Original Article
Differential expression of ACINUS variants in bone marrow in myelodysplastic syndromes

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Abstract: Hematopoietic cells in the bone marrow of myelodysplastic syndromes (MDS) exhibit a complex pattern of cellular dynamics, showing active cellproliferation, as well as frequent apoptosis. To determine the influence of ACINUS expression on the regulation of cellular dynamics in MDS, the expression of ACINUS in bone marrow samples from controls and patients with MDS, overt leukemia (OL) transformed from MDS and *de novo* acute myeloid leukemia (AML) was analyzed using immunohistochemistry and real-time quantitative PCR (RT-PCR). The expression of the ACINUS protein was up-regulated in the bone marrow of MDS, OL, and *de novo* AML. RNA of *ACINUS-S'* also up-regulated as the same pattern, whereas *ACINUS-L* did not. *In vitro* experiments demonstrated that overexpression of ACINUS-S' resulted in increased cell proliferation. These results suggest that ACINUS variants exhibit differential patterns of expression and function in the bone marrow of myeloid neoplasms, such as MDS, OL, and *de novo* AML.

Keywords: MDS, AML, ACINUS, cell proliferation, apoptosis

Introduction

The hematopoietic cell dynamics of myeloid neoplasms are positively and negatively regulated by multiple and complex factors in bone marrow. These factors can include genetic/epigenetic changes in stem cells/neoplastic cells [1-4]; humoral factors, such as cytokines and chemokinesproduced by various types of cells [5]; cognitive contact of hematopoietic cells with stromal cells constituting the so-called microenvironment [6]; and vascular supply [7]. In contrast to neoplastic cells in myeloid leukemia and/or myeloproliferative neoplasms, hematopoietic cells in myelodysplastic syndromes (MDS) are characterized by an apoptosis-prone nature, although they also show high proliferative activity [8, 9].

We previously showed that many kinds of biological signals regulated the apoptotic features of MDS bone marrow cells [10-15]. In a mouse model of retrovirus-induced leukemia in mice, we demonstrated that two proteins, minichromosome maintenance 2 (MCM2) and ACINUS, were key factors in host- specific induction of DNA-damage-induced apoptosis in hematopoietic cells of Friend leukemia virus-infected mice [16, 17]. MCM2 positively regulated the induction of apoptosis of hematopoietic cells in normal C3H mice but in other strains of mice [16, 17]. Normal C3H mice expressed higher levels of MCM2 in hematopoietic cells as compared with the levels in other strains of mice [18, 19]. MCM2 was also shown to be highly expressed in apoptosis-prone hematopoietic cells of MDS patients [20].

ACINUS, the other keyapoptotic protein examined in the above-mentioned experiments, was previously identified as a caspase-3-activated protein required for apoptotic chromatin condensation [21]. The ACINUS protein has different isoforms (ACINUS-L, ACINUS-S, and ACINUS-S'), which regulate cell dynamics in a variety of ways [22]. Previous research demonstrated that an upstream molecule, Akt, phosphorylated ACINUS and inhibited its proteolytic cleav-

age, preventing ACINUS-dependent chromatin condensation [23]. It also showed that ACINUS was phosphorylated on serine 422 and 573, making it resistant to caspase cleavage, and that this Akt-regulated process led to the survival of cells [23].

The potential influence of ACINUS on the cell dynamics of myeloid neoplasms is not known. Thus, the aim of the present study was to investigate the effects of ACINUS expression in bone marrow cells of patients with myeloid neoplasms and controls. An additional aim was to analyze functional aspects of ACINUS using cell line cells.

Materials and methods

Patients and bone marrow samples

For clinical samples, bone marrow clots were obtained by aspiration during routine pathological diagnostic procedures. Fresh frozen samples were obtained from the National Hospital Organization Kumamoto Medical Center. Formalin fixed, paraffin embedded (FFPE) bone marrow samples were obtained from patients at Tokyo Medical and Dental University Hospital. This study was approved by the Ethics Committees of Tokyo Medical and Dental University and National Hospital Organization Kumamoto Medical Center. Informed consent was obtained via disclosing information, according to the Ethical Guidelines for Clinical Studies of The Ministry of Health, Labour, and Welfare, Japan. All the procedures were conducted in accordance with the ethical standards established by these committees (approval no.: M2015-545).

