Apoptosis-inducing Factor, Mitochondrion-associated 2, Regulates Klf1 in a Mouse Erythroleukemia Cell Line

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Abstract. *Background/Aim: Apoptosis-inducing factor, mitochondrion-associated 2 (Aifm2), is a DNA-binding oxoreductase protein that promotes apoptosis. To assess its potential role in erythropoiesis we analyzed the effects of Aifm2 loss-of-function in the murine erythroleukemia line (MEL). Materials and Methods: MEL cells were transfected with siRNA targeting Aifm2 for 24 h and evaluated by cell counting, flow cytometry with annexin V and PI staining and gene expression analysis. Results: Aifm2 knockdown did not affect the apoptotic status of MEL cells. However, Aifm2 knockdown significantly increased expression of the erythropoietic transcription factor Klf1 (2.9±0.2-fold, $p<0.05$) and decreased α- and β-globin expression (0.6±0.2-fold, $p<0.05$ and 0.5±0.2-fold, $p<0.01$). Conclusion: Aifm2 may function in differentiation of erythroid MEL cells in vitro.*

Erythropoiesis is a process in which hematopoietic stem cells give rise to functional and mature erythrocytes, which are produced daily and circulate throughout the body to deliver oxygen (1). Abnormalities in erythropoiesis result in anemia, which impairs quality of life due to inadequate oxygen-associated symptoms, such as tiredness, headaches, lack of concentration and dizziness (2-4). Erythropoietic homeostasis is tightly regulated by intrinsic factors, such as the transcription factors Gata-binding protein 1 (Gata1) and Krüppel-like factor 1 (Klf1, also known as Eklf or Nan). The erythropoietic system also requires proper control of apoptosis to maintain a balance between erythroid cell production and destruction (5). Murine erythroleukemia (MEL) cells, an acute leukemic blast line with the capacity to differentiate into erythroid cells, has been a useful model for the delineation of molecular mechanisms underlying erythroid differentiation (6-9). For instance, doking protein 2, which provides a platform for assembly of signaling molecules, is a novel intrinsic factor to regulate *Klf1* transcription through *Klf1* promoter binding in the MEL cells (9).

Apoptosis-inducing factors or mitochondria-associated proteins are a family of flavoproteins implicated in caspase-independent apoptosis (10). The absence of poly (ADP-ribose) glycohydrolase reportedly promotes increased cell death mediated by these factors (11), which translocate from mitochondria to the cytoplasm and then to the nucleus in order to promote cell death. Consequently, apoptosis-inducing factors induce chromatin condensation and DNA fragmentation (12). Apoptosis-inducing factor mitochondrion-associated 2 (Aifm2), a member of this family, is present in the mitochondrial inter-membrane space (13) and functions in p53/TP53-dependent apoptosis (14). Our preliminary data showed that MEL cells express *Aifm2* mRNA and Aifm2 protein but its function in those cells remains unknown. To assess the potential function of Aifm2 in regulating erythropoiesis, we knocked-down *Aifm2* mRNA in the MEL cell line and evaluated expression of erythropoiesis-related genes. Our findings suggest that Aifm2 regulates expression of the erythroid transcription factor encoded by *Klf1*, thus suggesting a potential role in mouse erythroid differentiation.

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Materials and Methods

Cell lines. MEL cells (kindly provided by Dr. Brand, Ottawa Health Research Institute, Ottawa, Canada) were cultured in RPMI-1640 with L-glutamine and phenol red (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS) and

10 units/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). Cells were passaged every 3-4 days.

siRNA transfection. One hundred thousand cells were cultured for 12 h in 24-well plates in RPMI-1640 containing 1% FBS without antibiotics before siRNA transfection. siRNA-Lipofectamine™ 2000 (Life Technologies, Palo Alto, CA, USA) complexes were prepared according to the manufacturer's protocol. For *Aifm2* knockdown, we used pre-designed *Aifm2* siRNA (Sigma-Aldrich). Silencer® Negative Control No.1 siRNA (Ambion, Austin, TX, USA) served as control siRNA. Sixty pmole *Aifm2* siRNA was diluted in 50 μl of Opti-MEM® I Reduced Serum Medium (Life Technologies). In a separate tube, 2 μl of Lipofectamine™ 2000 was diluted in 50 μl of Opti-MEM® I Reduced Serum Medium (Life Technologies) and incubated at room temperature for 5 min. Then, siRNA and Lipofectamine™ 2000 were mixed and incubated at room temperature for 20 min to allow formation of complexes, which were then added to cells and incubated at 37˚C with 5% of CO_2 for 24 h. To induce erythroid differentiation, dimethyl sulfoxide (DMSO) was added at 2% final concentration at 24 h post-siRNA transfection and cells were collected 24 h later for gene expression analysis.

