**4-PBA**) 4-phenyl-butyrate; (**PAM**) the Prediction Analysis of Microarray; (**SAM**) (Significance Analysis of Microarray); (**SODD**) the silence of death domain; (**lncRNAs**) long noncoding RNAs; (**DNMT**) DNA methyltransferase; (**HDACs**) histone deacetylases; (**HATs**) histone acetyltransferases; (**SP**) specificity protein; (**ESR**) estrogen receptor; (**FSCN1**) fascin actin-bundling protein 1; (**BSG**) basigin; (**MMP**) matrix metalloproteinases; (**ITGB3**) integrin beta 3; (**MITF**) microphthalmia-associated transcription factor; (**MARCKS**) myristoylated alanine-rich C-kinase substrate .

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ABSTRACT

Acquired tumor resistance to cancer therapies pose major challenges in the treatment of cancers including melanoma. Among several signaling pathways or factors that affect neocarcinogenesis, cancer progression and therapies, altered microRNAs (miRNAs) expression has been identified as crucial players in modulating the key pathways governing these events. While studies on miRNA field has grown exponentially in the last decade, much remains to be discovered, particularly with respect to their roles in cancer therapies. As immune and non-immune signaling cascades prevail in cancers, identification and evaluation of miRNAs, their molecular mechanisms and cellular targets involved in underlying development of cancers as well as acquired therapeutic resistance would help in devising new strategies for the prognosis, treatment and an early detection of recurrence. Importantly, an in-depth validation of miRNAs-targeted molecular events could lead to the development of an accurate progression-risk biomarkers, improved effectiveness as well as patient's responses to standard therapies. The current review focuses on the roles of miRNAs with recent updates including on its regulated cell cycle and proliferation, immune responses, oncogenic/epigenetic signaling pathways, invasion, metastasis and apoptosis with broader attention on melanomagenesis and melanoma therapies.

Introduction:

MicroRNAs (miRNAs) are a class of evolutionally conserved single-stranded noncoding RNAs of 19-22 nucleotides (Bartel, 2004). miRNAs are encoded within the genome (from intronic, exonic or intergenic regions) and are initially part of an immature primary transcripts (pri-miRNAs) that can be of several kilobases in length. The biogenesis of miRNA involves the cleavage of pri-miRNAs by RNAse enzyme, Drosha followed by its transcription to 60-100 nucleotide hairpin precursor RNAs (pre-miRNA). The pre-miRNA then transport to the cytoplasm by nuclear export factor, exportin-5 and excised by RNA polymerase enzyme, Dicer to produce 70 nucleotide long precursor miRNAs. Finally, putative helicase unwind these precursor miRNAs to ~18-24 nucleotides mature miRNAs (Pillai et al., 2004). Single-stranded mature miRNAs associate with argonaute proteins (Ago) to form the core of a multicomponent gene regulatory complex known as RNA-induced silencing complex (RISC) (Bartel, 2004). This RISC facilitates miRNA-mediated regulation of gene expression through base-pairing between miRNA and sequence(s) within the 3`untranslated region (UTR) of target messenger RNA (mRNA, i.e., between the protein-coding region of mRNA and its poly(A) tail) (Pillai et al., 2004). The binding of miRNA to mRNA reduces translation rate and/or increases degradation of mRNA (Vasudevan et al., 2007). However, recent evidences suggest that miRNAs may also increase mRNAs translation when cells are undergoing cell cycle arrest (Vasudevan et al., 2007). In general, miRNA half-life ranges from hours to days and varies depending on the organs, body fluids, and cell types (van Rooij et al., 2007). In comparison to mRNA, miRNAs are highly stable in formalin-fixed paraffin-embedded tissue blocks or biobank stored animal or human biosamples, which allow its use for localization and expression studies as biomarkers even after years of storage (Hall et al., 2012; Samir and Pessler, 2016).

miRNAs play important roles in essentially all biological processes (Tufekci et al., 2014). The differential expression of host miRNAs during infection supported the idea that they may constitute key players in host responses to invading pathogens (Lee et al., 1993; Ambros, 2004). It is being recognized that the regulatory roles of miRNAs are much sophisticated than initially thought, due to the cooperativity (i.e., more than one miRNA species can target the same mRNA) and the multiplicity of their targets (i.e., one miRNA can target hundreds of mRNA species) (Scaria et al., 2006). Three basic mechanisms of miRNA-mediated gene regulation are present: translation repression, direct mRNA degradation and, miRNA-mediated mRNA decay (Guo et al., 2010). Importantly, recent data suggest that the repression mechanism is predominately govern by reduction in mRNA target stability (Guo et al., 2010). Notably, miRNAs have been implicated in mediating broad range of processes including cell cycle and differentiation, regulation of metabolic pathways involved in lipid metabolism, inflammation, neurological, cardiovascular and metabolic disorders, apoptosis, cancer development and metastasis (Friedman et al., 2009; Lynn et al., 2009; Lorenzen et al., 2010; Lages et al., 2012; O'Connell et al., 2012; Salta and De Strooper, 2012).

Discovery of miRNA: In early 1990s, during studies investigating the timing of embryonic development of different larval stages of the worm, *Caenorhabditis elegans* (*C. elegans*), authors observed that the RNA transcribed from lin-4 locus did not encode a protein but instead silenced the gene encoding Lin-14, an important protein in larval development (Lee et al., 1993). The lin-4 containing complementary sequences in the 3`UTR of lin-14 mRNA, illustrated a regulatory mechanism by which lin-4 could modulate lin-14 mRNA translation in *C. elegans* leading to temporal pattern formation during development (Lee et al., 1993; Wightman et al., 1993). Additionally, the discovery that miRNAs are involved in sensing nutrient stress in plants, or

mediating responses to environmental stress as one of the mechanisms deployed to reprogram the gene expression so that cells can adapt to changing environments, has opened new horizons on identifying their functions in human diseases (Chiou, 2007; Holtz and Pasquinelli, 2009; Leung and Sharp, 2010; Mendell and Olson, 2012).