Diagnoses were made according to the criteria of the World Health Organization [24, 25]. Informed consent was obtained from all the individuals.

FFPE samples were obtained from 21 patients with MDS before treatment (15 males and 6 females; median age of 64 years), 13 patients withovert leukemia (OL) transformed from MDS (9 males and 4 females; median age of 62 years), 8 patients withde novo AML (6 males and 2 females; median age of 62 years), and 8 controls with no hematologic disorders and no remarkable pathological changesin their bone marrow (3 males and 5 females; median age of 58.5 years).

Fresh frozen samples were obtained from 28 patients with MDS before treatment (23 males and 5 females; median age of 75.5 years), 11 patients with OL (7 males and 4 females; median age of 65 years), 19 patients withde novo AML (9 males and 10 females; median age of 73 years), and 18 controls with no remarkable pathological changesin theirbone marrow (6 males and 12 females; median age of 67 years).

Immunohistochemical staining methods

For immunohistochemical staining, 4-μm-thick FFPE bone marrow sections were used. After de-paraffinization, heat-based antigen retrieval, endogenous peroxidase blockade using 3% hydrogen peroxide, and blocking were performed. The following primary antibodies were used: ACINUS (Abcam), CD33, CD61 (NOVOCASTRA), and CD71 (Invitrogen). The primary antibodies were incubated overnight at 4°C. Detection of Immunohistochemical staining was performed using the following: an ABC kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (Nichirei Bioscience), a HISTOFINE simple stain AP series kit (Nichirei Bioscience), and a WarpRed chromogen kit (Biocare Medical, Concord, CA, USA). For double immunostaining, heat treatment and blocking were performed between each step. For negative isotype control, mouse IgG1 (Dako) and a rabbit immunoglobulin fraction (Dako) diluted to the same concentration as that of the primary antibodies were used.

RNA preparation and real-time (RT) quantitative polymerase chain reaction (PCR)