May-Grünwald-Giemsa staining. Twnety-four hours after siRNA transfection, MEL cells were spread onto glass slides (Matsunami Glass ind., Ltd., Osaka, Japan) using a CytoSpin™ 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 450 rpm for 7 min, rapidly air dried and then fixed and stained using May-Grünwald reagent (Muto Pure Chemicals, Tokyo, Japan) at room temperature for 5 min. After a brief wash in tap water, cells were incubated with PBS (pH=6.4) for 2 min and then stained with diluted Giemsa solution (1:18; Muto Pure Chemicals, Tokyo, Japan) at room temperature for 30 min. After an additional tap water wash, slides were air-dried and covered with glass coverslips with a drop of MGK-S mounting solution (Matsunami glass). Cells were imaged using an Olympus CKX41 microscope (Olympus, Tokyo, Japan).

Immunocytochemistry. Harvested cells were centrifuged onto glass slides (Matsunami Glass) using a CytoSpin4 (Thermo Fisher Scientific) at 450 rpm for 7 min and air-dried overnight. Cells were then fixed in 1% paraformaldehyde at 4˚C for 30 min. After washing three times with PBS, cells were incubated with PBS containing 0.05% Triton X-100 at room temperature for 15 min, washed again three times with PBS and then blocked with PBS containing 1% bovine serum albumin (BSA) at room temperature for 30 min to prevent non-specific antibody binding. Cells were incubated with rabbit anti-mouse Aifm2 (1:200; Thermo Fisher Scientific, Waltham, MA, USA) primary antibody at 4˚C overnight in the dark. After three PBS rinses, cells were covered with diluted goat anti-rabbit IgG antibody conjugated with AlexaFluor488 (1:400; Invitrogen, Carlsbad, CA, USA) and TOTO-3 iodide (1:1,500; Invitrogen) at room temperature for 30 min in the dark. After three PBS washes, cells were coverslipped with one drop of anti-fade mounting medium (Dako Corporation, Glostrup, Denmark). Images were acquired using a FluoView 1000 confocal microscope (Olympus).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from harvested cells using an RNAqueous-4PCR™ Kit (Life Technologies) and mRNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA™ Kit (Life Technologies). *Aifm2, Gata1, Klf1*, α-Globin (*Hba1*), β-Globin (*Hbb*) and β-actin (*Actb*) expression was evaluated using StepOnePlus™ real-time PCR (Life Technologies) reagent with TaqMan® Gene Expression Assays (Life Technologies). *Actb* served as an internal control. Relative expression of each gene was calculated using a standard curve method. Relative expression of *Bax, Bcl2, Mcl1, Myc* and *Ccnd1* was assessed using Fast SYBR® Green Master Mix (Life Technologies) with the following primers: *Bax*, forward: 5'- AGTGTCTCCGGCGAATTGG-3', reverse: 5'-AGCTGCCACC CGGAAGA-3'; *Bcl2*, forward: 5'-GAGGCTGGGATGCCTTTGT-3', reverse: 5'-CCAGGTATGCACCCAGAGTGA-3'; *Mcl1*, forward: 5'-GGGCTGGTCTGGCATATCTA-3', reverse: 5'-GCAGCTTCAA GTCCACCTTC-3'; *Myc*, forward: 5'-CCTAGTGCTGCATGAGG AGA-3', reverse: 5'-TCTTCCTCATCTTTTGCTCTTC-3'; *Ccnd1*, forward: 5'-CGCCCTCCGTATCTTACTTCAA-3', reverse: 5'-CTCA CAGACCTCCAGCATCCA-3'; and *Actb*, forward: 5'-GCTCTGGCT CCTAGCACCAT-3', reverse: 5'-GCC ACCGATCCACACAGAGT-3'.