The biogenesis and modes of miRNAs mechanisms have not been completely elucidated. However, miRNA-mediated translational repression has been reported to be involved in regulating almost every cellular processes. The regulatory patterns of several other miRNAs have now been identified in species ranging from viruses to humans (Berezikov et al., 2006). The latest version of miRBase (miRBase Version 16.0) has 1048 miRNA sequences annotated in the human genome, and additional miRNAs will likely to be validated in the future (Berezikov et al., 2005; Griffiths-Jones et al., 2008; Shao et al., 2010; Persson et al., 2011). The literature indicates that one-third of these miRNAs are located in 113 gene clusters and based on the evidence from miRNA profiling data in various tissues and cell lines, these clusters are mostly co-expressed. This observation led to questions about cistronic expression regulatory patterns in gene clusters (Berezikov et al., 2005; Griffiths-Jones et al., 2008; Shao et al., 2010; Persson et al., 2011). The current understanding is that deregulation of one member of the cluster is accompanied by similar deregulations of other miRNAs from the same cluster. Thus, it would be crucial to ascertain whether one miRNA in a cluster can be regulated independently of others, especially those miRNAs which are implicated in the pathophysiology of human diseases (Karius et al., 2012). Of several protein-coding genes, miRNAs are believed to target approximately one-third of human mRNAs, and due to differential target binding patterns a single miRNA may target approximately 200 transcripts simultaneously (Brennecke et al., 2005). Hence, an in-depth analysis of miRNAs regulation might provide effective strategies to control numerous genes simultaneously.

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MiRNAs in the regulation of cancer

In particular, miRNAs are often aberrantly expressed in several human cancers including melanoma (with numerous miRNAs being overexpressed in one type of cancer and downregulated in another) (Nelson et al., 2006; Nelson and Weiss, 2008; Cortez et al., 2011; Bonozzi et al., 2012). For example, miR-205 is upregulated in lung, bladder and pancreatic cancers (Nelson and Weiss, 2008; Cortez et al., 2011; Bonozzi et al., 2012). In contrast, miR-205 is significantly downregulated in prostate and esophageal squamous cell carcinomas, indicating that cancer-associated miRNAs cannot be generalized (Melo and Esteller, 2010). Nonetheless, cancer-specific miRNA expression signatures may prove useful as diagnostic and therapeutic tools. Interestingly, miRNA expression signatures have been linked to several clinicopathological variables such as tumor stage and metastasis, receptor status, disease recurrence, treatment resistance and patient survival (Andorfer et al., 2011; Jiang et al., 2012). According to the personalized medicine model, miRNAs-associated molecular taxonomy could help to predict the likelihood of patients developing resistance against a particular treatment. For example, studies in breast cancer patients revealed that both miR-451 and miR-27 were involved in developing resistance to doxorubicin (Andorfer et al., 2011). Additionally, overexpression of miR-125b was shown to induce resistance of breast cancer cells to paclitaxel (Zhou et al., 2010). Therefore, the analysis of miRNAs that affect drug sensitivity represents potentially important area of investigation in understanding mechanistic insights contributing to drug resistance and clinical management of cancers.

MiRNAs and regulation of melanomagenesis and progression

Melanocytes are skin cells that originate from the neural crest cells and have the ability to produce melanin pigment. Melanocyte differentiation occurs via series of steps, resulting in lineage

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specification of melanoblasts, and transportation of mature melanosomes to keratinocytes (Ernfors, 2010). Melanomagenesis is a stepwise metamorphic process in which normal melanocytes in the epidermis gradually transform into the vertical growth phase characteristic of malignant melanomas (Bevona et al., 2003). Cutaneous malignant melanoma is a highly aggressive and metastatic malignancy accounting for the majority of skin cancer-related deaths worldwide (Villanueva and Herlyn, 2008). Among several factors, exposure to ultraviolet (UV) light, melanocyte integrity, melanocyte homeostatic mechanisms play important roles in the transformation of melanocytes into melanomas (Gupta et al., 2005; Rigel., 2008).

The dysregulation of miRNAs has been linked with either the suppression or progression of an initiation, differentiation, development or prognostic biomarker for melanoma (Gaur et al., 2007; Mueller et al., 2009; Chan et al., 2011; Bonozzi et al., 2012; Poliseno et al., 2012; Kozubek et al., 2013; Guo et al., 2014; Hwang et al., 2014; Knoll et al., 2014; Sun et al., 2014; Liu et al., 2015; Saldanha et al., 2016; Varamo et al., 2017) (Table 1). In miRNAs systematic screens, miR-211 identified as the most differentially expressed miRNA between normal melanocytes, non-pigmented melanoma cell lines, and primary melanomas in patients (Xu et al., 2012; Bell et al., 2014). Bell et al using gene expression profiling of normal and melanoma cells, investigated relationships between transcription factors and miRNAs, crucial for melanoma proliferation/invasion and identified several miRNAs including miR-211, and its new target NUAKI (Bell et al., 2014). The ectopic expression of miR-211 in melanoma cells significantly inhibited its growth and invasion compare to parental cells, suggesting that miR-211 possess tumor suppressor functions (Xu et al., 2012; Bell et al., 2014). This hypothesis was supported by findings that miR-211 is encoded by a region in the sixth intron of TRPM1 (transcriptional regulation of the melanoma prognostic marker melastatin), a candidate suppressor of melanoma metastasis

