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Signaling by p38 MAPK Stimulates Nuclear Localization of the Microprocessor Component p68 for Processing of Selected Primary MicroRNAs

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Abstract

The importance of microRNAs (miRNAs) in biological and disease processes necessitates a better understanding of the mechanisms that regulate miRNA abundance. We showed that the activities of the mitogen-activated protein kinase (MAPK) p38 and its downstream effector kinase MAPK-activated protein kinase 2 (MK2) were necessary for the efficient processing of a subset of primary miRNAs (pri-miRNAs). Through yeast two-hybrid screening, we identified p68 (also known as DDX5), a key component of the Drosha complex that processes pri-miRNAs, as an MK2-interacting protein, and we found that MK2 phosphorylated p68 at Ser¹⁹⁷ in cells. In wild-type mouse embryonic fibroblasts (MEFs) treated with a p38 inhibitor or in MK2-deficient (MK2^{-/-}) MEFs, expression of a phosphomimetic mutant p68 fully restored pri-miRNA processing, suggesting that MK2-mediated phosphorylation of p68 was essential for this process. We found that, whereas p68 was present in the nuclei of wild-type MEFs, it was found mostly in the cytoplasm of MK2^{-/-} MEFs. Nuclear localization of p68 depended on MK2-mediated phosphorylation of Ser¹⁹⁷. In addition, inhibition of p38 MAPK promoted the growth of wild-type

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SUPPLEMENTARY MATERIALS

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MEFs and breast cancer MCF7 cells by enhancing the abundance of c-Myc through suppression of the biogenesis of the miRNA miR-145, which targets c-Myc. Because pri-miRNA processing occurs in the nucleus, our findings suggest that the p38 MAPK–MK2 signaling pathway promotes miRNA biogenesis by facilitating the nuclear localization of p68.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small RNAs that suppress gene expression posttranscriptionally by partial base pairing with the 3' untranslated regions of target mRNAs (1). They are predicted to regulate the expression of 20 to 30% of genes within the genome at any given time (2). Many of these miRNA-targeted mRNAs encode genes whose products are essential for cell proliferation, differentiation, survival, apoptosis, migration, and invasion (3, 4). miRNAs are initially generated in the nucleus as long primary transcripts known as primary miRNAs (pri-miRNAs). The biogenesis of functional mature miRNAs includes two consecutive steps. First, the Drosha- and DGCR8-containing processing machinery (also called the microprocessor) mediates the processing of pri-miRNAs to produce stem-loop-structured precursors known as precursor miRNAs (pre-miRNAs) of 60 to 70 nucleotides, which are exported to cytoplasm. Second, a complex containing Dicer and TARBP2 mediates the processing of pre-miRNAs to produce mature miRNAs of ~22 nucleotides (1, 5). This two-stepped process of miRNA biogenesis provides additional regulatory options for fine-tuning (6).

In human cancer tissues, the total cellular amount of miRNAs is reduced (7, 8), whereas pri-miRNAs accumulate (9), indicating that miRNA biogenesis is impaired in cancers. This notion is supported by studies showing that the amounts of Drosha, Dicer1, AGO2 [argonaute RISC (RNA-induced silencing complex) catalytic component 2], and other proteins essential for miRNA biogenesis are often reduced in cancers (10–12). Additionally, knockdown of Drosha or Dicer promotes oncogenesis (13). Mutations in the genes encoding TARBP2 and Dicer1 occur in colon and nonepithelial ovarian cancers, respectively (14–16), and contribute to tumorigenesis by impairing miRNA biogenesis (14, 17). In addition to regulating total cellular miRNA biogenesis, RNA binding proteins (RNPs) also regulate the biogenesis of specific miRNAs. For example, LIN28 suppresses the expression of the let-7 miRNA by binding to the terminal loop of pri-let-7, thus blocking its cleavage by Drosha (18). The hairpin of pri-miR-18a is recognized by heterogeneous nuclear RNP₁, which facilitates the biogenesis of miR-18a by recruiting the Drosha-containing complex to pri-miR-18a (19).

Mitogen-activated protein kinases (MAPKs) are involved in various biological processes, including cell proliferation, apoptosis, differentiation, migration, and cytoskeletal remodeling (20). The three major families of MAPKs are the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs. The importance of MAPKs in miRNA biogenesis is suggested by a study that found that ERK-mediated phosphorylation of TARBP2 facilitates pre-miRNA processing (21). Other studies have reported that the expression of several miRNAs is functionally associated with the p38 MAPK signaling pathway. For example, the activity of p38 MAPK is required for both DNA damage-induced production of miR-34c and hypoxia-induced production of miR-1 (22, 23); however, whether the p38 MAPK signaling pathway controls miRNA abundance by directly regulating miRNA biogenesis is unclear.

p68 [also known as DEAD (Asp-Glu-Ala-Asp) box helicase 5 (DDX5)] is a member of the DEAD box RNA helicase family, and it is capable of unwinding double-stranded RNAs (24). The identification of p68 as a component of the Drosha complex highlighted its potential involvement in miRNA biogenesis (25). A later study showed that p68-null mouse

embryonic fibroblasts (MEFs) were defective in the processing of a subset of pri-miRNAs (26). That p68 can unwind the let-7 miRNA duplex in vitro (27) suggests that p68 assists in the processing of pri-miRNAs by facilitating the loading of pri-miRNAs into the Drosha complex. Several studies showed that p68 also acts as a scaffold to bring Drosha and other regulators together. For example, p53 interacts with p68 to enhance the biogenesis of miRNAs that suppress cell growth (28). Binding of estrogen receptor to p68 blocks p68-dependent processing of pri-miRNAs (29). In addition, p68 shuttles between the nucleus and the cytoplasm, which involves both nuclear localization and nuclear export signals (30). Given that pri-miRNAs are processed to pre-miRNAs by the Drosha complex, it is imperative that p68 be present in the nucleus to play its role in regulating miRNA biogenesis; however, how the shuttling of p68 is regulated remains unclear.

