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SON Protein Regulates GATA-2 through Transcriptional Control of the MicroRNA 23a∼27a∼24-2 Cluster*

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Background: SON is a recently characterized splicing factor with potential functions in transcription.
Results: SON knockdown increases miR-27a through transcriptional activation, which in turn down-regulates GATA-2.
Conclusion: Transcriptional repression by SON on the miR-23a∼27a∼24-2 promoter controls the GATA-2 protein level.
Significance: Understanding SON regulation of microRNA transcription provides a mechanism of SON-mediated gene expression and its effect on hematopoiesis.

SON is a DNA- and RNA-binding protein localized in nuclear speckles. Although its function in RNA splicing for effective cell cycle progression and genome stability was recently unveiled, other mechanisms of SON functions remain unexplored. Here, we report that SON regulates GATA-2, a key transcription factor involved in hematopoietic stem cell maintenance and differentiation. SON is highly expressed in undifferentiated hematopoietic stem/progenitor cells and leukemic blasts. SON knockdown leads to significant depletion of GATA-2 protein with marginal down-regulation of GATA-2 mRNA. We show that miR-27a is up-regulated upon SON knockdown and targets the 3′-UTR of GATA-2 mRNA in hematopoietic cells. Up-regulation of miR-27a was due to activation of the promoter of the miR-23a∼27a∼24-2 cluster, suggesting that SON suppresses this promoter to lower the microRNAs from this cluster. Our data revealed a previously unidentified role of SON in microRNA production via regulating the transcription process, thereby modulating GATA-2 at the protein level during hematopoietic differentiation.

SON is a large, poorly characterized protein localized to nuclear speckles. Its multiple structural features include a large arginine/serine-rich (SR) domain, a glycine-rich motif (G patch), a double-stranded RNA binding motif, and long repeats of amino acids. The first SON cDNA fragment, named SON3, was cloned from a human embryonic cDNA library as a new member of the *mos*-related family of oncogenes (1). Later, several fragments of the SON gene were cloned and tested for functions *in vitro* and *in vivo*. Overexpression of a partial fragment of SON in a transformed cell line decreased the tumorigenic potential of the cell in nude mice (2) and protected yeast from apoptosis (3). However, partially due to its large size, the SON protein has not been well characterized for its functions. Recently, our group reported a function of SON in regulating a number of genes involved in cell cycle progression and DNA repair. Mechanistic studies revealed that SON facilitates splicing through the recruitment of SR proteins, such as SC35, to RNAP II complexes, and ensure efficient RNA processing of multiple genes that are necessary for mitotic progression and proper signaling pathways (4). Interestingly, SON was initially characterized as a DNA-binding protein. Through its region adjacent to the SR domain, SON interacts with the human hepatitis B virus genome and represses viral gene transcription; hence, it is also called negative regulatory element-binding protein (5). Despite this initial discovery on the SON DNA binding ability, there has been a lack of evidence for SON function in mammalian gene transcription. Recently, a mouse homolog of human negative regulatory element-binding protein was shown to repress the growth hormone secretagogue-receptor (GHS-R) promoter (6). Besides the murine *GHS-R* gene, the target genes regulated by SON at the transcriptional level are largely unknown.

During hematopoiesis, transcription factors play fundamental roles in hematopoietic cell fate decision by governing gene expression. Three members of the GATA family have emerged as key regulators of gene expression in hematopoietic cells. GATA-1 is highly expressed in erythroid, megakaryocytes, and mast cells and is essential for primitive and definitive hematopoiesis (7–9). Whereas GATA-3 is restricted in T lymphoid cells, GATA-2 is highly expressed in hematopoietic stem cells/progenitors as well as immature erythroid cells, mast cells, and megakaryocytes (10–12). GATA-2 acts at the earlier stage of hematopoietic stem cell generation and expansion. Gene targeting experiments demonstrated that *Gata-2* knock-out leads to embryonic lethality at embryonic day 10.5 due to anemia and a deficit in definitive hematopoiesis (13). *Gata-2*$^{+/-}$ embryos are not lethal, but the number of hematopoietic progenitors is

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reduced, and hematopoietic stem cells from *Gata-2*$^{+/-}$ mice showed a proliferation defect (14), demonstrating that the full dose of GATA-2 is necessary for normal hematopoietic stem cell generation and expansion. Although there is no doubt that controlling the GATA-2 level is critical in qualitative and quantitative development of hematopoietic stem cells, our knowledge of the mechanisms regulating the GATA-2 level has mainly been limited to transcriptional control.