RNA was extracted from fresh frozen bone marrow samples using an RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. cDNA was generated from RNA using TaqMan reverse transcription reagents (Applied Biosystems). The primers were as follows: *ACINUS-L*: 5'-GTTCGAAGGGCGTTGGCGGA-3' (forward) and 5'-CAGGGCACTCTTCTGCCCGC-3' (reverse); *ACINUS-S:* 5'-CAGAAAGCAAAGAAGGTGAGG-3' (forward) and 5'-TCA TCATTGCTGACTTGGTCT-3' (reverse); *ACINUS-S':* 5'-ACTCGGGGTTTGCCTGAG-3' (forward) and 5'-TCATCATTGCTGACTTGGTCT-3' (reverse); *beta-ACTIN:* 5'-AGCACAGAGCCTCGCCTTT-3' (forward) and 5'-CACGATGGAGGGGAAGAC-3' (reverse).
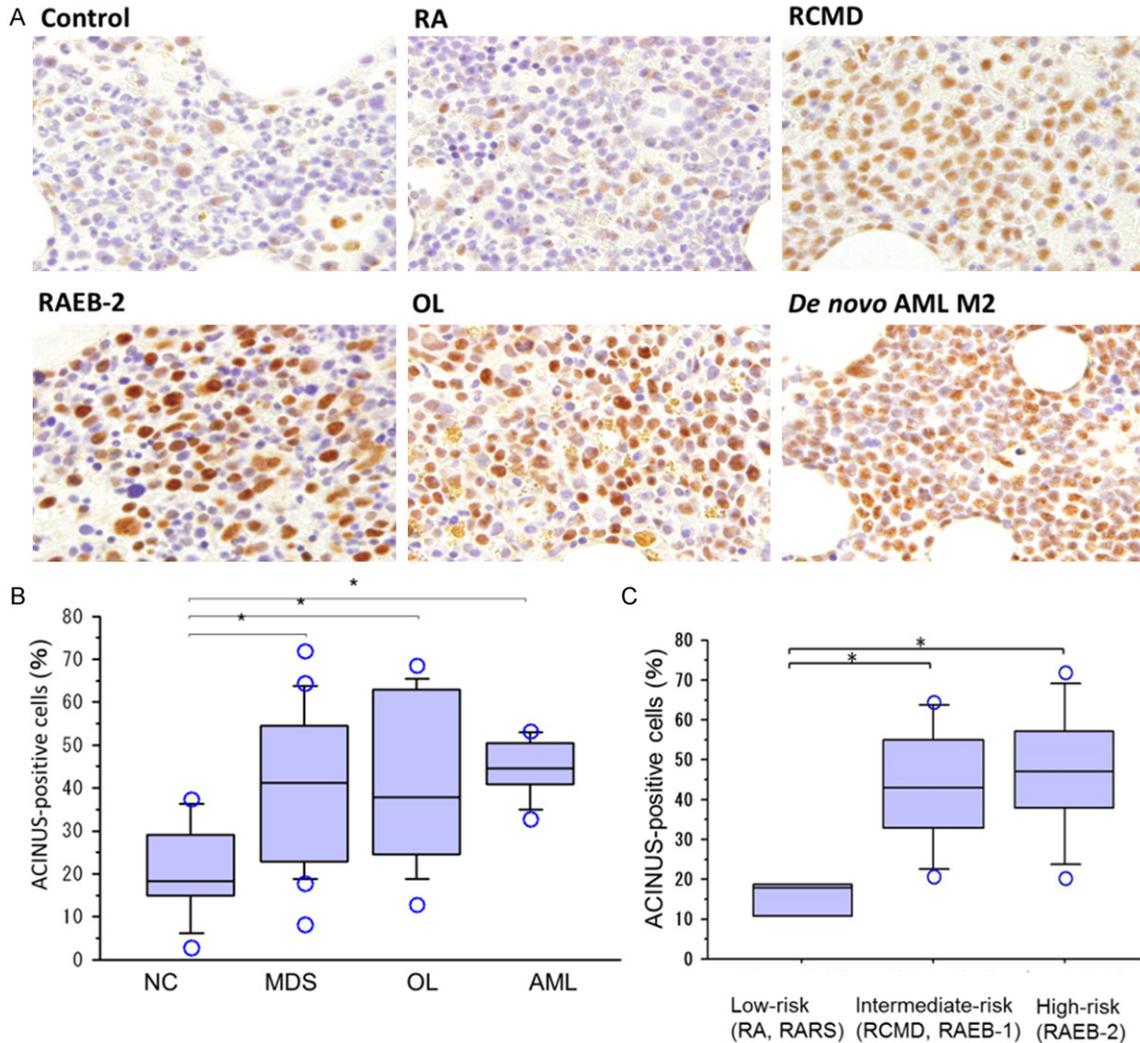
Figure 1. Strong expression of ACINUS in bone marrow cells of MDS and AML. A. Immunohistochemistry of ACINUS in control, MDS, MDS-OL, and *de novo* AML bone marrow (×400, original magnification). B. A box plot analysis of the percentage of ACINUS-positive cells in the bone marrow of each group. The percentages of positive cells in the MDS, OL, and *de novo* AML cases were significantly higher than those of the control cases (*P<0.05). C. A box plot analysis of the percentage of ACINUS-positive cells in the bone marrow from low-risk (RA, RARS), intermediate-risk (RCMD, RAEB-1), and high-risk MDS (RAEB-2) cases. Note the significantly higher numbers in the intermediate-risk and high-risk groups as compared with the ratio in the low-risk group (*P<0.05).

The PCR reactions were performed using Fast Start Universal SYBR Green Master (Rox) (Roche) and were monitored using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The mRNA level was analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized with glyceraldehyde 3-phosphate dehydrogenase as an endogenous control.

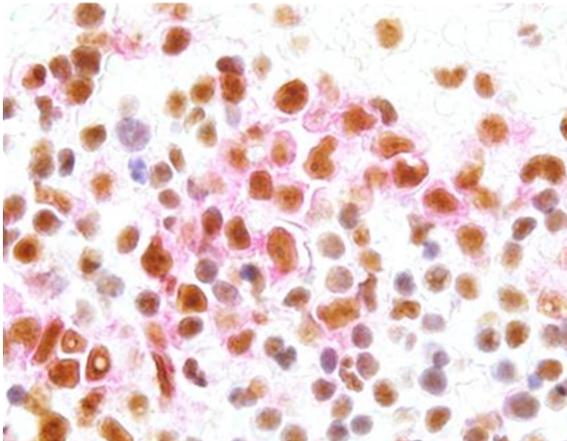*Cell culture and short hairpin (sh)-ACINUS and overexpression of ACINUS*