Here, we determined the effect of inhibiting p38 MAPK activity on miRNA biogenesis. The p38 MAPK inhibitor SB203580 reduced the abundances of both the precursor and the mature forms of miR-145, miR-181a1, and miR-199a by blocking the processing of their respective pri-miRNAs. In experiments with MEFs deficient in the p38 effector MAPK-activated protein kinase 2 (MK2), we further showed that MK2 was required for efficient processing of pri-miRNAs. Mechanistically, MK2 interacted with p68 and phosphorylated p68 at Ser¹⁹⁷ both in vitro and in cells. MK2-mediated phosphorylation of p68 was essential for pri-miRNA processing because a phosphomimetic variant of p68 fully restored pri-miRNA processing in wild-type MEFs treated with a p38 MAPK inhibitor and in MK2^{-/-} MEFs. We found that whereas p68 was present in the nuclei of wild-type MEFs, it was present in the cytoplasm of MK2^{-/-} MEFs. Moreover, a phosphomimetic variant of p68 was exclusively found in the nucleus even in MK2^{-/-} MEFs, whereas a mutant p68 that could not be phosphorylated by MK2 remained mostly cytoplasmic. These results suggest that MK2-mediated phosphorylation of p68 was required for its nuclear localization. Because pri-miRNA processing occurs in the nucleus, we conclude that the p38 MAPK–MK2 signaling pathway promotes miRNA biogenesis by inducing the nuclear localization of p68.

RESULTS

The p38 MAPK–MK2 signaling pathway promotes miRNA biogenesis at the level of pri-miRNA processing

To investigate the potential involvement of the ERK, JNK, and p38 MAPK signaling pathways in miRNA biogenesis, we treated wild-type MEFs with the MEK1/2 (mitogen-activated or extracellular signal-regulated protein kinase kinase 1/2) inhibitor U0126, the JNK inhibitor SP600125, or the p38 MAPK inhibitor SB203580 and then analyzed the amounts of the primary (pri-), precursor (pre-), and mature forms of miR-145, miR-181a1, and miR-199a, which are expressed in MEFs (26). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed that the amounts of mature miRNAs were decreased in cells treated with U0126, but that the amounts of their respective pri- and pre-miRNAs were not substantially affected (Fig. 1A). This observation is consistent with a previous finding that the ERK signaling pathway is involved in pre-miRNA processing (21). We found that SP600125 did not alter the amounts of pri-miRNAs, pre-miRNAs, or mature miRNAs (Fig. 1B). In contrast, SB203580 reduced the amounts of both pre-miRNAs and mature miRNAs without altering the amounts of pri-miRNAs (Fig. 1C). We then performed in vitro pri-miRNA processing experiments by incubating in vitro-synthesized radiolabeled pri-miR-145, pri-miR-181a1, and pri-miR-199a transcripts with whole-cell extracts collected from vehicle-treated (control) or SB203580-treated MEFs. Analysis by gel electrophoresis showed that all three pri-miRNAs were processed to their respective pre-miRNAs by extracts of untreated cells (Fig. 1D); however, the pri-miRNAs were processed to a lesser degree by the extracts of SB203580-treated cells (Fig. 1D). These results suggest that the activity of p38 MAPK is required for efficient pri-miRNA processing.

MK2 is a kinase that is directly downstream of p38 MAPK in the signaling pathway, and it executes p38 MAPK-mediated posttranscriptional gene regulation, including regulating mRNA stability and protein translation (31). The nature of miRNAs as posttranscriptional gene regulators led us to hypothesize that MK2 might serve as an effector of p38 MAPK to regulate pri-miRNA processing. To test this hypothesis, we analyzed the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in wild-type and MK2-deficient (MK2^{-/-}) MEFs. Our qRT-PCR analysis showed that the amounts of the precursor and mature forms of these miRNAs were at least 50% less in MK2^{-/-} MEFs than in those of their wild-type counterparts, whereas the amounts of their respective pri-miRNAs were similar between both cell types (Fig. 2A). Retroviral expression of MK2, but not the empty vector, in MK2^{-/-} MEFs increased the amounts of the pre- and mature miRNAs so that they were similar to those observed in wild-type MEFs (Fig. 2A). Transducing wild-type MEFs with lentivirus expressing an MK2-specific short hairpin RNA (shRNA), but not with lentivirus expressing a luciferase-specific shRNA, reduced the amounts of the pre- and mature miRNAs such that they were comparable to those in MK2^{-/-} MEFs (Fig. 2A). Consistent with these results, *in vitro* miRNA processing experiments also showed that all three pri-miRNA transcripts were poorly processed in extracts of MK2^{-/-} cells (Fig. 2B). These results show that the presence of MK2 is essential for efficient pri-miRNA processing. To substantiate these observations, we performed microarray analyses to examine the total cellular amounts of miRNAs in wild-type and MK2^{-/-} MEFs. Among 345 miRNAs that were detected in the array, 83 were decreased in abundance more than twofold, whereas only 13 were increased in abundance more than twofold, with the remaining miRNAs not being substantially altered, supporting the notion that MK2 is a stimulator of the biogenesis of at least a subset of miRNAs.