Previously, we identified SON as a protein interacting with the leukemogenic protein AML1-ETO (15). Given the information that SON is highly expressed in hematopoietic cells and organs (5) and SON has a broad impact on gene expression (4), we have been investigating SON function in regulation of hematopoiesis-related genes that potentially affect lineage determination. Here, we report that SON is involved in controlling GATA-2 expression. SON is highly expressed in hematopoietic stem cells and down-regulated during differentiation. SON depletion causes a marked reduction of GATA-2 protein, which is mediated by increased miR-27a[4] production. We further identified that SON depletion activates the promoter of the miR-23a∼27a∼24-2 cluster, indicating that SON acts as a transcriptional repressor on this promoter. Our results reveal a previously unidentified function of SON in microRNA transcription and controlling the GATA-2 protein level in hematopoietic cells.

EXPERIMENTAL PROCEDURES

Cell Culture—K562 cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) FBS. EML cells were maintained in Iscove's modified Dulbecco's medium containing 20% heat-inactivated horse serum and supplemented with 10% conditioned medium from the BHK/MKL cell line containing murine stem cell factor.

Bone Marrow RNAs from Normal Donors and AML Patients—Bone marrow RNA samples from normal donors or AML patients (FAB subtype M2) were obtained from the University of Chicago Medical Center and the University of New Mexico Cancer Center. Institutional Review Board-approved informed consent was obtained from each study participant (courtesy of Drs. Janet Rowley and Cheryl Willman).

Isolation of Mouse Bone Marrow Cells, Sorting, and Retroviral Infection for shRNA—Bone marrow cells from C57BL/6J mice were collected by flushing tibia and femur in PBS. Bone marrow mononuclear cells were separated by density gradient centrifugation, and lineage-negative cells were selected using the MACS® magnetic beads separation kit (Miltenyi Biotec) according to the manufacturer's instruction. Lineage-negative cells were cultured overnight in Iscove's modified Dulbecco's medium containing 15% FBS, 10 ng/ml mIL-3, 50 ng/ml mSCF, and 50 ng/ml mFLT3L, and then infected with retrovirus carrying control shRNA or Son shRNA by spinoculation (1,200 × *g* for 3 h at 32 °C) in an Allegra-12R centrifuge with a SX4750 rotor (Beckman Coulter). shRNAs were cloned in pSuper-Retro-Puro (OligoEngine), and the target sequence of shRNA#1 is 5′-AGGCTCAATTACTTGAAATA-3′, and the target of shRNA#2 is 5′-CAGCGCTGGAATCCTATA-ATA-3′.

Transfection of siRNA and miRNA Mimic—siRNA for human SON (catalog 16708, siRNA ID 143161), negative control siRNA, mirVana miRNA mimic for miR-27a and miR-24, negative control miRNA mimic were purchased from Invitrogen/Ambion. siRNA for mouse Son was synthesized and labeled with Alexa Fluor 546 (Qiagen), and the target sequence is 5′-CAGCGCTGGAATCCTATAATA-3′. Transfections of siRNA and miRNA mimic into K562 cells and EML cells were done by Nucleofector (Amaxa/Lonza).

RNA Isolation, Reverse Transcription, and Quantitative PCR (qPCR)—Total RNAs were isolated by the RNeasy kit (Qiagen) according to the manufacturer's protocol. To detect splicing efficiency, RNAs were isolated from HeLa cells transfected with control siRNA or *SON* siRNA and reverse transcribed using the Superscript III kit (Invitrogen). qPCR was performed with SYBR Green PCR Master Mix (Bioline) using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems). Primers used for qPCR are as follows: for SON (human) forward, 5′-CAGATT-TTTAGGTCTTTCGTGGT-3′ and reverse, 5′-TTTTTCTGGA-GCCCTCTTTC-3′; for Son (mouse) forward, 5′-GGCGGAAA-AGATCCAGGT-3′ and reverse, 5′-GCTCCTCCAGACTTTT-TAGCAA-3′; for GATA-2 (human) forward, 5′-CCTCCAGCT-TCACCCCTAA-3′ and reverse, 5′-CACAGGCATTGCACAG-GTAGT-3′; for Gata-2 (mouse) forward, 5′-TGACTATGGC-AGCAGTCTCTTC-3′ and reverse 5′-ACACACTCCCGG-CCTTCT-3′.

Quantification of MicroRNAs—Primary transcript of miR-23a∼27a∼24-2 was measured by qPCR using following primers: set 1 forward F1, 5′-CACCGAGGATGCTGCC-3′ and reverse R1, 5′-GGGCGGAACTTAGCCACT-3′; set 2 forward F2, 5′-AGCAGCCAGTTACCCAAGA-3′ and reverse R2, 5′-TGACAGTGCGAGACTCCATC-3′. Mature forms of microRNA were measured using TaqMan miRNA assays (Applied Biosystems) according to the manufacturer's instructions.

Plasmid Construction—Luciferase gene was cut from pSP-luc+NF Fusion Vector and inserted into NheI/XhoI site of pcDNA3.1(+) to generate pcDNA3.1(+)/luc. Then, human GATA-2 3′-UTR was amplified from K562 cDNA and inserted in the EcoRI/XhoI site of pcDNA3.1(+)/luc. Mutations in the miR-27a binding site were created using QuikChange site-directed mutagenesis kit (Stratagene). The luciferase reporter construct containing the miR-23a∼27a∼24-2 cluster promoter (−603 ∼ +36 fragment) was generously provided by Dr. V. Narry Kim (Seoul National University).