(Mazar et al., 2010; Xu et al., 2012; Bell et al., 2014). Additionally, TRPM1 and miR-211 expressions were regulated by MITF (microphthalmia-associated transcription factor), a transcription factor and master regulator of melanocyte development and function (Miller et al., 2004; Mazar et al., 2010). These findings indicate that the tumor suppressor activities of MITF and/or TRPM1 could be in-part mediated by miR-211. Recently, several miR-211 target genes including runt-related transcription factor 2 (RUNX2), insulin-like growth factor 2 receptor (IGF2R), TGF-beta receptor 2 (TGFBR2), POU domain-containing transcription factor BRN2, and nuclear factor of activated T cells 5 (NFAT5) have been identified (Aftab et al., 2014). It has been proposed that miR-211 may also directly regulate melanocyte pigmentation, and invasion as it is highly expressed in melanocytes and pigmented melanomas but not in non-pigmented melanomas (Aftab et al., 2014). Moreover, melanomas with greatly reduced miR-211 expression have shown to possess highly invasive characteristics (Levy et al., 2010; Mazar et al., 2010; Margue et al., 2013). In contrast, miR-211 highly expressing melanoma cells possess reduced invasive potential, independent of metastatin, inhibitor of tumor growth (Liu et al., 2001; Aftab et al., 2014; Bell et al., 2014). In the same context, miR-200c and miR-205 have been shown to be differentially expressed between benign nevi, and primary or metastatic melanoma, and act as tumor suppressors (Xu et al., 2012). Similarly, Braig et al., investigated that miR-196a downregulation, upregulated HOX-B7 and consequently stimulated basic fibroblast growth factor (bFGF) signaling resulting in upregulating ETS-1 transcription factor and bone morphogenetic protein 4 (BMP-4) expression, which play crucial roles in melanoma progression (Braig et al., 2010). Later, this group using high-throughput miRNA expression profiling approach in melanoma cells and tissue samples have shown that miR-196a expression was significantly reduced in malignant lesions (Mueller and Bosserhoff, 2011). Importantly, overexpression of miR-196a

significantly reduced melanoma cell invasiveness (Mueller and Bosserhoff, 2011). In addition, HOX-C8, cadherin-11, calponin-1, and osteopontin were identified as miR-196a targets (Mueller and Bosserhoff, 2011). Moreover, miR-149*, a p53-responsive miRNA has been shown to be overexpressed in human metastatic melanoma isolates, which targets glycogen synthase kinase -3 alpha (GSK3α) to induce resistance of melanoma cells to apoptosis via increasing the expression of Mcl-1 (Jin et al., 2011). Furthermore, miR-506-514 (a cluster of 14 miRNAs on X chromosome) and miR-218 have been demonstrated to play crucial roles in initiating melanocyte transformation (melanomagenesis) and/or promoting melanoma growth (Streicher et al., 2012; Guo et al., 2014).

MiRNA and regulation of cell cycle and proliferation in melanoma

As cell cycle regulation is controlled by several factors including cyclin-dependent kinases (CDKs), E2F transcription factor and proteins such as c-myc, p27 (a tumor suppressor protein that binds to, and inhibits the function of the cyclin D1-CDK4 complex), as well as PTEN (Mamillapalli et al., 2001; Walter et al., 2002; Suryadinata et al., 2010), one can postulate that miRNAs that regulate cell proliferation might directly target these cell cycle regulators (Table 2). In this regard, let-7b miRNA has shown to target cell cycle regulators as increased let-7b expression significantly decrease melanoma cell proliferation via reducing the expressions of CDK4, cyclin D1, and cyclin D3 (Schultz et al., 2008). Using miRNA microarrays, Chen et al analyzed the expression of 470 miRNAs in benign nevi and metastatic melanoma tissues, and observed 31 differentially expressed miRNAs, of which miR-193b was significantly downregulated in melanoma tissues (Chen et al., 2010). Furthermore, overexpression of miR-193b in Malme-3M melanoma cells resulted in inhibition of cell proliferation via downregulating eighteen genes including cyclin D1 (*CCND1*) (Chen et al., 2010). Similarly, downregulation of

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miR-206, miR-143 or miR-106b expression has been correlated with reduced growth and migration/invasion of several melanoma cell lines mediated via G1 cell cycle arrest resulting in an inhibition of CDK4, cyclin D1, cyclin C and syndecan-1 (Syn-1) or reactivation of p21/WAF1/Cip1 or as target genes (Georgantas et al., 2014; Li et al., 2014; Prasad et al., 2014). As cell cycle regulation controls the proliferation of cells, miR-221 and miR-222 have been shown to directly modulate the *in-vitro* and *in-vivo* proliferation of melanoma cells via targeting multiple signaling pathways including c-Kit or p27(Kip1) and its circulating levels malignant melanoma patients could be used as a new tumor marker (Felicetti et al., 2008; Igoucheva and Alexeev, 2009; Kanemaru et al., 2011). Notably, miRNAs, including miR-205, miR-149, miR-18b, miR-21, miR-203 and miR-26a have been documented to regulate cell cycle proteins in a cyclin-independent manner. In this regard, downregulation of miR-205 was reported in primary melanomas that regulates E2F1 and E2F5 transcription factors, which play crucial roles in the development of malignant melanoma (Nelson et al., 2006 & 2008; Umemura et al., 2009; Dar et al., 2011). Studies by Levati et al demonstrated that ectopic overexpression of miR-155 significantly inhibited the proliferation of melanoma cells via inducing apoptosis (Levati et al., 2011). Similarly, Liu et al identified downregulation of miR-9 in metastatic melanomas compared to primary melanomas, and its overexpression significantly decreased the proliferation and migration of melanoma cells in NF-kB1-dependent manner (Liu et al., 2012). Along similar lines, miR-145 expression was reported to be downregulated in canine melanoma cells and tissues and human melanoma cells. The ectopic expression of miR-145 significantly reduced the growth and migration of canine and human melanoma cells (Noguchi et al., 2012). Felli et al showed that miR-126 and 126* expression were downregulated during melanoma progression, and metalloproteases, domain 9 (ADAM9)

and 7 (MMP7) were identified as direct targets of miR-126 and 126* indicating their importance in melanoma progression (Felli et al., 2013).