We next blocked MK2 activity in wild-type MEFs by transducing the cells with a lentivirus expressing a dominant-negative MK2 (MK2-DN) (32, 33). Our qRT-PCR analysis showed that MK2-DN suppressed the amounts of pre- and mature miRNAs (Fig. 2C), thus confirming the necessity of the kinase activity of MK2 for pri-miRNA processing. In a subsequent experiment, we transduced wild-type MEFs with lentivirus expressing a constitutively active mutant MK2 (MK2-CA) (32, 33) and then treated the cells with SB203580 to inhibit p38 MAPK activity. Whereas MK2-CA did not substantially alter the amounts of pre- and mature miRNAs in control cells, it almost completely restored their amounts in SB203580-treated cells, such that they were similar to the amounts in vehicle-treated cells (Fig. 2D). Together, these data suggest that MK2 is a downstream effector of p38 MAPK that promotes pri-miRNA processing.

p68 physically interacts with MK2 and is a direct MK2 substrate

That the kinase activity of MK2 was necessary for pri-miRNA processing led us to hypothesize that MK2 might function by directly phosphorylating a protein that is essential for miRNA biogenesis. To test this hypothesis, we screened a mouse embryonic yeast two-hybrid library, with MK2 as the bait. Among the interacting proteins that we identified, p68 was a logical choice to focus on because it is a component of the Drosha complex (25) and is required for pri-miRNA processing (26). To identify a possible interaction between MK2 and p68 in cells, we performed co-immunoprecipitation experiments with wild-type MEFs with either antibody against MK2 (anti-MK2 antibody) or antibody against p68 (anti-p68 antibody). We detected MK2 in p68 immunoprecipitates (Fig. 3A) and detected p68 in MK2 immunoprecipitates (Fig. 3B).

To determine whether the activation status of MK2 affected the interaction between MK2 and p68, we transfected MK2^{-/-} MEFs with plasmid encoding Myc-tagged wild-type MK2, MK2-CA, or MK2-DN and then subjected the cells to coimmunoprecipitation with a polyclonal antibody against the Myc tag. Western blotting analysis of these

immunoprecipitates showed that MK2, MK2-CA, and MK2-DN all interacted with p68 (Fig. 3C). To map the region in p68 that was required for its interaction with MK2, we cotransfected MEFs with plasmids encoding the hemagglutinin (HA)-tagged N-terminal, core, or C-terminal regions of p68 together with a plasmid encoding Myc-tagged MK2 (Fig. 3D). Coimmunoprecipitation experiments showed that only the core region of p68 interacted with MK2 (Fig. 3D). Moreover, the helicase and adenosine triphosphatase (ATPase) activities of p68 were not required for its interaction with MK2 because both a helicase-defective mutant of p68 (in which the DEAD sequence was mutated to DQAD) and an ATPase-defective mutant of p68 (in which the SAT sequence was mutated to AAA) interacted with MK2 to a similar extent to that of wild-type p68 (Fig. 3E).

MK2 phosphorylates proteins at serine residues in the consensus motif Hyd-X-Arg-X-X-Ser, where Hyd is a hydrophobic residue (34, 35). Human p68 contains the corresponding sequence ACRLKS¹⁹⁷ at amino acid residues 192 to 197, and this sequence is conserved across various species (Fig. 3F). To determine whether MK2 phosphorylated Ser¹⁹⁷ of p68, we prepared recombinant wild-type p68 and p68 Ser¹⁹⁷ Ala mutant (p68A) proteins. In vitro kinase assays with either recombinant active MK2 or MK2 immunoprecipitated from wild-type MEFs showed that recombinant p68 protein, but not p68A, was phosphorylated (Fig. 3G). To confirm the phosphorylation of Ser¹⁹⁷ of p68 by MK2, we analyzed both recombinant p68 and MK2-phosphorylated p68 by mass spectrometry. Ser¹⁹⁷ was the only phosphorylated amino acid residue that we detected in MK2-phosphorylated p68. These results show that MK2 can directly phosphorylate p68 at Ser¹⁹⁷ in vitro.

To determine whether MK2 was capable of phosphorylating p68 in cells, we generated a polyclonal antibody specific for phosphorylated p68 with a synthesized Ser¹⁹⁷-phosphorylated peptide [RLK(pS)TCIYGGAPKG], which corresponded to amino acid residues 194 to 207 of the human p68 protein. Western blotting analysis with this antibody showed that Ser¹⁹⁷-phosphorylated p68 was readily detected in wild-type MEFs but was absent from SB203580-treated wild-type MEFs and from MK2^{-/-} MEFs (Fig. 3H). However, we observed Ser¹⁹⁷-phosphorylated p68 in MK2^{-/-} MEFs that were transfected with plasmid encoding wild-type MK2 but not with plasmid encoding MK2-DN (Fig. 3H). These results demonstrate that MK2 phosphorylates p68 at Ser¹⁹⁷ in cells.

Because the RNA helicase p72 contains an amino acid sequence that is similar to the RLKSTCIYGGAPKG sequence of p68, it was possible that MK2 might also interact with and phosphorylate p72. Coimmunoprecipitation experiments with the anti-MK2 antibody revealed a physical association between MK2 and p72 in wild-type MEFs. An in vitro kinase assay showed that recombinant p72 was a substrate of MK2. That MK2 could phosphorylate p72 also indicated that the band observed at the position above phosphorylated p68 in lysates of wild-type MEFs and MK2^{-/-} MEFs expressing exogenous MK2 most likely corresponded to phosphorylated p72 (Fig. 3H).

MK2-mediated phosphorylation of p68 is required for pri-miRNA processing

To investigate whether MK2-mediated phosphorylation of p68 was relevant for miRNA biogenesis, we initially confirmed the necessity of p68 for pri-miRNA processing. We transduced wild-type MEFs with lentivirus expressing p68-specific shRNA and then analyzed the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a. Our qRT-PCR analysis showed that knockdown of p68 led to a more than 50% reduction in the amounts of the precursor and mature forms of these miRNAs when compared with those of cells transduced with lentivirus expressing a luciferase-specific shRNA (control cells), whereas the amounts of their respective pri-miRNAs were not substantially altered (Fig. 4A), thus confirming the importance of p68 for efficient pri-miRNA processing.