Luciferase Reporter Assay—K562 cells were transfected with control or SON siRNA and then next day, transfected with the luciferase constructs containing WT or mutated 3′-UTR of GATA-2, along with pRL-null (*Renilla* plasmid for normalization). Luciferase activity was measured 48 h after plasmid transfection using Promega Dual Luciferase Assay System. Three independent experiments were performed and assayed in triplicate per group.

[4] The abbreviations used are: miR, microRNA; AML, acute myeloid leukemia; EML, erythroid myeloid lymphoid; qPCR, quantitative PCR; SR, arginine/serine-rich; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

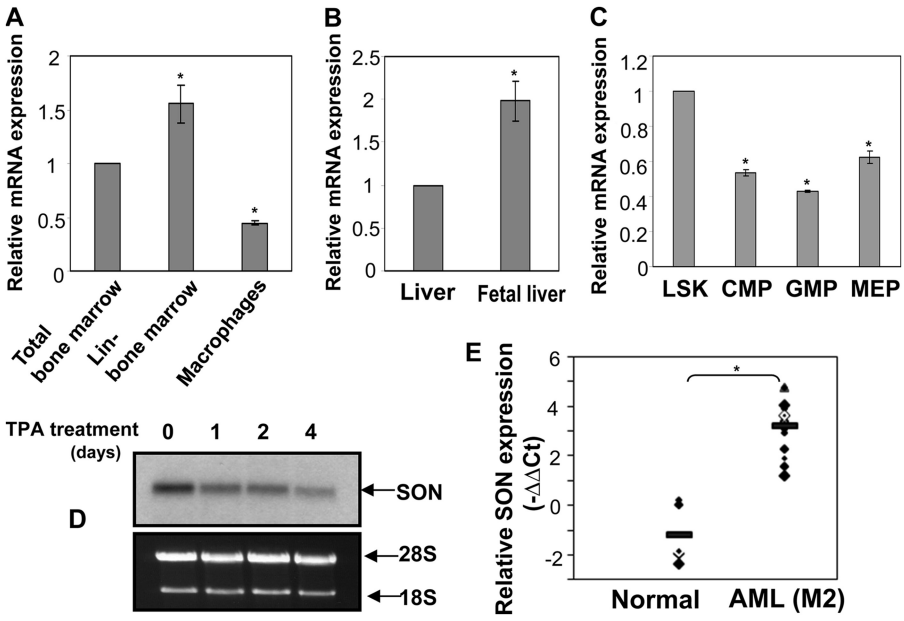

FIGURE 1. **Son expression in hematopoietic cells.** *A*, Son expression level in mouse total bone marrow cells, Lin$^-$ progenitor cells, and macrophages. RT-qPCR analysis of Son expression relative to GAPDH. The *bar* indicates the mean expression level ($n = 3$). Note that Son level is high in Lin$^-$ bone marrow cells and low in macrophages. *Error bars*, S.D. *, $p < 0.05$, compared with total bone marrow. *B*, Son is highly expressed in fetal mouse liver compared with that in adult mouse liver. *, $p < 0.02$, compared with the liver sample. *C*, Son expression in LSK cells and other hematopoietic progenitor populations, CMP, GMP and MEP. *, $p < 0.004$, compared with LSK. *D*, Son down-regulation during TPA-mediated differentiation of U937 cells, detected by Northern blotting. Signal intensities of EtBr-stained 18 S/28 S rRNA indicate the relative loading of total RNA. *E*, relative Son expression levels in normal human bone marrow and bone marrow cells from AML patients (M2 subtype). RNAs from normal bone marrow and AML patient bone marrow cells were used for qPCR of SON using the primer set targeting exon 1 (forward) and exon 3 (reverse) described under "Experimental Procedures." GAPDH qPCR was used as an internal control to calculate the relative levels of SON expression. The data are presented at log2 scale of the SON to GAPDH ratios ($-\Delta\Delta C_t$). AML patient bone marrow cells show higher Son expression compared with normal bone marrow cells (normal bone marrow; $n = 5$, AML patient bone marrow; $n = 16$). The average is indicated by a *black horizontal bar*. *, $p < 0.001$. The *p* value was determined by using Student's *t* test.

Antibodies—GATA-2 antibody was purchased from Santa Cruz Biotechnology (sc-267), and α-tubulin antibody was from Sigma (T9026).

Northern Blotting—U937 human monocytic leukemic cells were treated with 65 nM of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 0, 1, 2, and 4 days, and RNAs were harvested. Northern blotting was done as described previously (15) using the first 750 nucleotides of the SON coding sequence as a probe.