Flow cytometry and apoptosis analysis. For apoptosis analysis, cells were resuspended after two brief PBS washes in annexin V binding buffer (BioLegend, San Diego, CA, USA) at 1.0×10^6 cells/ml. After transferring 100 μl of the cell suspension to a test tube, cells were stained with allophycocyanin (APC)-conjugated annexin V and propidium iodide (PI) (Invitrogen) and then incubated at room temperature for 15 min in the dark. After addition of 400 μl annexin V binding buffer, stained cells were analyzing by BD FACS Area (BD Bioscience, San Jose, CA, USA). Cells were fractionated based on the following criteria: annexin V−/PI− were defined as non-apoptotic (living cells), annexin V+/PI− as early apoptotic and annexin V+/PI+ as late apoptotic cells.

Statistical analysis. Results are presented as means±standard deviation (SD). For statistical tests, we used Student's *t*-test to compare two samples: *Aifm2* siRNA-transfected and control siRNA-transfected cells. All *p*-values less than 0.05 were considered statistically significant.

Results

Aifm2 protein and mRNA expression in MEL cells. MEL cell did express *Aifm2* mRNA. When we compared the level of *Aifm2* mRNA with *Dok2* mRNA (9) by qRT-PCR, relative expression of *Aifm2* mRNA was 3.2±0.3-fold higher than that of *Dok2* mRNA in MEL cells ($p<0.05$; Figure 1A). These data show the possible role of Aifm2 in MEL cells. To investigate Aifm2 protein expression and localization, we performed immunocytochemical analysis of cultured MEL cells with an Aifm2 antibody. Confocal microscopy images revealed that Aifm2 protein was present in the cytoplasm and nucleus of MEL cells (Figure 1B).

Aifm2 loss-of-function in MEL cells. To assess *Aifm2* function, we knocked-down *Aifm2* mRNA in MEL cells using siRNA. Twenty-four hours after siRNA transfection, we assayed knockdown efficiency by qRT-PCR and found that *Aifm2* transcripts had decreased by 76.3±0.0% relative to control siRNA-transfected cells (Figure 2A). When we assessed cell morphology at 24 h post-transfection by light microscopy, we

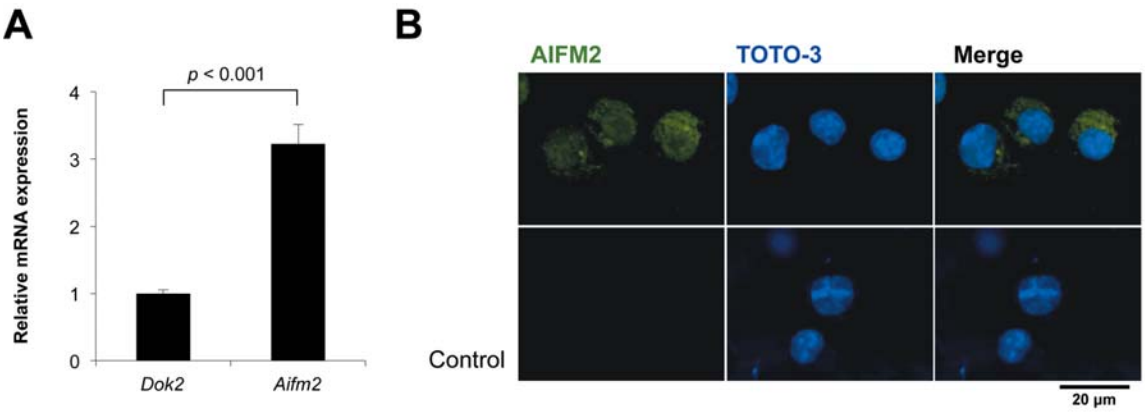

Figure 1. *Expression of Aifm2 in MEL cells. (A) Relative expression of Aifm2 and Dok2 mRNAs in MEL cells, as measured by quantitative real-time polymerase chain reaction (qRT-PCR). Aifm2 transcripts are expressed at levels 3.2±0.3-fold (p<0.05) higher than those of Dok2. (B) Immunocytochemical images of Aifm2 protein (green) in MEL cells. Scale bar=20 μm. Nuclear DNA is stained with TOTO-3 iodide (blue). Aifm2 protein is localized to both the cytoplasm and nucleus.*