We next reconstituted wild-type MEFs with wild-type p68, an MK2-phosphomimetic mutant form of p68 known as p68E (in which Ser¹⁹⁷ is replaced by Glu), or an MK2-nonphosphorylatable mutant form of p68 known as p68A (in which Ser¹⁹⁷ is replaced by Ala). Our qRT-PCR analysis showed that p68E fully restored the amounts of the precursor and mature forms of miR-145, miR-181a1, and miR-199a in SB203580-treated MEFs, whereas wild-type p68 had little effect, and p68A further decreased the amounts of the precursor and mature forms of miR-145, miR-181a1, and miR-199a in SB203580-treated MEFs (Fig. 4B). Similarly, the amounts of the precursor and mature forms of these miRNAs in MK2^{-/-} MEFs expressing p68E, but not p68A, were similar to those observed in wild-type MEFs (Fig. 4C). These results suggest that MK2-mediated phosphorylation of p68 is essential for efficient pri-miRNA processing.

To further determine the importance of MK2-mediated phosphorylation of p68 at Ser¹⁹⁷ in pri-miRNA processing, we investigated whether the assembly of p68 into the Drosha complex required its MK2-mediated phosphorylation. Coimmunoprecipitation studies with a polyclonal antibody against Drosha showed that p68 was detected in the Drosha complex in wild-type MEFs, but not in MK2^{-/-} MEFs or in SB203580-treated wild-type MEFs (Fig. 4D). Furthermore, the p68 that was in the Drosha complex was phosphorylated at Ser¹⁹⁷ (Fig. 4D), and preclearing the lysates of wild-type MEFs with a polyclonal antibody against phosphorylated p68 before performing the immunoprecipitation completely removed p68 from the Drosha complex (Fig. 4E). In a subsequent experiment, we transfected wild-type MEFs with plasmids encoding HA-tagged wild-type p68, p68E, or p68A. Coimmunoprecipitation with a polyclonal antibody against Drosha showed that HA-tagged p68 and p68E, but not p68A, were present in the Drosha complex (Fig. 4F). Similarly, we observed Drosha in immunoprecipitates of HA-tagged p68 and p68E, but not p68A (Fig. 4G). Together, these results suggest that only p68 phosphorylated at Ser¹⁹⁷ is assembled into the Drosha complex.

MK2-mediated phosphorylation determines the intracellular distribution of p68

Because pri-miRNA processing occurs in the nucleus, we would expect p68 as a component of Drosha complex to participate in pri-miRNA processing in the nucleus. Although p68 predominantly localizes in the nucleus in most cultured cells (36), p68 also shuttles between the nucleus and the cytoplasm (30). We postulated that the nuclear localization of p68 requires its MK2-mediated phosphorylation at Ser¹⁹⁷. To test this hypothesis, we performed immunofluorescence staining with an anti-p68 monoclonal antibody to determine the intracellular localization of p68 in wild-type and MK2^{-/-} MEFs. We detected p68 in the cytoplasm of MK2^{-/-} MEFs, and we observed it in the nuclei of wild-type MEFs and MK2^{-/-} MEFs reconstituted with MK2 (Fig. 5A). In parallel, we also prepared nuclear and cytoplasmic fractions of wild-type MEFs, MK2^{-/-} MEFs, and MK2^{-/-} MEFs expressing exogenous MK2. Western blotting analysis of these fractions revealed that p68 was mainly detected in the cytoplasmic fraction of MK2^{-/-} MEFs (Fig. 5B). In contrast, p68 was mostly detected in the nuclear fractions of wild-type MEFs and MK2^{-/-} MEFs expressing exogenous MK2 (Fig. 5B). Furthermore, p68 phosphorylated at Ser¹⁹⁷ was detected only in the nuclear fraction of wild-type MEFs (Fig. 5C). These results show that the presence of MK2 is essential for the nuclear localization of p68 and that p68 phosphorylated at Ser¹⁹⁷ resides in the nucleus.

To further examine the importance of MK2-mediated phosphorylation of p68 for its nuclear localization, we transduced wild-type and MK2^{-/-} MEFs with lentiviruses expressing enhanced green fluorescent protein (EGFP) fusion proteins of the p68A and p68E mutants. With a fluorescence microscope, we observed that EGFP-p68A was found in the cytoplasm of wild-type and MK2^{-/-} MEFs (Fig. 5D). In contrast, we observed EGFP-p68E only in the nuclei of these cells (Fig. 5D). Similarly, cellular fractionation followed by Western blotting

analysis showed that HA-tagged p68E was detected only in the nuclear fractions of wild-type and MK2^{-/-} MEFs, whereas p68A was detected only in the cytoplasm of these cells (Fig. 5E). These results suggest that MK2-mediated phosphorylation of Ser¹⁹⁷ is sufficient for the nuclear localization of p68.

Blocking p38 MAPK signaling promotes the proliferation of MEFs and MCF7 cells by reducing miR-145 abundance and increasing c-Myc abundance

The miRNA miR-145 suppresses the proliferation of MCF7 cells, a human breast cancer cell line, by decreasing the abundance of c-Myc (37, 38). Our observation that inhibiting the p38 MAPK–MK2 signaling pathway reduced the amount of miR-145 in MEFs (Figs. 1 and 2) suggested that blocking p38 MAPK activity might enhance the abundance of c-Myc and the extent of proliferation in MEFs and MCF7 cells through the suppression of miR-145 production. To examine this possibility, we first treated MCF7 cells with SB203580 for 1 day and then used qRT-PCR to analyze the amounts of the pri- and mature forms of miR-145. Similar to its effect on the processing of pri-miR-145 in wild-type MEFs (Fig. 1C), SB203580 reduced the amount of mature miR-145 in MCF7 cells by more than 80% compared to that in control cells, whereas the amount of pri-miR-145 was not substantially altered (Fig. 6A). Also similar to what we observed in MEFs (Fig. 4B), p68E, but not p68A, largely restored the production of mature miR-145 in SB203580-treated MCF7 cells (Fig. 6A). In a parallel experiment, we performed Western blotting analysis and showed that the amount of c-Myc was greater in MK2^{-/-} MEFs than in wild-type MEFs (Fig. 6B); however, reconstitution of these cells with MK2 or p68E, but not p68A, reduced the abundance of c-Myc in MK2^{-/-} MEFs to that observed in wild-type MEFs (Fig. 6B). These results suggest a functional connection between MK2-mediated phosphorylation of Ser¹⁹⁷ of p68 and c-Myc abundance.