MiRNA and regulation of melanoma immune responses

In addition to regulating cell cycle, miRNAs have been demonstrated to influence the host immunity against melanoma (Table 3). In this regard, the ectopic expression of miR-30b and miR-30d has been shown to target GalNAc transferase GALNT7 to enhance melanoma metastasis via promoting invasion, and increased synthesis of an immunosuppressive cytokine IL-10 and reduced immune cell activation & recruitment that resulted in induction of immunosuppression (Gaziel-Sovran et al., 2011). Similarly, miR-34a/c has been reported to regulate innate immune responses in melanoma cells via controlling ULBP2 expression, a stress-induced ligand of natural killer cell immunoreceptor (NKG2D) (Heinemann et al., 2012). As NKG2D detects early tumorigenesis, eliminates cytotoxic lymphocytes and provides an innate barrier to tumor development, overexpression of miR-34 downregulated ULBP2 expression, and removal of ULBP2 ligand protected malignant melanoma cells from NKG2D-mediated immune surveillance (Heinemann et al., 2012). Similarly, upregulation of NKG2D ligands, MICA/B and ULBP2 has been reported to mediate natural killer cell-induced cytotoxicity of melanoma cells by 1,25(OH)2D3 treatment mediated partly via the downregulation of miR-302c and miR-520c expression (Min et al., 2013). As suppressive immunophenotypes such as myeloid derived suppressor cells (MDSC) are involved in mediating immunosuppression and/or promoting tumor growth (Sahu et al., 2014). Liu et al has shown that TGF-β1-induced miR-494 expression in MDSCs favors the accumulation and functions of tumor-expanded MDSCs mediated by targeting of PTEN and activation of Akt pathway (Liu et al., 2012). Moreover, studies by Arts et al, demonstrated the role of miR-155 in

targeting the novel mechanisms of melanoma immune escape in inflammatory microenvironment mediated via the modulation of IL-1β-induced downregulation of endogenous microphthalmia-associated transcription factor (MITF-M) expression in melanoma cells (Arts et al., 2015).

MiRNA and epigenetic regulation of melanoma

Epigenetic refers to those biological processes by which changes in phenotype or gene expression occur without changes in DNA sequences. Several miRNAs have been shown to regulate or being regulated by epigenetic modification in melanoma (de Unamuno et al., 2015) (Table 3). In this regard, Mazar et al have demonstrated that miR-375 epigenetically regulate the development of melanoma in patients (Mazar et al., 2011). In this study, authors have shown that CpG island methylation regulate miR-375 expression in WM1552C stage 3 melanoma cells following treatment with demethylating agents, 5-aza-2-deoxycytidine and 4-phenyl-butyrate (4-PBA) (Mazar et al., 2011). Methylation of miR-375 CpG islands was stage dependent with significant levels in stage II and III melanoma tumors compared to stage I melanomas or benign melanocytes (Mazar et al., 2011). In another study, the expression of miR-34b was shown to be regulated by increase methylation of CpG islands, and this was apparent in stage III and IV melanoma tumors compared to stage I and II melanomas, melanocytes, and keratinocytes (Mazar et al., 2011). Similarly, epigenetic modulation has been shown to induce overexpression of miR-182 in human melanoma cells, and CpG islands upstream of mature miR-182 were found to be hyper-methylated in melanoma cells (Liu et al., 2013). In addition, decreased expression of miR-148a was reported in skin cancer patients when TGIF2 gene was targeted (Tian et al., 2015). In this study, authors found that DNA methylation regulate the expression and function of miR-148a, and concluded that this miR-148a methylation could serve as an independent potential indicator/marker in the

prognosis of skin cancer. However, due to the limitation of the number of samples and experimental conditions, as well as other unfavorable factors, further studies are still necessary (Tian et al., 2015). Importantly, studies by Gasque Schoof et al, demonstrated the roles of miR-26, miR-29 and miR-203 in the regulation of epigenetic reprogramming, and the involvement of Dnmt3a, Dnmt3b, Mecp2 and Ezh2 genes during melanocyte transformation (Gasque Schoof et al., 2015). Similarly, DNA methylation of CpG islands upstream of the miR-203 coding region (MIR203) was detected in both human and canine melanoma cells as well as canine clinical specimens, but not in human normal melanocytes. These findings indicate that demethylating MIR203 agents could be used as promising therapeutic target for the treatment of human and canine melanomas (Noguchi et al., 2015). The same group later demonstrated that miR-203 functions as a common tumor suppressor miRNA in human and canine melanoma cells via its ability to directly target CREB1, and its downstream targets, MITF and RAB27a (Noguchi et al., 2016).