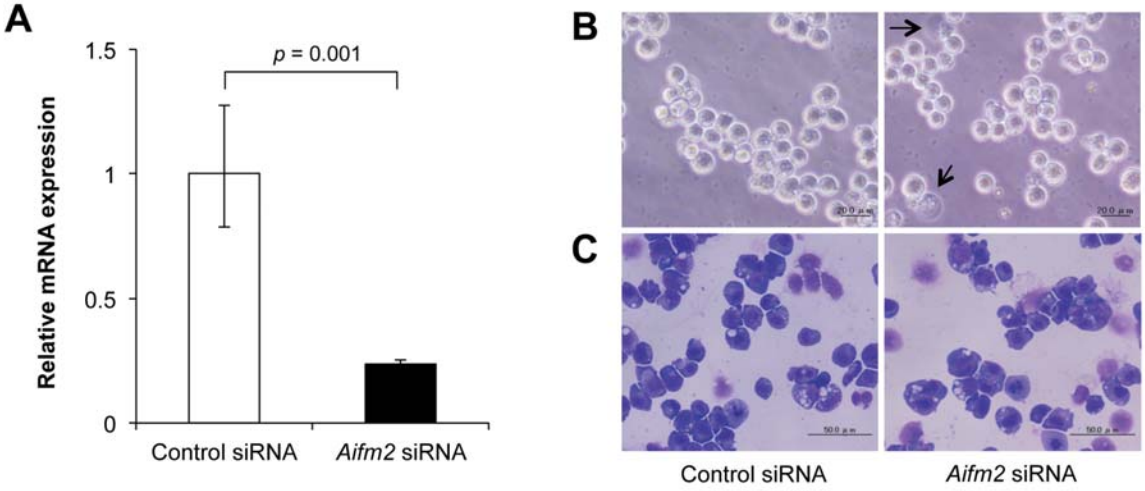

Figure 2. *Morphological analysis of MEL cells after Aifm2 knockdown. (A) Relative expression of Aifm2 mRNA 24 h after siRNA transfection. Aifm2 mRNA levels were decreased 76% relative to MEL cells transfected with control siRNA (p<0.05). (B) Microscopic images of MEL cells 24 h after transfection with control (left) or Aifm2 (right) siRNA. Arrows indicate swollen cells. Scale bar=20 μm. (C) May-Grünwald-Giemsa staining of MEL cells 24 h after transfection with control (left) or Aifm2 (right) siRNA. Scale bar=50 μm.*

found that most cells exhibited a round shape, same as before siRNA transfection. Relative to controls, some *Aifm2* siRNA-transfected cells appeared swollen (Figure 2B, arrows). May-Grünwald-Giemsa staining revealed that there was no obvious morphological differences between *Aifm2* siRNA-transfected and control samples after 24 h of siRNA transfection. Blue-colored cytoplasm, vacuoles and multi-nuclear cells were observed in both samples (Figure 2C).

Analysis of apoptotic status of Aifm2 siRNA-transfected MEL cells. After 24 h of siRNA transfection, we counted viable and dead cells in both control and *Aifm2* siRNA-transfected samples. While we observed no differences in the number of viable cells in control and *Aifm2* siRNA-transfected cells (199,200±31,146.8 cells and 205,166±11,918.2 cells, respectively; Figure 3A, upper pannel), we observed a significant difference in the number of dead cells in control and *Aifm2* siRNA-transfected cells (30,566±2,974.0 and 17,300±2,605.0, respectively, $p<0.05$; Figure 3A, lower pannel). To further assess the apoptotic status of the cells, we performed annexin V and PI staining (Figure 3B). Living cells (annexin V−/PI−), early apoptotic (annexin V+/PI−) and late apoptotic (annexin V+/PI+) cells were evaluated by flow cytometry. As shown in Figure 2C, most MEL cells were early apoptotic (annexin V+/PI−) cells, namely, 78.1±3.7% in *Aifm2* siRNA-transfected and 81.4±0.0% in control cells. Among the