We next examined the effect of SB203580 on c-Myc abundance and proliferation in wild-type MEFs and MCF7 cells. Western blotting analysis showed that SB203580 enhanced the amount of c-Myc in both cell lines (Fig. 6C), whereas MTT assays showed that SB203580 enhanced their proliferation (Fig. 6D). However, the increased c-Myc abundance and cell proliferation were abolished in cells expressing p68E, but not p68A (Fig. 6, C and D). To link SB203580-enhanced cell proliferation to decreased miR-145 abundance and increased c-Myc abundance, we introduced an miR-145 mimic into SB203580-treated wild-type MEFs and MCF7 cells. Western blotting analysis showed that the miR-145 mimic abolished the SB203580-induced increase in c-Myc abundance in both cell types without influencing the phosphorylation of Ser¹⁹⁷ of p68 (Fig. 6E). MTT assays further showed that the miR-145 mimic and a c-Myc-specific small interfering RNA (siRNA) abolished the ability of SB203580 to increase cellular proliferation (Fig. 6, F and G). These results suggest that inhibiting p38 MAPK stimulates the proliferation of wild-type MEFs and MCF7 cells by blocking the biogenesis of miR-145, leading to an increase in the abundance of c-Myc.

DISCUSSION

As the importance of miRNAs to physiological and pathological events continues to be realized, it is becoming critical to understand how miRNA amounts are determined. One way to achieve this is to identify the molecules and signaling pathways that are involved in miRNA biogenesis. Here, we demonstrated that the functional integrity of the p38 MAPK–MK2 signaling pathway is essential for the efficient processing of pri-miRNAs (Figs. 1 and 2). Our study further revealed that p38 MAPK–MK2 signaling participates in pri-miRNA processing by facilitating the nuclear localization of p68, a key component of the Drosha complex (Fig. 5). Thus, our study establishes a direct link between the p38 MAPK–MK2 signaling pathway and miRNA biogenesis.

Our knowledge about how miRNA amounts are regulated is rapidly expanding. Although transcription plays an important role in regulating miRNA abundance, the observation that the amounts of pri- or pre-miRNAs do not often correlate with the amounts of their respective mature miRNAs in cancer tissues suggests that miRNA biogenesis is a critical aspect that regulates miRNA abundance. Cellular control of miRNA biogenesis is regulated by the extent of expression and the functional integrity of genes that are essential for miRNA processing. For example, reduced Dicer1 abundance is found in ovarian, lung, and gastric cancers (10, 39, 40), and it is associated with the inhibition of total miRNA production (7, 8). Function-impairing mutations were identified in the genes encoding TARBP2 and Dicer1 in colon and nonepithelial ovarian cancers, respectively (14–16). Additionally, particular RBPs (ribose-binding proteins) can regulate the biogenesis of specific miRNAs. For example, LIN28 suppresses let-7 processing by blocking its access to Drosha and Dicer (18, 41). Our study showed that blocking the activities of p38 MAPK and MK2 led to reduced amounts of miRNAs by suppressing the processing of pri-miRNAs (Figs. 1 and 2), thus providing evidence that the p38 MAPK–MK2 signaling pathway is functionally essential for efficient miRNA biogenesis. This finding adds another layer of regulation to the process of miRNA biogenesis.

A study showed that the ERK signaling pathway facilitates pre-miRNA processing by directly phosphorylating TARBP2, a key component of the Dicer complex (21). Here, we showed that MK2, an effector of p38 MAPK, physically interacted with p68 and directly phosphorylated it at Ser¹⁹⁷ (Fig. 3). p68 is a key component of the Drosha complex, and its presence is essential for the processing of a subset of pri-miRNAs (25, 26). It is thought that p68 regulates miRNA biogenesis through the loading of pri-miRNAs into the Drosha complex (27) or by acting as a bridge to bring Drosha and other regulators together (28, 29). Regardless of how p68 is involved in pri-miRNA processing, the interaction between p68 and the Drosha complex appears to be essential for the pri-miRNA–processing capability of the Drosha complex. Our study showed that the p68–Drosha interaction was absent in MK2^{−/−} MEFs (Fig. 4) and that the phosphomimetic mutant form of p68 fully restored pri-miRNA processing in MK2-null cells and in MEFs in which p38 MAPK was inhibited (Fig. 4), demonstrating that the interaction between p68 and Drosha depended on MK2-mediated phosphorylation of Ser¹⁹⁷ in p68. Together with data from a study of the ERK signaling pathway (21), we conclude that two major MAPK signaling pathways act at two distinct steps of miRNA biogenesis: the p38 MAPK pathway at the stage of pri-miRNA processing and the ERK pathway at the stage of pre-miRNA processing.