HEK293T cells were maintained in Dulbecco's Modified Eagle medium, supplemented with 10% (v/v) fetal bovine serum. Short heparin RNA (Invitrogen) was ligated to pSUPER (Oligoengine). To determine the impact of overexpression of the variants, the three different variants were amplified using the PCR process and ligated with pTarget vectors (Promega) using TA cloning.
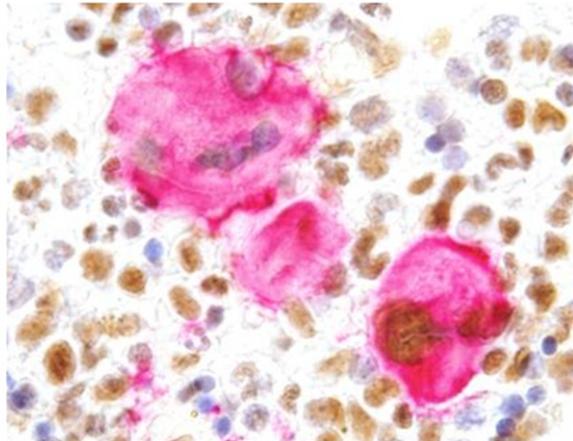
Statistical analysis

The Mann-Whitney *U*-test and Kruskal-Wallis test were used to analyze the RT-PCR and immunohistochemistry data obtained from the human bone marrow samples.
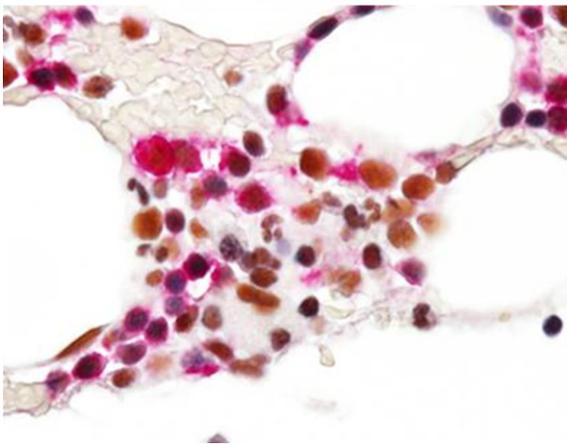
Figure 2. Double immunostaining of ACINUS with cell-lineage markers in the bone marrow ofan RCMD patient.The markers used were CD33 for myeloid cells (A), CD61 for megakaryocytes (B), and CD71 for erythroid cells (C).

Results

ACINUS was strongly expressed in the bone marrow of MDS and AML

The expression of ACINUS in the bone marrow cells ofthe MDS, OL, and *de novo* AML patients was stronger than in the bone marrow cells of the control group (**Figure 1A**). The numbers of ACINUS-positive cells were evaluated by counting the cells in five high-power fields (×400) under a microscope, and the median values were identified in each group. Statistically, the expression of ACINUS was significantly higher in the MDS, OL, and AML groups as compared with that of the control group (**Figure 1B**). The MDS cases were classified into the following three groups: lowrisk (refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS)), intermediaterisk (Refractory cytopenia with multilineage dysplasia (RCMD), Refractory

anemia with excess blasts (RAEB)-1), and highrisk (RAEB-2). Interestingly, the expression of ACINUS was significantly higher in the high-risk and the intermediate-risk groups compared to the low-risk group (**Figure 1C**). These results suggested that ACINUS might have an important role in the regulation of blastic cell proliferation in the pathogenesis of MDS.