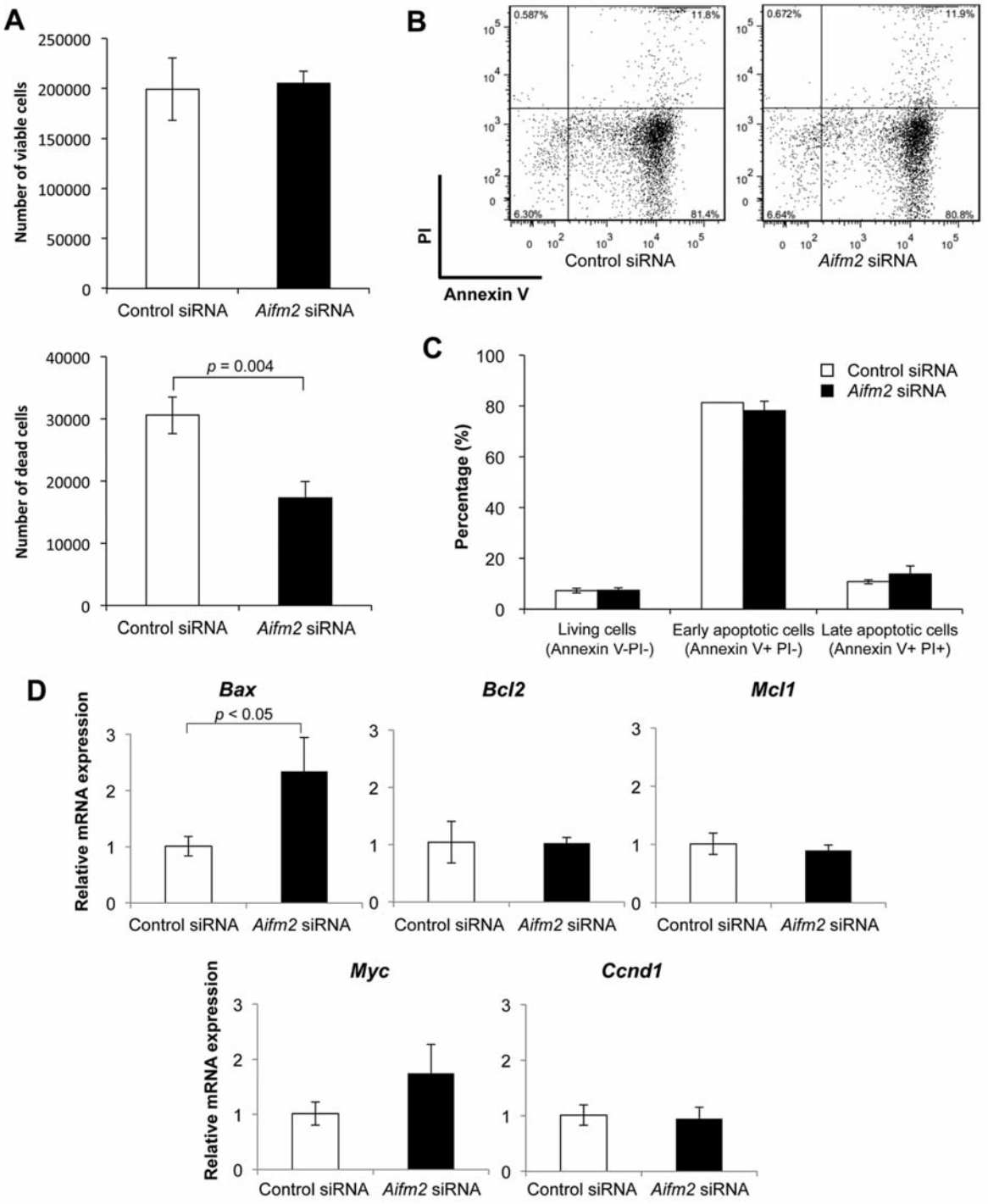

Figure 3. *Analysis of apoptosis in MEL cells after Aifm2 knockdown. (A) The number of viable and dead MEL cells 24 h after siRNA transfection. No significant difference was observed in the number of viable cells between groups (upper panel), while we observed a reduced number of dead cells (lower panel, p<0.05) in Aifm2 siRNA-transfected versus control siRNA-transfected cells. (B) Flow cytometry analysis of apoptosis using annexin V and propidium iodide (PI). Shown are representative flow cytometry plot and gate settings. Percentages were calculated with gates for the following cell populations: annexin V−/PI−, living cells; annexin V+/PI−, early apoptotic cells; and annexin V+/PI+, late apoptotic cells. (C) Percentage of cells shown in B. Most cells were early apoptotic cells in both Aifm2 siRNA-transfected (78.1±3.7%) and control siRNA-transfected (81.4±0.0%) cells. No significant difference was observed in any population between Aifm2 siRNA-transfected and control cells. (D) Relative expression of the pro-apoptotic genes Bax and Bcl2, the cell survival-related gene Mcl1 and the cell proliferation-related genes Myc and Ccnd1. By 24 h post siRNA transfection, Bax expression increased 2.3±0.6-fold (p<0.05) in Aifm2 siRNA-transfected versus control cells.*