Signaling by p38 MAPK inhibits proliferation in both normal and breast cancer cells. For example, p38^{−/−} MEFs show increased proliferation compared to that of wild-type MEFs, and mice with a liver-specific deletion of p38 display enhanced hepatocyte proliferation and tumor development in a chemically induced liver cancer model (42). The increased proliferation of p38^{−/−} null cells was attributed to activation of the JNK–AP1 (activating protein 1) pathway (42). Here, we revealed a distinct mechanism by which the p38 MAPK signaling pathway suppresses cell proliferation through the regulation of miRNA biogenesis. We showed that the inhibition of p38 MAPK activity decreased the amount of miR-145, a growth-inhibitory miRNA, in MEFs and MCF7 cells (Fig. 6). c-Myc mRNA was previously identified as a target of miR-145 (37, 38). The growth-stimulatory effect of inhibition of p38 MAPK was apparently achieved through an increase in the abundance of c-Myc because both the miR-145 mimic and the siRNA specific for c-Myc abolished growth stimulation induced by a p38 MAPK inhibitor (Fig. 6). miR-145 inhibits the growth of only noninvasive cancer cell lines (37, 38). This finding may explain why a p38 MAPK inhibitor accelerates the growth of only noninvasive breast cancer cell lines (43). We previously showed that the p38 MAPK signaling pathway is involved in the miRNA-dependent regulation of mRNA decay (44). Together with the present study, these data suggest that small molecules that

interfere with the p38 MAPK signaling pathway might function through miRNA-dependent pathways. Therapies based on miRNAs have shown promise in preclinical studies. For example, silencing miR-10b or forcing expression of miR-26a leads to suppression of tumorigenicity in experimental mouse models (45, 46). Our studies indicate that miRNA-based therapeutic interventions may also be accomplished by small molecules that produce targeted and coordinated reprogramming of miRNA abundance. Defining the signaling pathways and molecules involved in miRNA biogenesis is essential for an understanding of the regulation of the miRNA pathway. Similar to gene transcription, splicing, and pre-miRNA processing, the processing of pri-miRNAs can also be modulated through signaling-induced modifications.

MATERIALS AND METHODS

Cells, lentiviral expression plasmids, and reagents

Wild-type and MK2^{-/-} MEFs were provided by J. Han at The Scripps Research Institute and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MCF7 cells were obtained from the American Type Culture Collection and were cultured in DMEM supplemented with 10% FBS and insulin (10 µg/ml). Mutants of p68 were generated with the QuikChange Site-Directed Mutagenesis Kit (Agilent), and complementary DNAs (cDNAs) encoding these constructs were subcloned into either pcDNA3.1 (Invitrogen) or pCDH-CMV-MCS-EF1-Puro (System Biosciences). Sequences of the oligonucleotides used to generate these mutants can be found in the Supplementary Materials. MK2- and p68-specific shRNAs were designed with the Web-based Block-IT program and were subcloned into the pLV-mU6-[shRNA]-EF1 -GFP vector (Biosettia). The sequences of these shRNAs are included in the Supplementary Materials. Information on inhibitors, antibodies, and other reagents are also described in the Supplementary Materials.

Analysis of in vitro pri-miRNA processing

In vitro pri-miRNA processing was performed as described previously (47). Briefly, ³²P-labeled pri-miRNA transcripts were synthesized in vitro from pcDNA3.1 containing pri-miR-145, pri-miR-181a1, or pri-miR-199a and [³²P]uridine 5'-triphosphate. Labeled transcripts (~10⁵ cpm) were incubated with 100 µg of whole-cell extracts of MEFs, 1 U of RNasin, and 6.4 mM MgCl₂ in a total volume of 30 µl of reaction buffer [20 mM Hepes-KOH (pH 8.0), 100 mM KCl, 0.2 mM EDTA, 5% glycerol] at 37°C for 90 min. Reaction mixtures were extracted with a phenol-chloroform mixture and then precipitated with 3 M sodium acetate, glycogen, and 100% ethanol. The precipitated RNA was resolved by electrophoresis in a 15% denaturing polyacrylamide gel, and the gel was then dried and exposed to x-ray film. To determine the role of p38 MAPK activity in pri-miRNA processing, we treated wild-type MEFs with 10 µM SB203580 for 1 day before preparing whole-cell extracts. The sequences of the primers used for plasmid construction are provided in the Supplementary Materials.

qRT-PCR analysis

Total RNA was extracted from cells with TRIzol (Life Technologies), then treated with DNase I (Fermentas), and subsequently used for the quantification of the primary, precursor, and mature forms of miR-145, miR-181a1, and miR-199a. The amounts of mature miR-145, miR-181a1, and miR-199a were measured with the respective TaqMan MicroRNA Assay Kits (Life Technologies). The amounts of the precursor forms of these miRNAs were determined with the respective miScript pre-miRNA assay kits (Qiagen). To determine the amounts of the primary forms of miR-145, miR-181a1, and miR-199a, we reverse-transcribed total RNA with the TaqMan MicroRNA Reverse Transcription Kit (Life

Technologies) to generate cDNA that was then subjected to qRT-PCR analysis with primer sets specific for each miRNA. The amounts of the miRNAs were normalized to the amount of β -actin mRNA. To determine the effect of blocking p38 MAPK activity on the amount of miRNAs, we treated wild-type MEFs or MCF7 cells with 10 μ M SB203580 for 1 day before isolating total RNA. The sequences of the primers can be found in the Supplementary Materials.

Yeast two-hybrid assays

To identify MK2-interacting proteins, we subcloned cDNA encoding murine MK2 with that encoding the C terminus of the Gal4-binding domain into the pGBKT7 vector (Clontech) and used this to screen a Mouse 7-day Embryo Matchmaker cDNA Library as described by the manufacturer (Clontech). We screened a total of 10^7 transformants, and those clones that were identified were verified with the X-Gal (X-galactosidase) colony filter assay (Clontech) according to the manufacturer's instructions.

Immunofluorescence staining

Cells were cultured on coverslips overnight, fixed with 3% paraformaldehyde, and permeabilized with 1% Triton X-100. To visualize the subcellular localization of p68, we incubated the coverslips with p68-specific monoclonal antibody for 1 hour before incubating them with rhodamine-conjugated secondary antibody and visualized the cells under a fluorescence microscope (Axiovert 200M, Zeiss). We used DAPI to stain the nuclei in MEFs.

