SON regulates GATA-2 through transcriptional control of the miR-23a~27a~24-2 cluster*

Erin Eun-Young Ahn¹,², Tsunehito Higashi¹,⁵, Ming Yan¹, Shinobu Matsuura¹,⁶, Christopher J. Hickey², Mia-Chia Lo¹, Wei-Jong Shia¹, Russell C. DeKelver¹,⁴, and Dong-Er Zhang¹,³,⁴

¹Moores UCSD Cancer Center, University of California San Diego, La Jolla, CA 92093, USA
²Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604, USA
³Department of Pathology and ⁴Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA
⁵Current address: Department of Cellular Pharmacology, Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan
⁶Current address: Boston University School of Medicine, Center for Advanced Biomedical Research, Boston, MA 02118, USA

*Running title: SON regulates GATA-2 via microRNA

To whom correspondence should be addressed: Dong-Er Zhang, Ph.D., Moores UCSD Cancer Center, University of California San Diego, Mail Drop 0815, 3855 Health Sciences Drive, La Jolla, CA 92093, Tel.: (858) 822-5372, Email: d7zhang@ucsd.edu

Keywords: SON, GATA-2, microRNA, hematopoiesis

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.

SUMMARY
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 upregulated upon SON knockdown and targets the 3'UTR of GATA-2 mRNA in hematopoietic cells. Upregulation 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 (RS) domain, a glycine-rich motif (G-patch), a double-stranded RNA binding motif (DSRM) 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 RS domain, SON interacts with the human hepatitis B virus (HBV) genome and represses viral gene transcription, hence it is also called negative regulatory element binding protein (NREBP) (5). Despite this initial discovery on SON’s DNA-binding ability, there has been a lack of evidence for SON’s function in mammalian gene transcription. Recently, a mouse homolog of human NREBP 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). While 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 knockout leads to embryonic lethality at E10.5 due to anemia and a deficit in definitive hematopoiesis (13). GATA-2 +/- embryos are not lethal, but the number of hematopoietic progenitors is 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 HSCs, 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’s 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 production. We further identified that SON depletion activates the promoter of the miR-23a~27a~24-2 cluster, indicating 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 (IMDM) 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 University of Chicago Medical center and University of New Mexico Cancer Center. IRB-approved informed consent was obtained from each study participant (courtesy of Drs. Janet Rowley and Cheryl Willman).
SON regulates GATA-2 via microRNA

Isolation of mouse bone marrow cells, sorting and retroviral infection for shRNA - Bone marrow cells from C57BL6/J mice were collected by flushing tibia and femur in PBS and 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, Germany) according to manufacturer’s instruction. Lineage negative cells were cultured overnight in IMDM media 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 hours at 32°C) in an Allegra-12R centrifuge with a SX4750 rotor (Beckman Coulter Palo Alto, CA). shRNAs were cloned in pSuper-Retro-Puro (OligoEngine), and the target sequence of shRNA#1 is 5’-AGGCTCAATTACTTGAAA TA-3’, and the target of shRNA#2 is 5’-CAGCGCTGG AAATCCTATAATA-3’.

Transfection of siRNA and miRNA mimic - siRNA for human SON (Cat#16708, siRNA ID# 143161), negative control siRNA, mirVana miRNA mimic for miR-27a and miR-24, negative control miRNA mimic were purchased from Life Technologies/Ambion. siRNA for mouse Son was synthesized and labeled with Alexa-546 (Qiagen), and the target sequence is 5’-CAGCGCTGG AAATCCTATAATA-3’. Transfections of siRNA and miRNA mimic into K562 cells and EML cells were done by Nucleofector (Amaxa/Lonza).

RNA isolation, Reverse transcription and 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’-CACAGGGAATTACCCGTGAT-3’, reverse 5’-TCTTCTGGAGGCC TCTTTC-3’, for GATA-2 (human), forward 5’-CACAGGGAATTACCCGTGAT-3’, reverse 5’-CCCTCCAGACTTTTTAGCAA-3’, for GATA-2 (human), forward 5’-CCTCAGCCCTTC ACCCCCTAA-3’, reverse 5’-CAGAGGCAATTCGACG-3’; for Gata-2 (mouse), forward 5’-TGACTATGCGCAGTCTCTTC-3’, reverse 5’-ACACACTCCCGGCT TTCT-3’.