MiRNAs in melanoma cell invasion and metastasis:

Metastasis of melanoma tumors to distal organs including the brain is a complex process requiring several stages from local tumor invasion to intra and extravasation leading to the formation of macrometastases, which is the major cause of skin cancer-related mortality in the United States (Adler et al., 2017; Westphal et al., 2017). Several factors or signaling pathways have been shown to drive melanoma cell migration and invasion leading to metastasis including FSCN1, BSG, β3-integrin, GALANT7, MARCKS, c-MET, STAT3, PTEN and NFkB1 (Muramatsu et al., 2003; Boukerche et al., 2007; Estrada-Bernal et al., 2009; Elson-Schwab et al., 2010; Yang et al., 2011; Chattopadhyay et al., 2012; Lie et al., 2012; Li et al., 2016). Importantly, a wide array of miRNAs

have been identified to target the key signaling pathways including these above mentioned (Zhang et al., 2006). In this regard, studies by Migliore et al have shown that miR-34b/c act as suppressor of metastasis as ectopic expression of these miRNAs directly target the proto-oncogene MET, leading to inhibition of MET-induced signal transduction and invasive behavior of melanoma cells (Migliore et al., 2008). Given that enhanced expression of integrin beta 3 (*ITGB3)* increases the invasiveness of melanoma cells (Seftor et al., 1992), let-7a has been shown to reduce the invasive potential of melanoma cells via negatively regulating ITGB3 expression (Müller et al., 2008). Similarly, miR-182, a frequently amplified miRNA in melanoma tumors compared to benign melanocytes has been shown to promote melanoma metastasis via repressing FOXO3 and microphthalmia-associated transcription factor (MITF-M) (Sequra et al., 2009). Downregulation of miR-182 impeded the invasion via inducing apoptosis, and enhanced expression of FOXO3 or MITF-M blocked miR-182 induced proinvasive effects (Segura et al., 2009). While several miRNAs have been shown to either promote or reduce the invasiveness of melanoma cells, studies by Elson-Schwab *et al* demonstrated that expression of miR-200 family members do not suppress invasion but regulate morphological plasticity or lead to a switch between modes of invasion of melanoma cells. The expression of miR-200c resulted in a higher proportion of cells adopting the rounded or amoeboid-like mode of invasion mediated via reduced expression of MARCKS, miR-200a induced a protrusion-associated elongated mode of invasion via reducing actomyocin contractility (Elson-Schwab et al., 2010). As reduced *let-7b* expression in melanoma cells leads to increased metastases due to enhanced expression of basigin (BSG), an invasion-associated protein and consequently enhanced expression of extracellular matrix metalloproteinases (MMPs) (Fu et al., 2011). Overexpression of *let-7b* resulted in reduced BSG and MMP-9 protein expression and decreased distant metastases (Fu et al., 2011). Along similar lines, Gaziel-Sovran et al reported

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that expression of miR-30b/30d in human melanoma positively correlated with the stage, metastatic potential, shorter time to recurrence and reduced overall survival. Ectopic expression of miR-30b/30d in melanoma cells increased their metastatic behavior via direct targeting of GalNAc transferase GALNT7 that resulted in reduced immune cell activation and recruitment (Gaziel-Sovran et al., 2011). In addition, Yang et al demonstrated that the overexpression of miR-21 in human melanoma requires STAT-3 activation, and regulates the metastatic behavior of B16 melanoma cells via targeting tumor suppressor (PTEN and PDCD40) and anti-proliferative (BTG2) proteins (Yang et al., 2011). Specific miRNAs termed as metastamiRs were reported to regulate the migration, invasion and metastasis of melanoma cells, suggesting that they represent novel targets to inhibit melanoma progression (White et al., 2011; Segura et al., 2012). Moreover, miR-1908, miR-199a-5p and miR-199a-3p have been shown to target ApoE that leads to LRP1/LRP8-dependent melanoma metastasis and angiogenesis (Pencheva et al., 2012). Similarly, overexpression of miR-200c (in CD44+CD133+ cancer stem cells) and miR-145 has been demonstrated to downregulate zinc-finger E-box binding homeobox 1 (ZEB1) or fascin actin-bundling protein 1 (FSCN1), a known regulator of cell migration to inhibit the migration, invasion and/or tumorigenicity of melanoma cells in-vitro and in-vivo (Dou et al., 2013; Dynoodt et al., 2013). Using miR-30-based short hairpin RNAs (ShRNAs) against heparanase (HPSE) and lentiviral approaches, it was reported that miRNAs (miR-30) targeting HPSE could be used as an effective RNA interference (RNAi) agents to suppress melanoma metastasis (Liu et al., 2013). Interestingly, studies by Fu et al, demonstrated the role of miR-26a in enhancing the biogenesis of other miRNA, especially let-7 in various cancer models including melanoma via targeting Lin28B and Zcchc11 and suppresses the tumor growth and metastasis (Fu et al., 2014). Recent studies have demonstrated that the downregulation of miR-365, miR-203 and miR-124 or overexpression/

upregulation of miR-15a, miR-194 and miR-21 inhibit the growth/proliferation, invasion or metastasis of malignant melanoma cells via their abilities to target distinct signaling pathways such as neuropilin 1 (NRP1), BMI1, RLIP76, a stress-inducible non-ABC transporter, CDCA4, GEF-H1, STAT3, PTEN and PDCD4 (Bai et al., 2015; Chang et al., 2015; Alderman et al., 2016; Guo et al., 2016; Li et al., 2016; Saldanha et al., 2016; Zhang et al., 2016). Importantly, the differential expression of miR-339-3p in melanoma cells and health melanocytes has been correlated with reduced invasion associated with decreased MCL1 expression (Weber et al, 2016)

