Research Article

A Micro-RNA Connection in Braf\(^{V600E}\)-Mediated Premature Senescence of Human Melanocytes

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1. Introduction

Unregulated oncogene expression during cancer development causes cancer cells to senesce prematurely, a gene-directed program that irreversibly induces cell cycle arrest [1–4]. First described in cell culture [5], oncogene-induced senescence (OIS) has been confirmed in vivo as a vital mechanism that constrains the malignant progression of many tumors [3]. Unregulated oncoproteins promote senescence by activating effector pathways that are cell-type and oncogene specific. Recent progress identified different regulatory circuitries of OIS, but much remains to be learned [6]. High-throughput sequencing of the cancer genomes has identified Braf kinase as the most frequently mutated (50–70%) oncogene in melanoma [7]. About 90% of Braf gain-of-function mutations are at position 600 with glutamic acid (E) inserted for valine (V) [7]. Braf is a serine/threonine protein kinase that functions directly downstream of the small GTPase Ras and upstream of the MEK and ERK mitogen-activated protein kinase (MAPK) cascade. This mutation significantly increases Braf kinase activity toward MEK, causing constitutive Braf-MEK-ERK signaling [7, 8]. The Braf gain-of-function Val to Glu mutation is required for cell viability, anchorage-independent growth, and proliferation of Braf\(^{V600E}\)-positive melanoma cell lines in both cell-based studies and in vivo mouse models [9–11]. Furthermore, a Phase I clinical trial with a potent inhibitor of Braf\(^{V600E}\) kinase demonstrates that Braf\(^{-}\)mutant melanomas are highly dependent on Braf kinase activity [12]. Besides its frequent presence in melanoma, Braf\(^{V600E}\) mutations are also present in up to 82% of melanocytic nevi with classical hallmarks of senescence [13]. Importantly, sustained Braf\(^{V600E}\) expression in human melanocytes induces premature senescence [13]. The gain-of-function mutation in Braf may be the cause of growth
2 Results and Discussion

To identify putative microRNAs involved in OIS, we detected microRNAs induced by \( \text{BRaf}^{V600E} \) melanocytes using a focused PCR-based microarray system that enabled assessment of 52 microRNAs (SABIosciences). As expected, expression of \( \text{BRaf}^{V600E} \) in primary human melanocytes induced constitutively high levels of ERK1/2 phosphorylation (Figure 1(a)) promoting growth arrest (Figure 1(b)) and senescence as detected by the expression of senescence markers \( \text{p}16^{\text{INK4a}}, \text{p}15^{\text{INK4b}}, \text{Dec}1, \text{p}16, \text{p}15, \) and \( \text{DcR}2 \) (Figures 1(a) and 1(d)). At a time point prior to the detection of senescence, we collected total RNA and enriched for small RNAs as recommended by the manufacturer. By enriching for small RNAs, this system is designed to detect mature microRNA expression levels. The levels of each microRNA were quantified relative to the levels of four control small RNAs. Among the 52 microRNAs that we examined, 13 were significantly induced and three (miR-10b, -15b, and -16) were greatly repressed by \( \text{BRaf}^{V600E} \) in primary melanocytes (data not shown). Subsequently, quantitative real-time PCR (qRT-PCR) was used to further examine the effect of \( \text{BRaf}^{V600E} \) on the expression of these microRNAs with specific primers for each individual microRNA. Eight out of the 13 that were stimulated in the original screen were consistently induced more than 2-fold by \( \text{BRaf}^{V600E} \) (Figure 2). Based on the documented expression of these microRNAs in clinical melanocytic nevi, we selected four of them for further study [20, 24, 25].