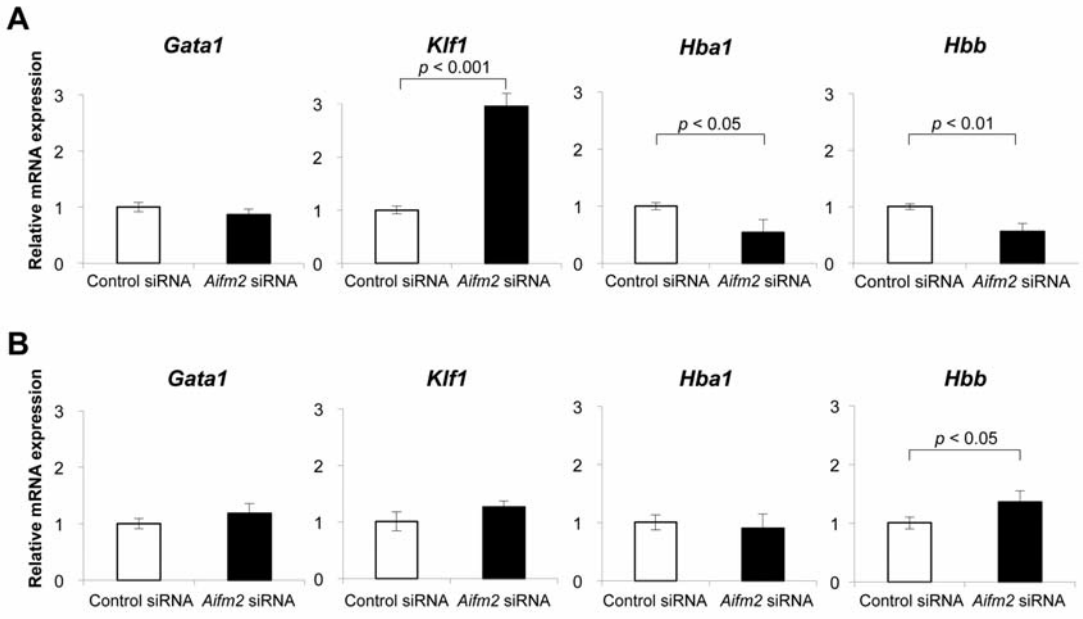

Figure 4. *Expression of erythroid differentiation-related genes following Aifm2 knockdown. (A) Relative mRNA expression of the erythroid differentiation-related genes Gata1, Klf1, α-Globin and β-Globin 24 h after siRNA transfection. Relative Klf1 expression increased 2.9±0.2-fold (p<0.05) and that of α- and β-Globin mRNA decreased 0.6±0.1-fold (p<0.05) and 0.5±0.2-fold (p<0.05), respectively, in Aifm2 siRNA-transfected cells compared to controls. (B) Relative expression of differentiation-related genes after dimethyl sulfoxide (DMSO) treatment of Aifm2 siRNA-transfected cells. Twenty-four hours post siRNA transfection, DMSO was added to cultures and gene expression was analyzed 24 h later. β-Globin mRNA expression significantly increased by 1.4±0.2-fold in Aifm2 siRNA-transfected versus control cells. (p<0.05).*

three populations evaluated, the percentage of living (annexin V−/ PI−) cells was the lowest: 7.6±0.8% in *Aifm2* siRNA-transfected cells and 7.3±0.9% in controls. We observed no significant change in percentages of early apoptotic cells in the *Aifm2* siRNA-transfected and control cells 24 h post-transfection (p=0.20; Figure 3C). Finally, we did not observe any significant difference in the percentage of late apoptotic cells between *Aifm2* siRNA-transfected and control cells (13.8±3.3% and 10.8±0.8%, respectively, p=0.20; Figure 3C).

To further investigate Aifm2 function in apoptosis, cell survival and cell proliferation, we examined expression of the apoptosis-related genes *Bax* and *Bcl2*, (15-18), the cell survival-related gene *Mcl1* (19, 20) and the proliferation-related genes *Myc* and *Ccnd1* (21-24) by qRT-PCR (Figure 3D). *Bax* mRNA increased by 2.3±0.6-fold in *Aifm2* siRNA-transfected cells (p<0.05) compared to controls. However, there was no significant difference in expression of Bcl2 (p=0.95) and *Mcl1* transcripts (p=0.41) nor was there a significant difference in *Myc* and *Cdnd1* transcript levels 24 h post-siRNA transfection (p=0.09 and p=0.71, respectively).