RESULTS

SON Is Highly Expressed at the Early Stages of Hematopoiesis and Decreased during Hematopoietic Differentiation—Although SON is expressed ubiquitously, its expression level is noticeably higher in hematopoietic organs/tissues and blood cells compared with other tissues (refer to the BioGPS Web site), suggesting important roles of SON in the hematopoietic system. To examine whether SON expression is regulated during hematopoietic differentiation, we measured relative mRNA levels of mouse Son at different stages of hematopoiesis. Quantitative PCR showed that the Son mRNA level is higher in lineage marker-negative (Lin$^-$) mouse bone marrow cells compared with total bone marrow cells. Macrophages showed less expression of Son, suggesting that Son is down-regulated along the hematopoietic differentiation (Fig. 1*A*). Son expression of the fetal liver, which is actively involved in hematopoiesis, was higher than that of the adult liver (Fig. 1*B*). We further sorted lineage marker-negative hematopoietic stem/progenitor cells (Lin$^-$ cells) and measured the Son level in LSK (Lin$^-$, Sca1$^+$, cKit$^+$), CMP (common myeloid progenitors), GMP (granulocyte/monocyte progenitors) and MEP (megakaryocyte/erythroid progenitors) populations. LSK cells, which precede other progenitors, showed the highest level of Son (Fig. 1*C*). In addition, we confirmed that SON is down-regulated during TPA-induced monocytic differentiation of U937 myeloid cells (Fig. 1*D*), and bone marrow cells from AML patients (AML M2), which have more immature blasts, showed significantly higher level of SON compared with normal bone marrow cells (Fig. 1*E*). Taken together, SON is more abundantly expressed in immature hematopoietic cells, such as stem/progenitor cells and leukemic blasts, and down-regulated during myeloid differentiation.

SON Knockdown Causes Depletion of the GATA-2 Protein—Because SON is differentially expressed during hematopoietic differentiation, we next examined whether SON is involved in regulation of hematopoietic transcription factors that are key dictators of hematopoietic differentiation. We isolated Lin$^-$ mouse bone marrow cells, infected them with control shRNA or two different shRNAs targeting mouse Son, and measured the mRNA level of Son. Whereas we previously observed strong cytotoxicity in cells transfected with highly effective human SON siRNA (4), the level of Son in mouse Lin$^-$ cells was reduced by 20~35% by shRNAs, so we could still maintain cells without massive growth arrest during the study. Among the several transcription factors critical to hematopoietic differentiation, interestingly, we found that GATA-2 mRNA was consistently reduced by both Son shRNAs in Lin$^-$ bone marrow cells (Fig. 2*A*). Down-regulation of the GATA-2 mRNA level