To examine their causal role in cellular senescence in melanocytes, we expressed individual microRNAs in primary human skin melanocytes by retroviral transduction. The expression of microRNAs was confirmed by qRT-PCR with specific primers (Figure 3(a)). The effects of individual microRNA expression on the expression of five senescence markers were determined by qRT-PCR (Figures 3(b)–3(e)). Three out of the four microRNAs tested had an observable effect on the expression of at least one of the senescence markers. These are microRNAs 143, 34a, and 29a. While expression of miR-34a increased \( \text{p}16^{\text{INK4a}}, \text{p}15^{\text{INK4b}}, \text{DcR}2, \) and \( \text{Dec}1 \) transcripts, expression of miR-143, or 29a increased the \( \text{p}15^{\text{INK4b}} \) and \( \text{DcR}2 \) transcripts (Figures 3(c)–3(e)). To investigate directly their role in cellular senescence, we measured cell proliferation by quantifying the number of melanocytes expressing the proliferation marker protein ki-67 due to the expression of miR-143, 34a, or 29a. Expression of miR-143 or -34a but not -29a or -100 led to a decrease in melanocyte proliferation as measured by the expression of proliferation marker protein ki-67. Importantly, the decreased expression of ki-67 correlated with intense activity of SA-\( \beta \)-Gal (Figure 3(f)).

Oncogenic \( \text{BRaf} \) induced premature senescence in melanocytes with a concomitant increase in the expression of senescence markers \( \text{p}16^{\text{INK4a}}, \text{p}15^{\text{INK4b}}, \text{Dec}1, \text{and DcR2}. \) In addition, we showed that oncogenic \( \text{BRaf} \) also significantly increased the expression of miR-143, miR-34a, let-7c, miR-15a, miR-29a, miR-100, miR-181a, and miR-181d. By specific Watson-Crick base pairing between the seed region of microRNA and sites within the mRNA 3′UTR, each microRNA can potentially target different groups of mRNAs for regulation. Expression of miR-143, -34a, or -29a led to the increased expression of some of the senescence markers. It is therefore possible that a combined expression of the identified microRNAs is responsible for the observed upregulation of senescence markers in oncogenic \( \text{BRaf} \) expressing melanocytes.

Among the microRNAs that are induced by the \( \text{BRaf} \) gain-of-function mutation, only miR-143 or -34a is sufficient to induce growth arrest and senescence when ectopically expressed in primary melanocytes. miR-34a negatively regulates cell cycle G1/S transition genes cyclin D1, cyclin E2, CDK4, and CDK6 and causes G1 arrest [26–28] when expressed in human primary diploid fibroblasts. In human cancer cells, miR-143 is one of the most notable tumor suppressor miR-RNAs, which can directly inhibit oncogene KRAS translation and block the downstream signal pathways [29]. Restoring miR-143 expression inhibited proliferation and induced apoptosis by targeting BCL2 [30, 31]. In agreement with these studies, we showed that miR-34a or -143 induced premature senescence in primary human melanocytes. Ectopic expression of miR-34a increased expression of p16\(^{\text{INK4a}}\), p15\(^{\text{INK4b}}\), Dec1, and DcR2. Unlike miR-34a, miR-143 only increased expression of p15\(^{\text{INK4b}}\) and DcR2. Since both miR-34a and -143 expression caused senescence, it can be inferred that expression of p16\(^{\text{INK4a}}\) or
Figure 1: Melanocytes were infected with control retroviral empty vector (EVC) or with a vector expressing BRafV600E. Four days after infection, cells were harvested for (a) immunoblotting with specific Abs as indicated or (d) total RNA. The expression levels of the indicated senescence markers in BRafV600E expressing or control cells were quantified by qRT-PCR and normalized to the level of b-actin. The values represent the means and standard deviation from three independent experiments. Cells were also isolated 4 days after infection, fixed, and stained for (b) ki-67 or (c) SA-β-Gal activity. Insets: representative fields are shown for control EVC and B-RafV600E expressing cells stained with ki-67 Ab or positive for SA-β-Gal activity.
Dec1 is not essential for oncogenic B Raf-mediated premature senescence. Concordantly, the presence of p16\textsuperscript{INK4a} was also previously found not to be required for senescence induced by gain-of-function B Raf mutation in melanocytes [13]. It is presently not clear which mRNAs, miR-34a or -143, target for repression in melanocytes, nor do we know how oncogenic B Raf induces their expression.