Localization of the ACINUS protein in the bone marrow of MDS

To identify which lineage of hematopoietic cells expressed ACINUS in MDS, double immunostaining was performed to localize ACINUS in lineage marker-positive cells. The following lineage markers were used: CD33 for myeloid cells, CD71 for erythroid cellsand CD 61 for megakaryocytes. Almost all CD33-positive cells were also positive for ASINUS (**Figure 2A**). In
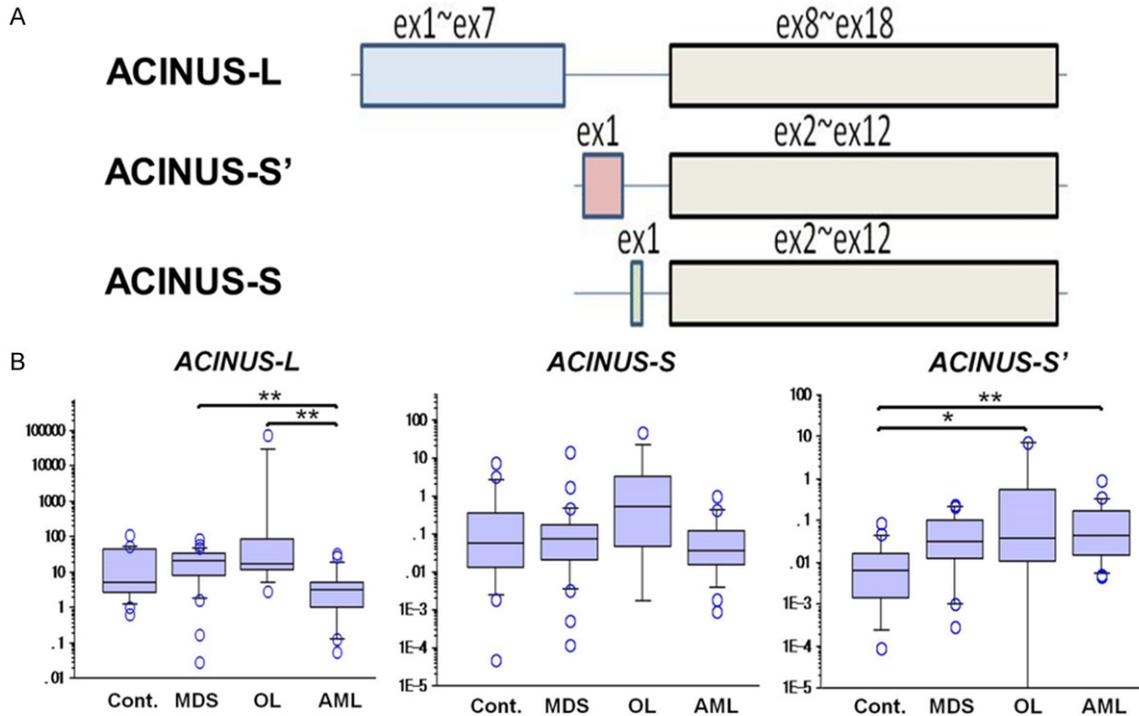
Figure 3. RT-PCR analyses of the expression of *ACINUS* isoforms in the bone marrow. A. Schematic illustration of the splicing variants ACINUS-L, ACINUS-S, and ACINUS-S'. B. Expression analysis of each variant in bone marrow cells from the control, MDS, OL, and *de novo* AML cases. The expression of *ACINUS-L* was significantly different higherin MDS versus AML and OL versus AML, and the expression of ACINUS-S' was significantly different higher in the OLversus control and AML versus control. *P<0.05, **P<0.01.

contrast, few CD61-positive or CD71-positive cells were positive for ACINUS (**Figure 2B**, **2C**).

mRNA expression of ACINUS variants in the bone marrow of MDS

Three variants of the ACINUS protein were identified with the regulation by splicing machinery (**Figure 3A**). The expression of these three variants was examined in the control, MDS, OL, and *de novo* AML samples by quantitative RT-PCR. As shown in **Figure 3B**, *ACINUS-L* was more strongly expressed in the MDS and OL groups than in the *de novo* AML group. Although the expression level of *ACINUS-S* was not significantly different among the groups, the expression of *ACINUS-S'* was significantly upregulated in the bone marrow of the OL and *de novo* AML groups as compared to the controlgroup. Generally, bone marrow cells inOL and AML contain blast cell fractions and have high cell proliferation activity. Thus, these results suggested that *ACINUS-S'* might contribute to cell proliferation of blastic cells in MDS and

AML and that *ACINUS-L* might have a different role in the regulation of cell dynamics in these myeloid neoplasms.

The hematopoietic cells of the MDS and OL groups showed many features of apoptosis as compared with those of the control group and *de novo* AML group. Further studies are necessary to clarify the role of ACINUS-L in the induction of apoptosis in hematopoietic cells of MDS.