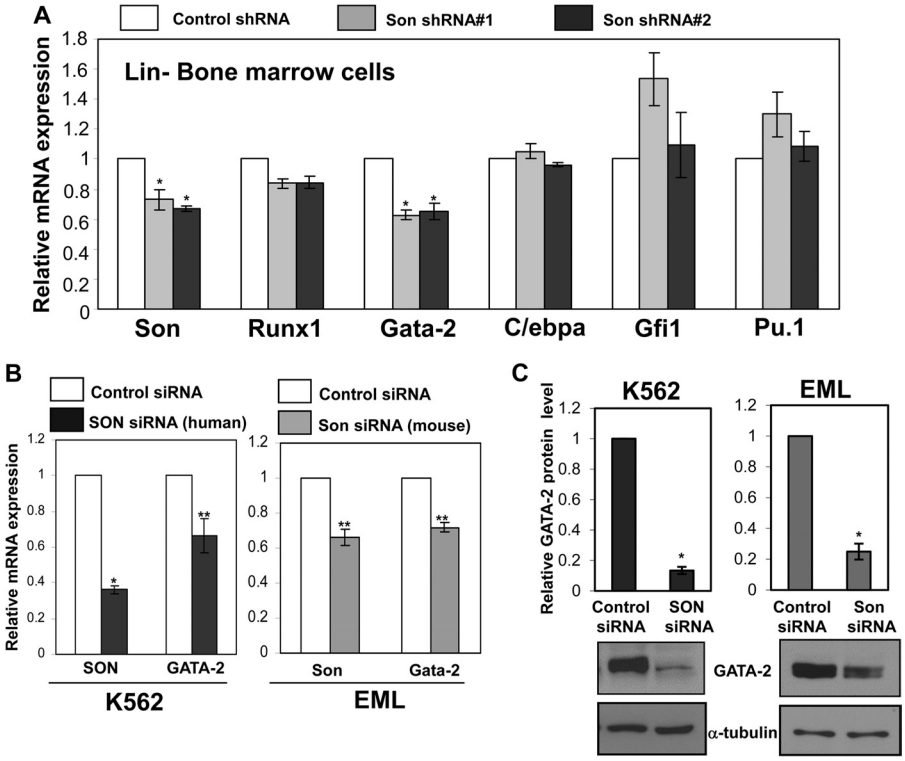

FIGURE 2. **Down-regulation of Gata-2 expression upon Son knockdown.** *A*, down-regulation of Gata-2 mRNA upon Son knockdown in mouse Lin$^-$ bone marrow cells. Two different shRNAs for mouse Son were introduced by retroviral infection, and mRNA for Son and several hematopoietic transcription factors were measured by qPCR. *, $p < 0.03$, compared with control shRNA. *Error bars*, S.D. *B*, down-regulation of SON mRNA upon SON knockdown with siRNAs in K562 and EML cells. *, $p < 0.001$; **, $p < 0.005$, compared with control siRNA. *C*, reduction of the GATA-2 protein in SON-depleted cells. SON protein level was determined by Western blotting and measuring the density of the band. The *bar graphs* represent relative density of the band (average ± S.D.) from two independent experiments. Representative results of Western blotting are shown. *, $p < 0.001$.

was further confirmed in human K562 leukemic cell line and mouse EML cell line transfected with human SON siRNA and mouse Son siRNA, respectively (Fig. 2*B*). More interestingly, although having 30~40% reduction of mRNA level, the GATA-2 protein level is more remarkably down-regulated upon SON knockdown, resulting in 75~90% reduction measured by Western blotting (Fig. 2*C*). These results indicate that upon SON knockdown, the GATA-2 protein level is substantially regulated at the post-transcriptional steps.

The 3′-UTR of GATA-2 Is Targeted by miR-27a—Reduction of the GATA-2 protein level with an unequal change in mRNA level could be achieved by microRNA inhibition of GATA-2 transcripts. Sequence analysis of the 3′-UTR of the human GATA-2 gene predicted several candidates of targeting microRNAs (Fig. 3*A*). Among them, we confirmed that the mature forms of miR-27a and miR-24 are up-regulated upon SON knockdown in both K562 cells and EML cells (Fig. 3*B*), raising the possibility that these miRs target the 3′-UTR of GATA-2. Next, we tested the effect of overexpression of miR-27a and miR-24 on luciferase expression from control or full-length GATA-2 3′-UTR-containing constructs in K562 cells. Luciferase activity was reduced ~40% by miR-27a expression, but not by miR-24 expression (Fig. 3*C*), confirming that miR-27a indeed targets the 3′-UTR of GATA-2 mRNA in this cell type. Reduction of endogenous GATA-2 protein by transfection of miR-27a mimic was also confirmed (Fig. 3*D*).

GATA-2 is one of the potential targets of miR-27a predicted by several programs, including TargetScan and PicTar. Nucleotide 155–162 of the 3′-UTR of GATA-2 is complementary to eight consecutive nucleotides in the 5′ end of miR-27a, which includes the seed region (position 2–8 of mature miRNA) (Fig. 4*A*). In addition, 10 additional nucleotides at the 3′ sequence of miR-27a are predicted to pair with further 5′ sequences of GATA-2 3′-UTR, forming a complex with three bulges (Fig. 4*B*). To verify the importance of the seed sequence of miR-27a, we mutated the potential miR-27a-targeting site in the 3′-UTR of GATA-2 and inserted it downstream of the luciferase gene. Compared with the wild type 3′-UTR, the mutated 3′-UTR showed significantly less inhibition in SON siRNA-transfected cells (Fig. 4*C*). Taken together, these results demonstrate that SON knockdown up-regulates miR-27a, which, in turn, targets the 3′-UTR of GATA-2 and down-regulates GATA-2 expression.

SON Knockdown Up-regulates miR-23a~27a~24-2 Cluster through Activation of Its Promoter—miR-27a is a member of the miR-23a~27a~24-2 cluster, and the primary microRNA is transcribed by RNA polymerase II (16) and further processed to generate miR-23a, miR-27a, and miR-24-2. Interestingly, quantitative PCR data demonstrated that primary microRNA of the miR-23a~27a~24 cluster is up-regulated upon SON knockdown (Fig. 5*A*), and the mature forms of miR-23a is also increased (data not shown) in addition to miR-27a and miR-24 (Fig. 3*B*). To test whether the increase of primary microRNA of this cluster is due to promoter activation, we used a reporter construct containing the promoter sequence of the miR-23a~27a~24-2 cluster (23P639, the promoter fragment con-