By targeting different subset of genes for regulation, microRNAs can function either as oncogenes or tumor suppressor genes. As expected, all the identified induced miRNAs have tumor suppressive functions. For instance, miR-29, which has three family members, negatively impinges on cancer cell survival by directly targeting the mRNAs of the regulatory subunit of PI3 kinase (p85a) and CDC42 for regulation. miR-29a could also directly decrease CDK6 expression and cause G1/S growth arrest in [32]. Consistent with its tumor suppressive role, miR-29 expression is frequently downregulated in multiple cancers. However despite its proven role in cell cycle regulation, expression of miR-29a is not sufficient to induce senescence in melanocytes. It is possible that miR-29a is required but not sufficient for oncogenic B Raf-induced senescence. It is also possible that the effect of miR-29a on cell cycle progression is cell-context dependent.
We reason that if microRNA is part of the mechanism that integrates the diverse components into a coordinated response to the B Raf$^{V600E}$ mutation, we should also observe downregulation of oncogenic microRNAs. Indeed, miR-10b, which increases the expression of oncogene RhoC by directly targeting its transcriptional repressor HOXD10 [33], is consistently repressed in melanocytes harboring the B Raf$^{V600E}$ mutation. In addition to miR-10b, we also observed expression levels of miR-15b and -16 downregulated. miR-15/16 belongs to a very unique group of microRNAs. miR-16 has 2 transcripts—one is called miR-16-1 located on Chromosome (Chr) 13 and shares the same transcript with miR-15a forming the miR-15a/16 cluster. The other miR-16 is named miR-16-2, which together with miR-15b is located at Chr 3 as the miR-15b/16 cluster. Interestingly while miR-15b and 16 were downregulated, the miR-15a was upregulated in melanocytes expressing B Raf$^{V600E}$. Since our miR-16 primer detected both miR-16-1 and -16-2 transcripts, it is unknown at present which miR-16 was downregulated by oncogenic B Raf expression. The miR-15/16 family is known to have a negative effect on cell proliferation by targeting miRNAs for various cell-growth-associated genes [34–36]. It is therefore not clear why the downregulation of miR-15b and -16 is favored in melanocytes, which are undergoing senescence. Alternatively it is also possible that miR-3b/16 has unidentified oncogene targets.

Although it is clear that B Raf induces senescence in melanocytes by activating MEK and ERK, the downstream effectors of its cytostatic effect remain to be identified [13, 37]. B Raf$^{V600E}$-mediated senescence also requires additional effector pathways involving several immune and growth mediators such as IL6, IL8, and IGFBP7 [6]. However, the mechanism that integrates the diverse pathways into a coordinate response to oncogenic stimulation has not been defined. In this study, we identified several microRNAs whose expression rates were stimulated by oncogenic B Raf. Expression of four of these microRNAs induced the expression senescence-surrogate markers. Among them, miR-143 or -34a alone can induce growth arrest and senescence when expressed in primary melanocytes.

Melanoma is a cancer of melanocytes and is the most deadly form of skin cancer. The transformation of melanocytes to malignant melanoma is a stepwise process fueled by the accumulation of mutations in critical growth and survival regulatory genes. The development of benign nevi from melanocytes is the first phenotypic change that can activate the oncogenic B-Raf-mediated senescence pathways. As such a thorough understanding of how oncogenic B Raf induces senescence will allow us to elucidate the molecular mechanisms that drive the conversion of melanocytes to preneoplastic nevi and transition to melanoma.

3. Materials and Methods

3.1. Cell Lines and Reagents. Human melanocytes were from Cascade Biologics (Portland, OR, USA) or Yale Cell Culture Core Facility. Human melanocytes were grown in Media 254 with added growth supplements (Cascade Biologics).