MiRNA and apoptotic induction in melanoma

Multiple studies have highlighted the role of microRNAs, including miR-205, miR-155, miR-26a, miR-21, miR-15b and miR-149* in apoptosis induction (Satzger et al., 2010; Dar et al., 2011; Jin et al., 2011; Levati et al., 2011; Satzger et al., 2012; Reuland et al., 2013; Jiao et al., 2015; Mao et al., 2017) (Table 5). In this regard, Satzger et al, determined the expression levels of 16 miRNA in normal melanocytes versus 10 melanoma cell lines, and FFPE tissues of 11 melanocytic nevi versus 16 melanoma (Satzger et al., 2010). In this study, the levels of miR-15b and miR-210 were significantly upregulated, and miR-34a was significantly downregulated. However, upon further evaluation of these 3 miRs in 128 primary melanomas from patients with detailed clinical follow-up information, only miR-15b was found to be significantly associated with poor recurrence free survival, and overall survival. The downregulation of miR-15b in two melanoma cell lines with higher miR-15b expression resulted in reduced tumor cell proliferation, and increased apoptosis, indicating the important role of miR-15b in melanoma and associated poor prognosis and tumorigenesis (Satzger et al., 2010). In another study, reduced miR-205 expression was identified in melanoma cells compared to benign nevi (Dar et al., 2011). Further analysis showed that miR-

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205 targets E2F1 and reduces its expression by decreasing the proliferation via inducing apoptosis mediated through the activation of p73 family members in advanced malignant melanomas (Dar et al., 2011). Importantly, miR-149* was found to be directly regulated by p53 which targets glycogen synthase kinase-3 alpha (GSK-3α) to induce resistance of melanoma cells to apoptosis mediated via increased expression of Mcl-1 (Jin et al., 2011). In addition, downregulation of miR-155 was found to be downregulated, and its ectopic expression induces apoptosis via inhibition of SKI gene expression (Levati et al., 2011). Interestingly, miR-21 expression was reported to be significantly increased in primary and malignant melanoma tissues and melanoma cells compared to benign nevi, normal skin and melanocytic cell preparation, and that downregulation of miR-21 in melanoma cells induces apoptosis without significantly affecting the cell proliferation or via targeting programmed cell death 4 (PDCD4) (Satzger et al., 2012; Jiao et al., 2015). Moreover, miR-26a was found to be significantly downregulated in human melanoma cell lines compared to primary melanocytes, and overexpression of miR-26a resulted in significant and rapid cell death and repressed silencer of death domain (SODD) expression that rescued melanoma cells from undergoing apoptosis suggesting miR-26a as potential therapeutic molecule in the treatment of melanoma (Reuland et al., 2013). Furthermore, miR-21 was reported to also regulate ERK/NF-kB pathway, and miR-21 inhibitors inhibit the proliferation, migration and invasion of A375 human melanoma cells via inducing increased apoptosis by targeting SPRY1, PDCD4 and PTEN (Mao et al., 2017).

MiRNA and melanoma therapy

Malignant melanoma often develop resistance to most standard chemotherapeutic agents and radiation therapy (Terando et al., 2003). While new targeted therapies such as vemurafenib, which

target a BRAFV600 activating mutant kinase has shown initial promising anti-tumor responses in melanoma patients, tumor-resistance remains a significant therapeutic challenge (Baukari et al., 2017; Kim et al., 2016; Sosman et al., 2012; Wagle et al., 2011; Zhao et al., 2017). Among several oncogenic signaling pathways that contribute in tumor-resistance, our recent studies have shown that platelet-activating factor-receptor mediated pathway play crucial roles in the development and negatively impacting the efficacy of standard chemotherapy and radiation therapy in preclinical and clinical studies including melanoma (Hackler et al., 2014; Sahu et al., 2012; 2014; 2015; 2016). Thus, further investigation of molecular mechanisms underlying melanoma development and/or therapeutic resistance is required to design new strategies to improve the clinical outcomes in melanoma patients. Despite various reports correlating miRNAs involvement with or without BRAF-mutated melanoma tumors and/or therapies in preclinical and clinical studies (Caramuta et al., 2010; Shi et al., 2014; Lankenau et al., 2015; Pinto et al., 2015; Foth et al., 2016; Mannavola et al., 2016; Saldanha et al., 2016), more research is needed to develop sensitive and specific molecular tests to identify novel miRNAs which are modulated in response to resistance to standard melanoma therapies (Table 5). Fattore et al, in a recent review have highlighted the roles of miRNA in inducing the development of resistance to BRAF and MEK inhibitors (Fattore et al., 2017). Importantly, studies by Kozar et al have identified the differential expression of several new and previously reported miRNAs in BRAF inhibitors (vemurafenib and dabrafenib) resistant melanoma cells (Kozar et al., 2017). In addition, Jiang et al reported that miR-21 status was an independent prognostic factor in cutaneous melanoma patients (Jiang et al., 2012). Importantly, antisense-mediated miR-21 silencing inhibited melanoma growth via increasing apoptosis and also enhanced the chemo- or radiosensitivity of human cutaneous melanoma cells, suggesting its potential in the treatment of human cutaneous malignant melanoma (Jiang et al., 2012). Poell and