Overexpression of ACINUS-S' induced cell proliferation

To evaluate the function of the ACINUS variants, three constructs of each variant were transduced to HEK293T cells. The expression of each variant after transduction was validated by Western blot analyses (**Figure 4A**). As expected, ACINUS-S' induced cell proliferation in the transduced cells as compared with the control cells (**Figure 4B**). Although the transduction of ACINUS-L and ACINUS-S also induced cell proliferation in HEK293T cells, the cell pro-
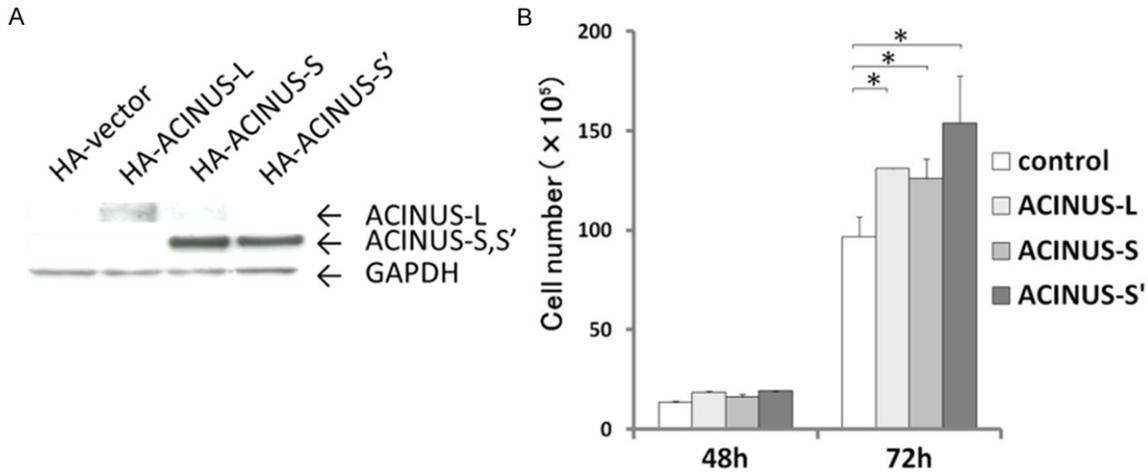
Figure 4. *In vitro* analyses of the effects of transduction of *ACINUS* isoformsinto HEK293T cells. A. Western blot analysis of the expression of ACINUS proteins in the variant-transduced HEK293T cells. B. Overexpression of the *ACINUS* isoforms, especially *ACINUS-S'*, induced cell proliferation in HEK293T cells. *P<0.05.
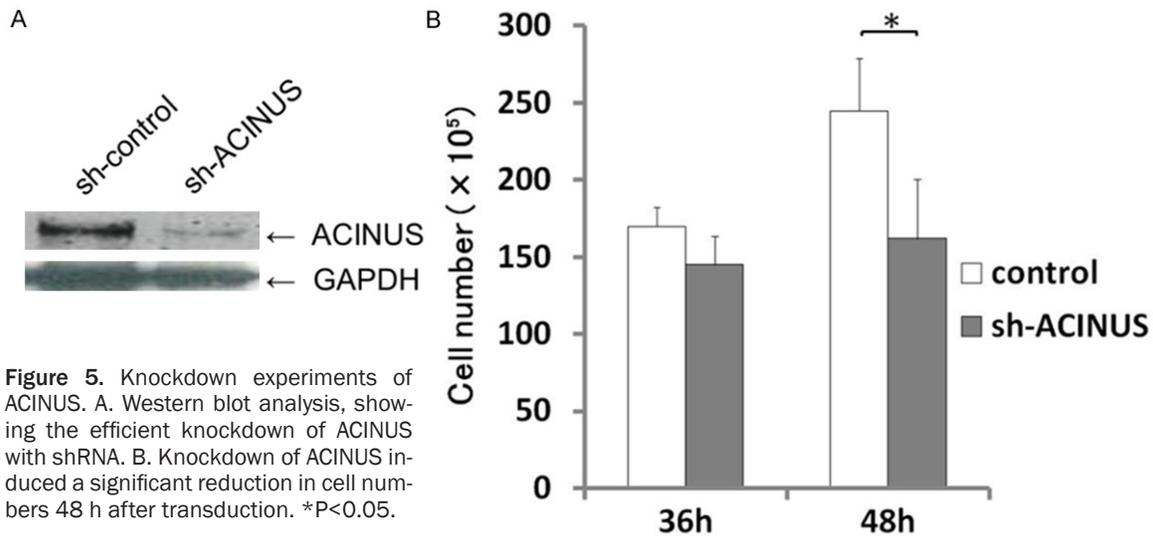