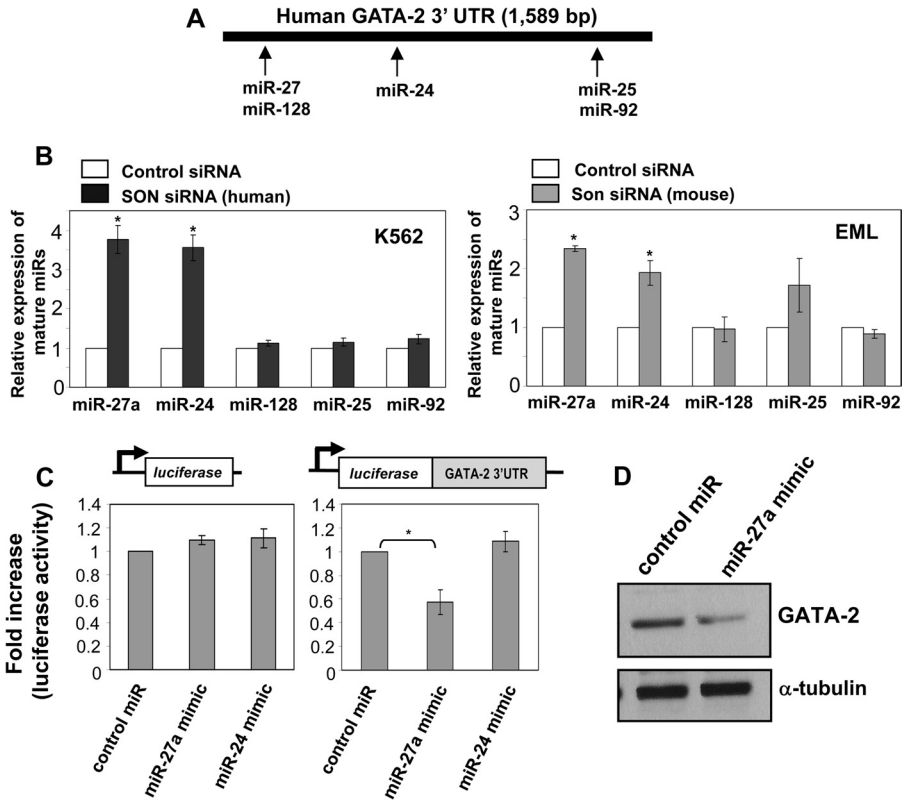

FIGURE 3. **miR-27 is up-regulated upon SON knockdown and targets the 3′-UTR of GATA-2 mRNA.** *A*, miRs are predicted to target human GATA-2 3′-UTR. *B*, miR-27a and miR-24 are up-regulated by SON siRNA in human K562 cells and murine EML cells. *, $p < 0.001$, compared with control siRNA. *Error bars*, S.D. *C*, miR-27a, but not miR-24, targets 3′-UTR of human GATA-2. 3′-UTR of human GATA-2 was cloned at the downstream of the CMV promoter and the luciferase gene. This construct was transfected into K562 cells that were pretreated with microRNA mimics of miR-27a and miR-24, and relative luciferase activity was measured. *, $p < 0.05$. *D*, reduction of endogenous GATA-2 protein by miR-27a mimic in K562 cells is shown.

taining the nucleotide position −603 ∼ +36) fused to the downstream luciferase gene (16). Interestingly, this reporter assay demonstrated that expression of luciferase driven by this promoter is significantly elevated by 2.5-fold upon SON knockdown (Fig. 5*B*). This result suggests that SON normally suppresses this promoter, and depletion of SON releases the transcriptional machinery from the inhibitory components.

To determine which region of the promoter is required for SON-mediated suppression, the promoter fragment was serially deleted and inserted into the reporter construct, and luciferase activity was measured in control and SON-depleted K562 cells. Although luciferase activity is significantly increased with full-length promoter upon SON knockdown, we observed diminished increase of luciferase activity in SON-depleted cells when testing the −403 ∼ +36 and −203 ∼ +36 promoter fragments (Fig. 5*C*). These data suggest that the upstream region with 200 nucleotides (−603 ∼ −403) is required for SON-mediated promoter repression. Taken together, our data demonstrated that SON functions to repress transcription of the miR-23a∼27a∼24-2 cluster, thereby relieving GATA-2 mRNA from targeting by miR-27a, and contributes to maintaining the GATA-2 protein level (Fig. 5*D*).

DISCUSSION

SON is an SR-like, nuclear speckle-localized protein with unique amino acid repeats, an SR domain, and two RNA binding motifs. A function of this protein in RNA splicing was recently revealed. SON governs proper cell cycle progression and genome stability through mediating efficient RNA splicing of a set of genes necessary for microtubule dynamics, mitotic progression, and DNA repair (4). It has also been reported that knockdown of SON causes exon skipping in pre-mRNA of several chromatin-modifying enzymes (17). Although SON is known to be expressed ubiquitously (5), we observed that the level of SON is precisely regulated in different hematopoietic organs and during hematopoietic differentiation. Importantly, we noticed that SON is expressed abundantly in hematopoietic stem cells and immature cells and down-regulated in differentiated cells, implicating its role in hematopoietic differentiation. Our data revealed that reduction of the SON level leads to depletion of the protein level of hematopoietic transcription factor GATA-2 and this is mediated by miR-27a targeting GATA-2 3′-UTR. RUNX1 is also shown to be targeted by miR-27a (18, 19), and we also detected a decrease of the RUNX1 protein upon SON knockdown (data not shown). These results provide a novel evidence of SON-mediated microRNA regulation and the subsequent effect on controlling a key hematopoietic transcription factor GATA-2.