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colleagues using functional assays highlighted the importance of miR-15/16, miR-141/200a, miR-96/182 and miR-203 as potent inhibitors of melanoma cell proliferation as ectopic expression of these miRNAs resulted in long-term inhibition of melanoma cell expansion, both in vitro and in vivo (Poell et al., 2012). This study provided a comprehensive interrogation of miRNAs that interfere with melanoma cell proliferation and viability, and offered a selection of miRNAs that are promising candidates in melanoma therapy (Poell et al., 2012). Wagenseller et al studied global miRNA expression profiles using microarrays in melanoma tissues from combination-targeted therapy of temsirolimus and bevacizumab treated patients, and detected significant upregulation of 15 miRNAs in treated vs. non-treated melanoma tissues, 12 of which possess tumor suppressor functions via their ability to target 15 different oncogenes (Wagenseller et al., 2013). Of these miRNAs, miR-125b, miR-7b and miR-29c were differentially expressed after temsirolimus and bevacizumab combination treatment. Similarly, differential expression of miR-659-3p based on progression free survival (PFS) was reported to predict the clinical outcome of carboplatin/paclitaxel-based therapy in metastatic melanoma patients (Villaruz et al., 2015). In particular, miR-514a which plays important role in initiating melanocyte transformation and promotion of melanoma growth has been reported to modulate the sensitivity of BRAF-targeted therapy via regulating the tumor suppressor NF1 gene (Stark et al., 2015). These findings indicate that these miRNAs could serve as attractive candidates for melanoma intervention (Stark et al., 2015; Villaruz et al., 2015; Wagenseller et al., 2013). In addition, miR-32 replacement therapy as a single agent has been demonstrated to suppress the growth of melanoma tumors in preclinical models via targeting MCL-1, and exhibit synergistic effects with vemurafenib (Mishra et al., 2016). Moreover, while miR-579-3p has been shown to be associated with the development of melanoma resistance (Fattore et al., 2016), miR-7, miR-34a, miR-100 and miR-125b were

demonstrated to reverse/restore melanoma resistance (Sun et al., 2016; Vergani et al., 2016) to targeted therapies via targeting distinct signaling pathways. Importantly, recent studies have implicated the functions and clinical significance of long non-coding RNA (lncRNAs) in melanoma (Aftab et al., 2014; Richtig et al., 2017). Thus, the future combinatorial approaches should focus on oncogenes that can be targeted by miRNAs and lncRNAs in cancer detection and treatment including melanoma**.**

Conclusion:

miRNAs were a paradigm shift in science from its discovery till to date. miRNAs may assist in the diagnosis and early detection of melanoma recurrence, and in predicting patient's outcomes/responses to therapies. Thus, the development of miRNAs as an accurate progression risk biomarkers would greatly enhance the clinical management of melanoma.

Conflict of Interest Disclosures

Authors declare no competing conflicts of interest.

Author's Contributions

All authors participated equally in writing and approving the final version of the article.

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Legends for Figure and Tables:

Figure 1. Schematic representation of miRNAs roles in melanoma and melanoma therapy

Table 1. miRNAs in melanomagenesis and progression.

Table 2. miRNAs in melanoma cell cycle regulation and proliferation.

Table 3. miRNAs in the regulation of immune responses and epigenetics in melanoma.

Table 4. miRNAs in melanoma cell invasion and metastasis.

Table 5. miRNAs in melanoma apoptosis and melanoma therapy.

Tables:

Table 1: miRNAs in melanomagenesis and progression.

miRNA	Function(s)	Expression of miRNA	Target(s)	References
miR-211	Several functions; tumor suppressor, regulation of melanocytic pigmentation, increase or decrease invasiveness	Upregulation or Downregulation	NUAKI, RUNX2, TGFBR2, BRN2, NFAT5, KCNMA1, TRPM1, MITF,	Levy et al., 2010 Mazar et al., 2010; Zhou et al., 2010 Boyle et al., 2011 Xu et al., 2012; Margue et al., 2013 Bell et al., 2014
miR-200c, miR-205	Tumor suppressor	Differential expression		Xu et al., 2012
miR-196a	Progression and invasiveness	Downregulation	HOX-B7, bFGF, ETS-1, BMP-4, HOX-C8, Cadherin-11, Calponin-1, Osteopontin,	Braig et al, 2010 Mueller and Bosserhoff, 2011
miR-149*	Oncogenic regulator	Upregulation	GSK3α, Mcl-1	Jin et al., 2011
miR-506-514 cluster, miR-218	Melanocyte transformation (melanomagenesis) & melanoma growth	Overexpression	MITF	Streicher et al., 2012; Guo et al., 2014

Table 2: miRNAs in melanoma cell cycle regulation and proliferation.

miRNA	Function(s)	Expression of miRNA	Target(s)	References
miR let-7b	Cell cycle regulation and proliferation	Upregulation	CDK4, cyclin D1, cyclin D3	Schultz et al., 2008
miR-193b	Cell cycle regulation and proliferation	Downregulation	Cyclin D1	Chen et al., 2010
miR-206	G1 cell cycle arrest and inhibition of proliferation	Downregulation	CDK4, cyclin D1, cyclin C	Georgantas et al., 2014
miR-143	G1 cell cycle arrest and induction of apoptosis	Downregulation	Syn-1	Li et al., 2014
miR-106b	G1 cell cycle arrest and inhibition of growth	Downregulation	P21/WAF1/Cip1	Prasad et al., 2014
miR-221 and miR-222	Cell proliferation	Downregulation and Upregulation	PLZF, c-Kit, p27(Kip1) /CDKN1B	Felicetti et al., 2008; Igoucheva and Alexeev, 2009; Kanemaru et al., 2011
miR-205	Cell proliferation	Downregulation	E2F1 and E2F5	Dar et al., 2011

miR-155	Cell proliferation	Downregulation	SKI	Levati et al., 2011
miR-9	Cell proliferation and migration	Downregulation	E-cadherin, NF-kB1-Snail1	Liu et al., 2012
miR-145	Suppression of cell proliferation and migration	Downregulation	c-MYC	Noguchi et al., 2012
miR-126 and miR-126*	Melanoma progression	Downregulation	ADAM9 MMP7	Felli et al., 2013

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Table 3: miRNAs in the regulation of immune responses and epigenetics in melanoma.