Aifm2 knockdown increases Klf1 expression. To further investigate Aifm2 function in erythropoiesis, we assessed expression of the erythroid differentiation-related genes *Gata1, Klf1,* as well as α- and *β-Globin* by qRT-PCR 24 h after siRNA transfection (Figure 4A). Among these genes, *Klf1* showed a significant increase (2.9±0.2-fold relative to controls) (p<0.001) in *Aifm2* siRNA-transfected cells. Relative α-Globin (*Hba1*) and β-Globin (*Hbb*) expression significantly decreased by 0.6±0.2-fold (p<0.05) and 0.5±0.2-fold (p<0.01), respectively.

We next examined expression of these genes after harvesting MEL cells that had been treated with DMSO to induce erythroid differentiation (Figure 4B). After 24 h of DMSO treatment, *Hbb* mRNA expression significantly increased by 1.6±0.2-fold (p<0.05) in *Aifm2* siRNA-transfected cells relative to controls, although we observed no significant differences in *Gata1, Klf1* and *Hba1* mRNA levels.

Discussion

Herein we report that *Aifm2* loss-of-function alters the expression of erythroid differentiation-related genes in MEL cells *in vitro*. Aifm2 is well-known as an apoptosis-inducing protein localized primarily to the mitochondrial outer membrane (25). Aifm2 protein contains oxidoreductase domain, which functions in induction of large-scale DNA breaks in apoptosis and Aifm2 protein levels increase with apoptotic activity (26). In our study, we observed high *Aifm2* mRNA expression in MEL cells (Figure 1A) and that Aifm2 protein resides in both the

cytoplasm and nucleus (Figure 1B). Mouse Aifm2 reportedly translocates from mitochondria to the nucleus to initiate apoptosis (12) suggesting a similar mechanism in MEL cells. To further a potential Aifm2 function in MEL cell apoptosis, we performed loss-of-function analysis by transfecting MEL cells with siRNA targeting *Aifm2*. Despite its characterization as an apoptosis-inducing protein (25), we did not observe significant changes in cell morphology, in the number of viable cells, or in apoptotic status, as determined by annexin V and PI staining, between *Aifm2* siRNA-transfected and control siRNA cells (Figures 2 and 3). We also observed no change in expression of genes that antagonize apoptosis (*Bcl2*) or enhance cell survival (*Mcl1*) or proliferation (*Myc* and *Ccnd1*). By contrast, we observed an increased expression of pro-apoptotic *Bax* mRNA. It is reported that Bax induces apoptosis of human K562 erythroleukemia cells in a caspase-dependent manner (27). Relevant to apoptosis, the discrepancy between ours and previous reports may be explained by compensatory activity of other members of the apoptosis-inducing factor family, such as Aifm1 or Aifm3. Although, apoptosis is a fundamental process in all metazoans (28), it requires diverse signaling pathways (29). Therefore, additional approaches will be required to assess potential roles played by other apoptosis-inducing factors at additional time points in MEL cells.

Given that we observed no apparent difference in apoptosis in *Aifm2* knockdown *versus* control MEL cells, we focused on Aifm2's effect on erythroid differentiation since MEL cells constitute a suitable model to study that process (9, 30). Intrinsic factors, such as the transcription factors Gata1 and Klf1 (7), regulate erythropoiesis to maintain cellular homeostasis. Gata1 reportedly regulates *Klf1* transcription, which is further linked to the activation of Globin transcription (7). In our study, *Aifm2* knockdown by 76.3% resulted in a 2.9±0.2-fold increase in *Klf1* mRNA expression compared to controls (Figure 4A) suggesting that Aifm2 antagonizes *Klf1* transcription. *Klf1* functions in regulation of erythroid commitment, globin switching and maturation of erythrocytes (31). Unlike *Klf1*, expression of *Globin* mRNAs decreased in *Aifm2* knockdown cells, although *Klf1* reportedly activates *Globin* transcription (32). To further evaluate the effect of Aifm2 on Globin expression, we induced erythroid differentiation of MEL cells by DMSO treatment (8) and observed a significant increase in *β-Globin* mRNA (1.4±0.2-fold, $p<0.05$) in *Aifm2* knockdown relative to control cells (Figure 4B).

In conclusion, we show that Aifm2 potentially regulates mouse erythroid differentiation in a MEL cell model. Further investigations are required to define mechanisms underlying Aifm2's activity in regulating erythropoiesis.

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