GATA-2 is expressed predominantly in adult and developing stem cells and mast cells (12, 20, 21). Although its function in maintaining hematopoietic stem cells and cell fate decision has been well documented (13, 14, 22–25), how GATA-2 expression is regulated remains unclear. A majority of the studies has

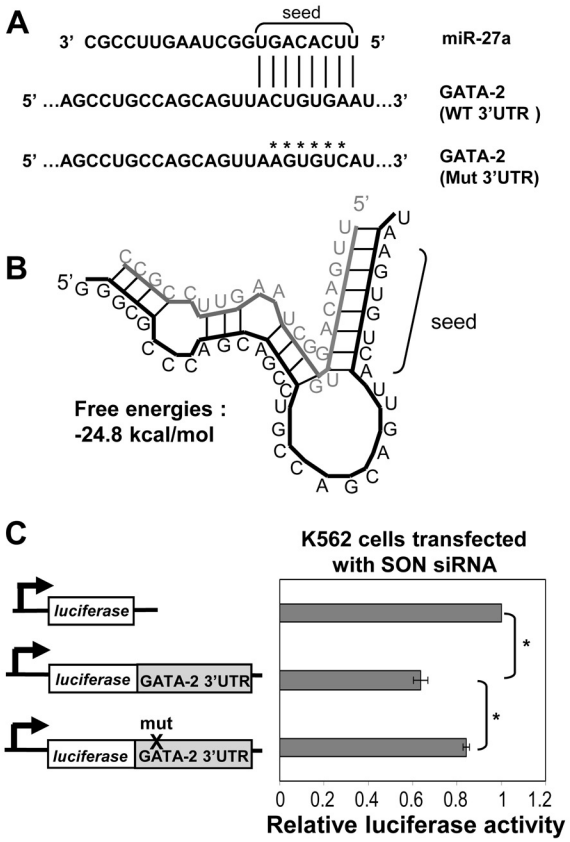

FIGURE 4. **The 3′-UTR of GATA-2 mRNA contains miR-27a-binding sites.** *A*, schematics showing the predicted miR-27a-binding sites in the 3′-UTR of human GATA-2 mRNA. The sequences altered in the mutant form of 3′-UTR are shown with *asterisks*. *B*, predicted structure of GATA-2 3′-UTR interacting with miR-27a. *Gray line*, miR-27a; *black line*, GATA-2 3′-UTR. *C*, sequence mutation at the predicted miR-27a binding site of GATA-2 3′-UTR alleviating the repressive effect of SON knockdown on luciferase expression. *, $p < 0.04$.

focused on regulation of GATA-2 promoter, and transcription and the transcriptional regulation seem to involve distinct pathways in diverse tissues (21, 26–28). However, post-transcriptional regulation of GATA-2 has not been explored much. In this report, we found that GATA-2 protein level is remarkably reduced with minimal changes in mRNA level upon SON knockdown, demonstrating that the GATA-2 protein level is not only controlled by transcription, but also fine-tuned at the post-transcriptional level. Down-regulation of SON during hematopoietic differentiation likely functions in fine tuning process through modulating microRNAs to maintain the low level of GATA-2 protein as cells exit the stem cell pool. Moderate, but significant, reduction of GATA-2 mRNA by SON siRNA (Figs. 2*A* and 2*B*) may be due to miR-27a-mediated speed up of mRNA deadenylation and decay (29). We also observed that the 3′-UTR of GATA-2 with miR-27a binding site mutated was still affected, albeit less, by SON siRNA (Fig. 4*C*), which suggests other miRNAs, in addition to miR-27a, regulated by the SON level also target this 3′-UTR to contribute to fine-tuning of the GATA-2 protein level.

All AML patient samples we tested were of the M2 subtype, because we originally obtained samples with an intention to examine whether M2 AML with specific chromosomal translocation, such as t(8;21), shows different SON expression level from other M2 AML. We did not find any correlation between the SON level and t(8;21) (data not shown). Instead, we observed that SON expression is in general higher in bone marrow cells from M2 AML patients compared with normal bone marrow, which is consistent with other data showing higher SON level in undifferentiated, immature cells. Recently, GATA-2 was reported to be overexpressed in AML patients, and overexpression of GATA-2 is associated with poor prognosis (30). Besides the transcriptional activation of GATA-2, it is likely that high SON level in AML patients contributes to maintain high GATA-2 protein.

A couple of recent studies validated the role of miRNAs in regulating GATA-2. In zebrafish, miR-451 targets gata-2 during erythroid maturation (31). miR-24 was also shown to target GATA-2 in human endothelial cells (32). Although we observed that miR-24 is up-regulated upon SON knockdown in both K562 cells and EML cells, overexpression of miR-24 did not target the 3′-UTR of GATA-2 in hematopoietic cells (Fig. 3*C*). This discrepancy between the two studies may be due to different cell types used for the assays, because the difference in endogenous gene expression can affect the availability and efficiency of microRNAs targeting a specific gene (33).