Cell fractionation

Cells were washed in ice-cold phosphate-buffered saline (pH 7.4) and lysed in buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT)] supplemented with protease inhibitors. Cell fractionation was performed as previously described (48). Briefly, cell lysates were passed through 26G needles 20 times with 1-ml syringes, after which they were centrifuged at 2000g for 10 min to obtain a crude nuclear pellet and a cytoplasmic supernatant (cytoplasmic fraction). The crude nuclear pellets were purified further by resuspension in buffer B [20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 0.45 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT] supplemented with protease inhibitors. The lysates were subjected to high-speed centrifugation at 18,000g for 60 min at 4°C, and supernatants were collected in a new tube to obtain nuclear fractions.

Cellular proliferation assays

Cellular proliferation was analyzed with the MTT assay as previously described (49). To determine the effect of p38 MAPK inhibition on the proliferation of MCF7 cells, we cultured cells in 24-well plates overnight and then treated them with 10 μ M SB203580 in the presence of 1% FBS for various times (1 to 3 days) followed by incubation with MTT solution for 2 hours at 37°C. After removing the media, we dissolved crystals of MTT formazan in DMSO and measured the samples with a Bio-Rad plate reader at a wavelength of 560 nm.

Statistical analysis

Statistical analyses were performed on data collected from at least three independent experiments. Intraindividual variation between control and treatment (Figs. 1, A to C, 2C, 4A, and 6, F and G) was analyzed by Student's *t* test (two-sided). Interindividual variation (Figs. 2, A and D, 4, B and C, and 6, A and D) was analyzed by two-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The activity of p38 MAPK is essential for pri-miRNA processing. (A to C) qRT-PCR analysis of the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in wild-type (WT) MEFs treated with (A to C) dimethyl sulfoxide (DMSO; vehicle) or (A) 10 μ M U0126, (B) 10 μ M SP600125, or (C) 10 μ M SB203580 for 1 day. Data are means $\pm$ SE from three experiments. * $P < 0.005$ versus vehicle-treated cells. (D) In vitro pri-miRNA processing in whole-cell extracts of WT MEFs treated with vehicle or 10 μ M SB203580. Data are representative of two independent experiments.

Fig. 2. MK2 is required for pri-miRNA processing. (A) qRT-PCR analysis of the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in WT MEFs, MK2^{-/-} MEFs, MK2^{-/-} MEFs transfected with empty vector (con), MK2^{-/-} MEFs transfected with plasmid encoding MK2, WT MEFs transfected with luciferase-specific shRNA (sh Luc), and WT MEFs in which MK2 was knocked down. Data are means ± SE from three experiments. **P* < 0.005 versus WT MEFs. (B) In vitro pri-miRNA processing with whole-cell extracts of WT and MK2^{-/-} MEFs. Data are representative of two independent experiments. (C) qRT-PCR analysis of the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in WT MEFs transfected with empty vector (control) or with plasmid encoding MK2-DN. Data are means ± SE from three experiments. **P* < 0.005 versus control. (D) WT MEFs were transfected with plasmid encoding MK2-CA and then

were treated for 1 day with solvent (vehicle) or 10 μ M SB203580. Total RNA was isolated and subjected to qRT-PCR to analyze the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a. Data are means $\pm$ SE from three experiments. * P < 0.005 versus vehicle alone.

Fig. 3.

MK2 physically interacts with p68 and phosphorylates it at Ser¹⁹⁷. (A) WT MEFs were subjected to immunoprecipitation with mouse immunoglobulin G (IgG) or monoclonal antibody against p68, and immunoprecipitates were subjected to Western blotting analysis to detect MK2. Data are representative of two independent experiments. (B) WT MEFs were subjected to immunoprecipitation with rabbit IgG or polyclonal antibody against MK2, and immunoprecipitates were subjected to Western blotting analysis to detect p68. Data are representative of two independent experiments. (C) WT MEFs were transfected with plasmid encoding Myc-tagged MK2, MK2-CA, or MK2-DN and then subjected to coimmunoprecipitations to detect their interaction with p68. Data are representative of three

independent experiments. **(D)** WT MEFs were transfected with empty plasmid (vector) or with plasmids encoding HA-tagged full-length p68 (FL) or the N-terminal (NT), core, or C-terminal (CT) regions of p68, and cell lysates were subjected to coimmunoprecipitations to detect interactions between the p68 proteins and MK2. Data are representative of two independent experiments. **(E)** WT MEFs were transfected with plasmids encoding HA-tagged WT p68 or p68 mutants, and then cell lysates were subjected to coimmunoprecipitation experiments to detect interactions between the p68 proteins and MK2. Data are representative of two independent experiments. aa, amino acid. **(F)** Alignment of p68 protein sequences from different species. The conserved MK2 phosphorylation target motif is underlined. Amino acid residue numbers are to the left and right of the sequences. **(G)** Left panel: Coomassie blue staining of recombinant p68 and p68A. Right panel: In vitro kinase assay with either recombinant active MK2 or MK2 immunoprecipitates (MK2-IP) to determine the ability of MK2 to phosphorylate p68. Data are representative of three independent experiments. **(H)** Western blotting analysis to detect Ser¹⁹⁷-phosphorylated p68, total p68, MK2, and β -actin in WT MEFs treated with solvent (vehicle) or 10 μ M SB203580 for 1 day; MK2^{-/-} MEFs; and MK2^{-/-} MEFs transduced to express either WT MK2 or MK2-DN. Data are representative of two independent experiments.