Quantification of microRNAs - Primary transcript of miR-23a~27a~24-2 was measured by qPCR using following primers; set 1, forward F1 5’-CACCAGGATGCTGCC-3’, reverse R1 5’-GGCCGGAACCTTGGACCT-3’; set 2, F2 5’-AGCAGCCAGTTACCCCAAGA -3’, reverse R2 5’-TGACAGTGGAGACTCCATC-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(+), then Human GATA-2 3’UTR was amplified from K562 cDNA and inserted in EcoRI/XhoI site of pcDNA3.1(+). 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 hours after plasmid transfection using Promega Dual Luciferase Assay System. Three independent experiments were performed and assayed in triplicate per group.

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 leukemia cells were treated with 65 nM of 2-O-tetradecanoylphorbol-13-acetate (TPA) for 0, 1, 2, and 4 days and RNAs were harvested. Northern blotting was done as described previously.
SON regulates GATA-2 via microRNA

**RESULTS**

SON is highly expressed at the early stages of hematopoiesis and decreased during hematopoietic differentiation - While SON is expressed ubiquitously, its expression level is noticeably higher in hematopoietic organs/tissues and blood cells compared to other tissues (refer to BioGPS, http://biogps.org), suggesting important roles of SON in the hematopoietic system. To examine whether SON expression is regulated during hematopoietic differentiation, we measured relative mRNA level 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 when compared to total bone marrow cells. Macrophages showed less expression of Son, suggesting that Son is down-regulated along the hematopoietic differentiation (Fig. 1A). Son expression of the fetal liver, which is actively involved in hematopoiesis, was higher than that of the adult liver (Fig. 1B). 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. 1C). In addition, we confirmed that SON is down-regulated during TPA-induced monocytic differentiation of U937 myeloid cells (Fig. 1D), and bone marrow cells from AML patients (AML M2), which have more immature blasts, showed significantly higher level of SON compared to normal bone marrow cells (Fig. 1E). 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 - Since 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. While 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. 2A). Down-regulation of the GATA-2 mRNA level 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. 2B). More interestingly, while 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 blot (Fig. 2C). These results indicate that upon SON knockdown, 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. 3A). Among them, we confirmed that the mature form of miR-27a and miR-24 are up-regulated upon SON knockdown in both K562 cells and EML cells (Fig. 3B), raising the possibility that these miRs target the 3’UTR of GATA-2. Next, we tested the effect of over-expression 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. 3C), 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. 3D).

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. 4A). 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 3 bulges (Fig. 4B). 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 to the wild type 3'UTR, the mutated 3'UTR showed significantly less inhibition in SON siRNA-transfected cells (Fig. 4C). Taken together, these results demonstrate that SON knockdown upregulates miR-27a, which, in turn, targets the 3'UTR of GATA-2 and down-regulates GATA-2 expression.

**SON knockdown upregulates 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 (pri-miR) 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 pri-miR of the miR-23a-27a-24-2 cluster is upregulated upon SON knockdown (Fig. 5A), and the mature forms of miR-23a is also increased (data not shown) in addition to miR-27a and miR-24 (Fig. 3B). To test whether the increase of pri-miR 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 containing 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. 5B). 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, inserted into the reporter construct, and luciferase activity was measured in control and SON-depleted K562 cells. While 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. 5C). These data suggest that the upstream region with 200 nucleotides (-603 ~ -403) are 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. 5D).

**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 abundantly expressed in hematopoietic stem cells and immortal 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 predominantly expressed in adult and developing stem cells and mast cells (12,20,21). While its function in maintaining hematopoietic stem cells and cell fate decision has
SON regulates GATA-2 via microRNA

been well documented (13,14,22-25), how GATA-2 expression is regulated remains unclear. A majority of the studies has focused on regulation of GATA-2 promoter and transcription and the transcriptional regulation seems 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. 2A and 2B) 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. 4C), 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, since 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 to 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 upregulated 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. 3C). This discrepancy between the two studies may be due to different cell types used for the assays, since 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 downregulated at the protein level by miR-23a, miR-27a and miR-24-2 that are upregulated 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 upregulated 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 HBV. 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 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.