3.2. Plasmid Constructs. Retroviral expression vectors (miR-vec) for miR-100, miR-15a/16-1, and -181a were a kind gift of Reuven Agami. The miRNA minigenes -29a, -34a, -143, -181d, -15b, and -15b/16-2 were PCR amplified from genomic human DNA, cloned downstream of the CMV promoter in miR-vec, and sequence verified. The primers for the miRNA minigenes cloning were the following:

- miR-29a Forward: 5'-gcGGATCCCTGGAACCAATCCCTCAA
- miR-29a Reverse: 5'-gcGAATTCGCTCTTCTCCACATCATCT
- miR-34a Forward: 5'-gcGGATCCCGGCTGGTCTTGAACCTC
- miR-34a Reverse: 5'-gcGAATTCGCTCTTCTCCACATCATCT
- miR-143 Forward: 5'-gcGGATCCTCAAGGTGGTGTCCTGGGTTG
- miR-143 Reverse: 5'-gcGAATTCGTCGTAAGCGATCTG
- miR-15 Forward: gcggatccAAGGATGATTATGAG
- miR-15 Reverse: gcgaattcAGTGGAAACAAATGTTAATG
- miR-15b/16 Forward: gcggatccGACTGCAACCATA
- miR-181d Forward: gcggatccGAGCTTGAAGTGGACACC
- miR-181d Reverse: gcgaattcTCCAGCCAGGAGCCCATCC

3.3. Isolation of Genomic DNA from Primary Melanocytes. One 10 cm cultured plate of subconfluent primary melanocytes was harvested by trypsin digestion. Trypsin was subsequently neutralized and cells were collected by centrifugation and washed with PBS. The washed cell-pellet was resuspended with 1.2 mL cell resuspension buffer (10 mM Tris-HCL, 10 mM NaCl, 1.5 mM MgCl$_2$) by gently
Figure 3: Continued.
Figure 3: Melanocytes were infected with EVC or with vector expressing the indicated miRNA. (a) Quantification of miRNAs expression by qRT-PCR in indicated miRNA expressing cells. (b)–(e) The expression levels of the indicated senescence markers in the indicated miRNA expressing cells were quantified by qRT-PCR and normalized to the level of β-actin. The values represent the means and standard deviation from three independent experiments. Cells infected with the indicated miRNA expressing virus were also isolated after selection, fixed, and stained for (f) ki-67 or (g) SA-β-Gal activity.

3.4. Analysis of SA-β-Galactosidase Activity. The senescence-associated β-galactosidase activity in culture cell was detected with a staining kit purchased from Cell Signaling Technology according to the manufacturer’s specifications.

3.5. Ki-67 Staining. Cells were plated on laminin-coated cover slips (Laminin, L 2020, SIGMA). After 48 hours, cells were washed with PBS and fixed with 100% cold Methanol for 10 minutes. Fixed cells were then incubated in blocking solution (0.1% BSA in PBS) for 30 minutes. Primary antibodies, 200–300 μL (1:100 in block solution), specific for Ki-67 (sc-15402, Santa Cruz) were added onto each cover slips and incubated overnight at 4°C. Primary antibody-stained cells were washed 3 times for 3 minutes each with 0.1% BSA-PBS blocking solution. Washed cells were subsequently incubated with FITC-conjugated secondary antibody (1:200 diluted in blocking solution) for 1 hour at room temperature followed by one wash with blocking solution for 3 minutes, two PBS washes for 3 minutes each, and two washes with dH2O. The nuclei of Ki-67 stained cells were visualized with DAPI (4 μg/mL in dH2O) staining for 10 min. Cells were rinsed with dH2O twice before mounting onto glass slides with DAKO fluorescent mounting medium (Dako, s3032). The glass slides were dried in the hood for 5–10 minutes.