Figure 5. Knockdown experiments of ACINUS. A. Western blot analysis, showing the efficient knockdown of ACINUS with shRNA. B. Knockdown of ACINUS induced a significant reduction in cell numbers 48 h after transduction. *P<0.05.

liferation tended to be greatest with ACINUS-S' transduction. These results were consistent with the *in vivo* data, which suggested that ACINUS-S' seemed to play an important role in cell proliferation in the bone marrow of MDS.

Effects of ACINUS knockdown on cell growth

To further evaluate the function of ACINUS *in vitro*, knockdown experiments were performed using short hairpin RNAs for *ACINUS*. The expression of the ACINUS protein was reduced in the HEK293T cells treated with *sh-ACINUS* (**Figure 5A**). ACINUS knockdown also reduced the total cell number of HEK293T cells 48 h

after the transduction of shRNA. These results suggested that ACINUS may be a key regulator of cell proliferation in MDS.

Discussion

Research has demonstrated the roles of ACINUS in apoptosis, RNA processing, regulation of retinoic acid-responsive (RAR)-dependent splicing, and RAR-dependent transcription [26, 27]. ACINUS has three different isoforms, termed ACINUS-L, ACINUS-S, and ACINUS-S'. Although they have different patterns of subnuclear localization [26], their differential roles in cellular biology are not clear. The present study

indicated that ACINUS-S' appeared to positively regulate the proliferation of hematopoietic cells in the bone marrow of MDS and *de novo* AML and that ACINUS-L had a role in MDS/OL-specific cell dynamics of bone marrow hematopoietic cells, possibly through the induction of apoptosis. As shown by the *in vitro* experiments, overexpression of ACINUS-S' strongly supported the proliferation of cell line cells.

During normal erythroblast differentiation, apoptosis occurs via a mitochondria-related process, which includesthe activation of caspases and proteins, including ACINUS, that are involved in nucleus integrity (lamin B) and chromatin condensation [28]. The ACINUS protein has been shown to be associated with the apoptotic induction of monocytes, resulting from differentiation-associated caspase activation during their differentiation into macrophages [29]. Thus, the physiological induction of apoptotic processes in hematopoietic cells seems to be strongly associated with the functions of ACINUS proteins.

The apoptotic induction of tumor cells associated with ACINUS expression has been demonstrated in prostatic cancer [30, 31]. However, little is known about howthe ACINUS protein regulates cellular dynamics during the process of oncogenesis and/or tumor progression.In the present study, in MDS and *de novo* AML, ACINUS expression was up-regulated, indicating that the ACINUS variants (ACINUS-L and ACINUS-S') might not only contribute to apoptotic induction in myeloid neoplasms but also differentially regulate the proliferation/apoptosis of hematopoietic cells. Although the mechanisms involved are likely complex and need to be clarified in a future study, a therapeutic strategy targeting the function of ACINUS might be effective in controlling the cellular dynamics of hematopoietic cells in bone marrow of MDS and AML patients.

Previous studies have clarified a number of gene mutations in MDS. However, the spectrum of those mutations overlapped largely with that in AML. Thus, MDS-specific mutations have not yet identified. Several groups reported frequent mutations of multiple components of the RNA-splicing machinery in MDS [32-34]. These splicing factor mutations suggested that the pathogenesis of MDS differed from that of *de novo* AML [34]. Little is known about the influence of these mutations on RNA splicing of MDS cells. However, in a newly proposed subclassification of MDS, RNA splicing-related mutations were one important criterion, together with gene mutations relating to epigenetic regulation, DNA-damage responses, transcriptional regulation, and signal transduction [35]. A previous study demonstrated the role of ACINUS in RNA binding and splicing regulation [36]. Thus, the expression profiles of ACINUS in the present study might be associated with specific RNA-splicing mutations in MDS/AML. ACINUS proteins may also have a supplementary role in loss-of-function mutations of RNA splicing-related mutations. A further study is needed to develop a targeting therapeutic strategy for the newly proposed subclassification of MDS based on the regulation of the functions of splicing-related proteins, including ACINUS.

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Disclosure of conflict of interest

None.

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