Fig. 4. Phosphorylation of Ser¹⁹⁷ of p68 is essential for pri-miRNA processing. **(A)** qRT-PCR analysis of the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in WT MEFs that were transduced with lentiviruses to express luciferase-specific shRNA (control) or p68-specific shRNA. Data are means $\pm$ SE from three experiments. $*P < 0.005$ versus control. **(B)** WT MEFs were transfected with plasmids encoding WT p68 or the p68E or p68A mutants and then were treated for 1 day with solvent (vehicle) or 10 μ M SB203580. Total RNA was isolated and subjected to qRT-PCR analysis to determine the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a1. Data are means $\pm$ SE from three experiments. $*P < 0.005$ versus vehicle. **(C)** qRT-PCR analysis of the amounts of the pri-, pre-, and mature forms of miR-145, miR-181a1, and miR-199a in WT MEFs, MK2^{-/-} MEFs, and MK2^{-/-} MEFs transfected with plasmids encoding either p68E or p68A. Data are means $\pm$ SE from three experiments. $*P < 0.005$ versus WT MEFs. **(D)** WT MEFs, MK2^{-/-} MEFs, and WT MEFs treated with 10 μ M SB203580 for 1 day were subjected to immunoprecipitation with polyclonal antibody against Drosha, and immunoprecipitates were subjected to Western blotting analysis with antibodies specific for total p68 and p68 phosphorylated at Ser¹⁹⁷ (pp68). Data are representative of three independent experiments. **(E)** Lysates of WT MEFs were precleared with or without polyclonal antibody against phosphorylated p68 followed by immunoprecipitation with polyclonal antibody against Drosha. Immunoprecipitates were subjected to Western blotting analysis with an antibody against p68. As a control, MEF lysates were subjected to immunoprecipitation with rabbit IgG. Data are representative of two independent experiments. **(F)** WT MEFs were transfected with plasmids encoding HA-tagged WT p68 or the p68E or p68A mutants, and then cell lysates were subjected to immunoprecipitation with polyclonal antibody against Drosha. Immunoprecipitates were analyzed by Western blotting with antibody against HA-tagged p68. As a control, WT MEFs were transfected with empty vector. Data are representative of two independent experiments. **(G)** WT MEFs were

transfected with plasmids encoding HA-tagged WT p68 or the p68E or p68A mutants, and cell lysates were subjected to immunoprecipitation with monoclonal antibody against the HA tag. Immunoprecipitates were analyzed by Western blotting with an antibody against Drosha. As a control, WT MEFs were transfected with empty vector. Data are representative of two independent experiments.

Fig. 5. Phosphorylation of Ser¹⁹⁷ of p68 is essential for its nuclear localization. **(A)** WT MEFs, MK2^{-/-} MEFs, and MK2^{-/-} MEFs transfected to express MK2 were subjected to immunofluorescence staining to detect the subcellular localization of p68. Cell nuclei were visualized with DAPI (4',6-diamidino-2-phenylindole). Data are representative of four independent experiments. **(B)** Nuclear and cytoplasmic fractions of WT MEFs, MK2^{-/-} MEFs, and MK2^{-/-} MEFs transfected to express MK2 were analyzed by Western blotting to detect p68. The cytoplasmic (Cy) and nuclear fractions (Nu) were verified with antibodies specific for paxillin and lamin A/C, respectively. Data are representative of two independent experiments. **(C)** The nuclear and cytoplasmic fractions of WT and MK2^{-/-} MEFs were analyzed by Western blotting with antibodies specific for total p68 and for p68 phosphorylated at Ser¹⁹⁷ (pp68). Data are representative of two independent experiments. **(D)** WT or MK2^{-/-} MEFs were transfected with plasmids encoding GFP-fused p68E or p68A mutants. One day later, live cells were monitored with a fluorescence microscope. Data are representative of three independent experiments. **(E)** WT and MK2^{-/-} MEFs were transfected with plasmids encoding either HA-tagged p68E or HA-tagged p68A. One day later, nuclear and cytoplasmic fractions were prepared and subjected to Western blotting analysis to detect HA-tagged p68E or p68A. Data are representative of three independent experiments.

Fig. 6. SB203580 enhances cell growth by suppressing miR-145 production and increasing the abundance of c-Myc protein. (A) Untransfected MCF7 cells and MCF7 cells transfected with plasmids encoding the p68E or p68A mutants were treated with 10 μ M SB203580 for 1 day and then were analyzed by qRT-PCR to measure the amounts of the pri- and mature forms of miR-145. Data are means $\pm$ SE from three experiments. $*P < 0.005$ versus vehicle. (B) WT MEFs, MK2^{-/-} MEFs, and MK2^{-/-} MEFs transfected with plasmids encoding MK2, p68E, or p68A were subjected to Western blotting analysis with an antibody against c-Myc. We used β -actin as a loading control. Data are representative of two independent experiments. (C) WT MEFs and MCF7 cells were transfected with plasmids encoding p68E or p68A. Cells were treated with SB203580 for 1 day before being analyzed by Western blotting to detect c-Myc. We used β -actin as a loading control. Data are representative of three independent experiments. (D) WT MEFs and MCF7 cells were transfected with plasmids encoding p68E or p68A. Cells were then treated with SB203580 for the indicated times before being analyzed by the MTT assay to determine the extent of cell proliferation. Data are means $\pm$ SE from three experiments. $*P < 0.005$ versus vehicle. (E) WT MEFs and

MCF7 cells were transfected with the miR-145 mimic (33 nM). After 3 days, the cells were treated with SB203580 for 1 day. Cells were then analyzed by Western blotting to detect c-Myc, total p68, p68 phosphorylated at Ser¹⁹⁷ (pp68), and β -actin. Data are representative of three independent experiments. **(F)** WT MEFs and MCF7 cells were transfected with the miR-145 mimic (33 nM). Three days later, the cells were treated with 10 μ M SB203580 for 2 days. The cells were then analyzed with the MTT to determine cell proliferation. Data are means $\pm$ SE from three experiments. * P < 0.005 versus vehicle. **(G)** WT MEFs and MCF7 cells were transfected with a c-Myc-specific siRNA pool (33 nM). Three days later, the cells were treated with 10 μ M SB203580 for 2 days. The cells were then analyzed with the MTT assay to determine the extent of cell proliferation. Data are means $\pm$ SE from three experiments. * P < 0.005 versus vehicle.