The cover slip edges were sealed with nail enamel polisher and were dried in the hood for an addition 10–20 min.

3.6. Antibodies. The BRaf polyclonal antibodies were from Upstate (number 07-453). The Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) Mouse mAb (number 9106) and p44/42 MAP Kinase Antibodies (number 9102) were from Cell Signaling. Dec1 (s-8) (sc-101023), p16 (JC8) (sc-56330), and p15 (C-20) (sc-612) antibodies were from Santa Cruz. DcR2 Polyclonal Antibody (AAP-371) was from Assay designs. Monoclonal anti-α Tubulin Clone B-5-1-2 (T-5168) was from SIGMA.

3.7. Quantitative Real-Time RT-PCR. Total cellular RNA was extracted with the Trizol reagent (Invitrogen) and reverse transcribed using random hexamer primers (Applied Biosystems). The resulting cDNAs were used for PCR using SYBR-Green Master PCR mix (QIAGEN) in triplicates. PCR and data collection were performed on ABI7500 (Applied Biosystems). β-actin, GAPDH, and HPRT were used as an internal standard. The gene-specific primers were as follows:

**Human p14**
- Forward: 5′-CATGACCTGTTCTCTAGGAAAGC-3′
- Reverse: 5′-CCCTCGTGTAGTGCTACTGA-3′

**Human p15**
- Forward: 5′-CATGCCTGTTCTCTAGGAAAGC-3′
- Reverse: 5′-CCCTCGTGTAGTGCTACTGA-3′

**Human p16**
- Forward: 5′-GGGAAGAAGGAAGAAGATGTTGCTGTTACT-3′
- Reverse: 5′-GGGAAGAAGGAAGAAGATGTTGCTGTTACT-3′

**Human p16**
- Forward: 5′-GGGAAGAAGGAAGAAGATGTTGCTGTTACT-3′
- Reverse: 5′-GGGAAGAAGGAAGAAGATGTTGCTGTTACT-3′
human DcR2
R: 5’-CCGGGGGATGGTGCCAGAGT-3’
F: 5’-CGCTCAGGACAGGAGCCTATC-3’
human Dec1
R: 5’-CGATGAGCCGGTGCGGAAT-3’
F: 5’-CCGGGACTGGAGCCAGGAG-3’
b-actin
F: 5’-ATCTGCGCACAGACCTCTGAC-3’
R: 5’-CGTCATACCTGCTTGATC-3’
glyceraldehyde-3-phosphate dehydrogenase
F: 5’-TGCAACCACTGCTTTAGC-3’
R: 5’-GGCATGGACTGTGGTCATG-3’

3.8. Real-Time PCR to Check Expression Levels of miRNAs. cDNA from the obtained miRNAs was synthesized using RT2 miRNA first strand kit (SABiosciences). Amount of miRNA used was 200 ng. The cDNA was then amplified using miRNA-specific primer. RNU6, SNORD 44, and SNORD 48 used were as controls. Real-time PCR results were analyzed using the ddCT method.

3.9. Isolation of miRNA. Small RNA from primary melanocytes was prepared using RNeasy Mini kit protocol (Qiagen). Cells were harvested for RNA using TRIzol (Invitrogen). Chloroform was added to the tubes containing homogenate (20% of the volume of TRIzol used). After shaking the tubes vigorously, the tubes were incubated at RT for 2-3 minutes and centrifuged (12,000 g) for 15 mins at 4°C. The upper aqueous layer was transferred to new tubes and 1 volume of 70% ethanol was added. The sample was mixed and applied to the RNasy minispin column and centrifuged at $8000 \times g$ (10,000 rpm) for 15 s at RT. The larger RNAs are bound to the column filter. The flow-through contained miRNAs and was further purified according to the manufacturer’s specifications (Qiagen).

Author’s Contribution
Gang Ren and Jingwei Feng are contributed equally to this work.

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