It is important to emphasize that SON is involved in miRNA production, resulting in changes in gene expression. Previously, our group and others reported that SON functions as an SR-like protein in constitutive and alternative splicing. In addition to acting at the splicing step, it is apparent that SON regulates gene expression through modulating the microRNA level. Three microRNAs that are encoded by the miR-23a∼27a∼24-2 cluster have been implicated to function in cell cycle and apoptosis, and the expression of this microRNA cluster is altered in many cancers, including breast cancer, hepatocellular cancer, and leukemia (34). Although we only focused on GATA-2 regulation by miR-27a in hematopoietic cells for the current study, it is certainly predictable that many other target genes involved in multiple cellular processes are down-regulated at the protein level by miR-23a, miR-27a, and miR-24-2 which are up-regulated when SON is reduced. Consistent with our observation of reduction in SON expression during TPA-induced U937 differentiation, miR-27a has been reported to be up-regulated during TPA-induced differentiation of HL-60 cells (35). Substantial information on SON-regulated microRNA signature is certainly of interest and will be investigated in the future.

Although our group and others recently reported that SON is a splicing factor, SON was originally reported as a DNA-binding protein (5) and shown to be a transcriptional repressor that binds to the viral genome of hepatitis B virus. The consensus sequence for optimal SON binding was determined by PCR-assisted binding site selection, which concludes the sequence to be GA(G/T)AN(C/G)(A/G)CC. We could not find any sequence that shows perfect match with the consensus sequence. However, GAGGAGACC at the −443 ∼ −435 region contains a sequence close to the consensus and might be a potential SON binding site. Such further detailed analysis, although beyond the scope of this report, could be explored in later studies. As it is dedicated in both microRNA production

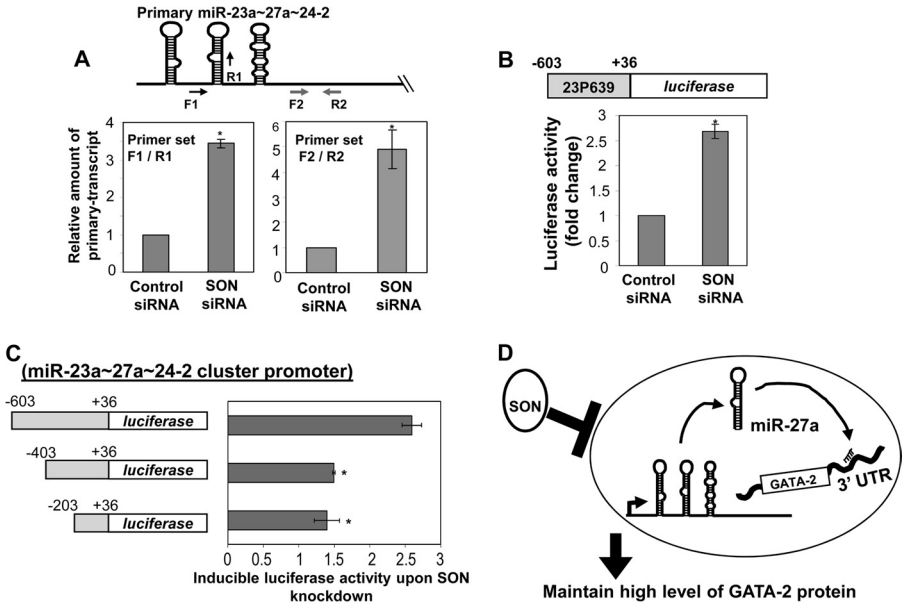

FIGURE 5. **SON depletion activates the promoter of the miR-23a~27a~24-2 cluster.** *A*, primary transcript of miR-23a~27a~24-2 is up-regulated upon SON knockdown, measured by qPCR. Locations of two primer sets used for qPCR are indicated with *arrows* in the figure of the transcript. *, $p < 0.001$. *B*, the promoter of the miR-23a~27a~24-2 is activated upon SON knockdown. The promoter contains the nucleotides from the positions bp −603 to +36 of the miR-23a~27a~24-2 gene (23P639). *, $p < 0.001$. *C*, the nucleotide sequences from −603 to −403 of the promoter contribute to SON-mediated suppression. Luciferase activity upon SON siRNA transfection was normalized by the luciferase activity of control siRNA-transfected cells to calculate -fold activation. *, $p < 0.05$, compared with −603 ~ +36. *D*, schematic shows SON function in regulating the GATA-2 protein level through inhibition of the miR-23a~27a~24-2 cluster.

and RNA splicing, SON is emerging as a multifunctional regulator of gene expression acting at the post-transcriptional level. In addition, our report demonstrates that SON is involved in transcriptional repression of mammalian genes, supporting the multifunctional feature of this protein. Further investigation on mechanisms of SON functions and identification of more SON target genes will expand our knowledge on the precise control of gene expression.

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