miRNA	Function(s)	Expression of miRNA	Target(s)	References
miR-30b and miR-30d	Cell invasion and Immune suppression	Upregulation	GalNAc transferases	Gaziel-Sovran et al., 2011
miR-34a and miR-34c	Regulation of innate immunity	Upregulation	NKG2DL ULBP2	Heinemann et al., 2012
miR-494	Functions of tumor-expanded MDSCs	Upregulation	PTEN	Liu et al., 2012
miR-302c and miR-520c	Natural killer cell-mediated toxicity to tumor cells	Downregulation	NKG2D, MICA/B and ULBP2	Min et al., 2013
miR-155	Melanoma immune escape	Upregulation	IL-1β, MITF-M	Arts et al., 2015
miR-375	Cell proliferation, invasion, and cell motility	Epigenetic regulation	Minimal CpG island methylation in melanocytes, keratinocytes, normal skin and nevus Hypermethylation of CpG island in patients primary,	Mazar et al., 2011

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			regional, distant and nodular metastatic melanoma tissues	
miR-34b	Cell invasion and motility	Epigenetic regulation	CpG island methylation	Mazar et al., 2011
miR-182	Epigenetic modulation	Upregulation	CpG island hypermethylation	Liu et al., 2013
miR-148a	Regulation of DNA methylation	Downregulation	TGIF2	Tian et al., 2015
miR-26, miR-29 and miR-203	Melanocyte transformation/ antioncogenic	Epigenetic regulation	Dnmt3b CREB1/MITF/RAB27a	Gasque Schoof et al., 2015 Noguchi et al., 2015 & 2016

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Table 4: miRNAs in melanoma cell invasion and metastasis.

miRNA	Function(s)	Expression of miRNA	Target(s)	References
miR-34b/c	Suppresses invasiveness and metastasis	Ectopic expression	MET	Migliore et al., 2008
Let-7a	Cell Invasion	Downregulation	ITGB3	Muller et al., 2008
miR-182	Cell invasion and metastasis	Upregulation	FOXO3, MITF	Segura et al., 2009
miR-200 family (miR-200c/a)	Morphological plasticity of cells	Downregulation	MARCKS	Elson-Schwab et al., 2010
Let-7b	Cell Invasion	Downregulation	BSG	Fu et al., 2011
miR-30b/d	Cell invasion and metastasis	Upregulation	GALNT7	Gaziel-Sovran et al., 2011
miR-21	Metastasis	Upregulation	BTG2	Yang et al., 2011
miR-1908, miR-199a-5p and miR-199a-3p	Metastasis		ApoE, DNAJA4, LRP1/LRP8	Pencheva et al., 2012
miR-200c	Cell migration and invasion	Overexpression	ZEB1	Duo et al., 2013
miR-145	Cell migration and invasion	Overexpression	FSCN1	Dynoodt et al., 2013

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miR-30-based RNAi	Metastasis		HPSE	Liu., 2013
miR-26a	Tumor growth and metastasis	Downregulation	Lin28B, Zcchc11	Fu et al., 2014
miR-365	Inhibition of cell invasion and metastasis	Downregulation	NRP1	Bai et al., 2015
miR-203	Metastasis	Downregulation	BMI1	Chang et al., 2015
miR-15a	Inhibition of growth and invasiveness	Upregulation	CDCA4	Alderman et al., 2016
miR-194	Inhibition of cell proliferation and metastasis	Overexpression	GEF-H1	Guo et al., 2016
miR-21	Metastasis	Upregulation	STAT3, PTEN PDCD4	Li et al., 2016; Saldanha et al., 2016
miR-339-3p	Tumor suppressor/ cell invasiveness	Differential expression in melanoma cells & melanocytes	MCL-1	Weber et al., 2016
miR-124	Inhibition of cell Invasion	Downregulation	RLIP76	Zhang et al., 2016

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Table 5: miRNAs in melanoma apoptosis and melanoma therapy.

miRNA	Function(s)	Expression of miRNA	Target(s)	References
miR-15b, miR-205, miR-149*, miR-155, miR-21, miR-26a, ,	Apoptosis induction	Upregulation of 15b, 149* & 21 Downregulation of 155, 205 & 26a	E2F1 (205) SODD (26a) GSK-3α (149*) SKI (155) PDCD4 (21), SPRY1 (21) & PTEN (21)	Satzger et al., 2010 Dar et al., 2011; Jin et al., 2011 Levati et al., 2011 Satzger et al., 2012 Reuland et al., 2013 Jiao et al., 2015 Mao et al., 2017
miR-21	Inhibition of growth and augmentation of chemo- and radiosensitivity	Upregulation	Bax/Bcl-2 ratio	Jiang et al., 2012
miR-15/16, miR-41/200a, miR-96/182 family of miRNAs and miR-203	Inhibition of cell viability	Downregulation	Survivin	Poell et al., 2012
miR-125b, miR-7b, miR-29c	Regulation of mechanisms of	Differential expression after	AKT, CCND1, DNMT3A/B	Wagenseller., 2013

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	action of Temsirolimus and Bevacizumab combination	treatment with Temsirolimus and Bevacizumab combination		
miR-659-3p	Predicts clinical outcome of carboplatin/ paclitaxel-based therapy	Differential expression based on PFS	NFIX	Villaruz et al., 2015
miR-514a	Modulates BRAFi sensitivity	Overexpression	NF1	Stark et al., 2015
miR-32	Tumor suppressor & exhibit synergistic effects with vemurafenib	Poor expression	MCL-1	Mishra et al., 2016
miR-579-3p	Resistance to targeted therapy	Low expression (downregulation)	BRAF, MDM2	Fattore et al., 2016
miR-7	Reversal of resistance to targeted therapy	Downregulation	EGFR/IGF-1R /CRAF	Sun et al., 2016

miR-34a, miR-100 and miR-125b	Restoration of resistance to vemurafenib	High expression (upregulation)	CCL-2	Vergani et al., 2016

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Figure 1: Schematic representation of miRNAs roles in melanoma and melanoma therapy.