ACKNOWLEDGEMENTS
We are grateful to Dr. Janet Rowley (University of Chicago Medical Center) and Dr. Cheryl Willman (University of New Mexico Cancer Center) for providing acute myeloid leukemia patient bone marrow samples, and Dr. V. Narry Kim (Seoul National University) for the luciferase construct containing the miR-23a~27a~24-2 cluster promoter.

REFERENCES
SON regulates GATA-2 via microRNA


FOOTNOTES
* This work was supported by CA096735 from the National Institutes of Health of USA (to D.E.Z.), and University of South Alabama Mitchell Cancer Institute Startup Fund (to E. A.).

To whom correspondence should be addressed: Dong-Er Zhang, Moores UCSD Cancer Center, University of California San Diego, Mail Drop 0815, Room 5328, 3855 Health Sciences Drive, La Jolla, CA 92093, Tel.: (858) 822-5372; Fax: (858) 822-5433; Email: d7zhang@ucsd.edu

The abbreviations used are: AML, acute myeloid leukemia; UTR, untranslated region; miR, microRNA

FIGURE LEGENDS
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. * p < 0.05, compared with total bone marrow. (B) Son is highly expressed in fetal mouse liver compared to that in adult mouse liver. * p < 0.02, compared with the liver sample. (C) Son expression in LSK and other hematopoietic progenitor populations, CMP, GMP and MEP. * p <
SON regulates GATA-2 via microRNA

0.004, compared with LSK. (D) Son is down-regulated during TPA-mediated differentiation of U937 cells, detected by Northern blot. Signal intensities of EtBr stained 18S/28S 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 quantitative PCR (qPCR) of SON using the primer set targeting exon 1 (forward) and exon 3 (reverse) described in 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 (-ΔΔCt). AML patient bone marrow cells show higher Son expression compared to normal bone marrow cells (normal bone marrow; n = 5 , AML patient bone marrow; n = 16). Average is indicated by a black horizontal bar. * \( p < 0.001 \). The \( p \) value was determined by using Student’s \( t \)-test.

**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. (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 ± SD) from two independent experiments. Representative results of Western blot were shown. * \( p < 0.001 \)

**FIGURE 3.** miR-27 is upregulated upon SON knockdown and targets the 3’UTR of GATA-2 mRNA. (A) miRs predicted to target human GATA-2 3’UTR. (B) miR-27a and miR-24 are upregulated by SON siRNA in human K562 cells and murine EML cells. * \( p < 0.001 \), ** \( p < 0.005 \), compared with control siRNA. (C) miR-27a, but not miR-24, target 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 pre-treated 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.

**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. **Grey line**, miR-27a; **Black line**, GATA-2 3’UTR. (C) Sequence mutation at the predicted miR-27a binding site of GATA-2 3’UTR alleviate the repressive effect of SON knockdown on luciferase expression. * \( p < 0.04 \).

**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 upregulated upon SON knockdown, measured by qPCR. Locations of two primer sets used for qPCR were 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) A schematic showing SON’s function in regulating the GATA-2 protein level through inhibition of the miR-23a~27a~24-2 cluster.
Figure 1
Figure 2
Figure 3
GATA-2

Figure 4

A

3' CGCCUUGAAUCCUGUGAGCACU 5'

5' ...AGCCUGCCAGCAGUUAAGUGUCAU...3' GATA-2 (WT 3'UTR)

5' ...AGCCUGCCAGCAGUUAAGUGUCAU...3' GATA-2 (Mut 3'UTR)

B

Free energies : -24.8 kcal/mol

C

K562 cells transfected with SON siRNA

Relative luciferase activity

Figure 4
Figure 5
SON regulates GATA-2 through transcriptional control of the miR-23a~27a~24-2 cluster
Erin Eun-Young Ahn, Tsunehito Higashi, Ming Yan, Shinobu Matsuura, Christopher J. Hickey, Miao-Chia Lo, Wei-Jong Shia, Russell C. DeKelver and Dong-Er Zhang

J. Biol. Chem. published online January 14, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.447227

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2013/01/14/jbc.M112.447227.full.html#ref-list-1