VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 13 September 2010
Final form accepted: 08 December 2010
Published online: 27 December 2010

Volume 16 (2011) pp 89-100
DOI: 10.2478/s11658-010-0041-1

Short communication

ZFAT IS A CRITICAL MOLECULE FOR CELL SURVIVAL IN MOUSE EMBRYONIC FIBROBLASTS

KEIKO DOI[1,2], TAKAHIRO FUJIMOTO[1,2], MIDORI KOYANAGI[1,2], TOSHIYUKI TSUNODA[1,2], YOKO TANAKA[1, 2], YASUHIRO YOSHIDA[1], YASUO TAKASHIMA[2], MASAHIDE KUROKI[2], TAKEHIKO SASAZUKI[3] and SENJI SHIRASAWA[1,2]*

[1]Department of Cell Biology, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan, [2]Center for Advanced Molecular Medicine, Fukuoka University, Fukuoka 814-0180, Japan, [3]Kyushu University, Fukuoka 812-8581, Japan

Abstract: ZFAT was originally identified as an immune-related transcriptional regulator containing 18 C2H2-type zinc-finger domains and one AT-hook. ZFAT is highly conserved among species and functions as an anti-apoptotic molecule in the lymphoblastic leukemia cell line, MOLT-4. We recently demonstrated that ZFAT is an essential molecule for hematopoietic differentiation in blood islands through the direct regulation of particular transcriptional factors, including *Tal1*, for endothelial cell assembly, and for the branch point formation of capillary-like structures. However, the molecular mechanisms underlying the anti-apoptotic function of ZFAT remain unknown. Here, we report that ZFAT knockdown by small interfering RNA induced apoptosis in mouse embryonic fibroblasts (MEFs). This response had been similarly observed for MOLT-4 cells. To explore the molecular mechanisms for ZFAT in anti-apoptotic function in both MEFs and MOLT-4 cells, microarray expression analysis and quantitative RT-PCR were done. Of interest was that *Bcl-2* and *Il6st* were identified as commonly down-regulated genes by the depletion of ZFAT for both MEFs and MOLT-4 cells. These results suggest that ZFAT is a critical molecule for cell survival in MEFs and MOLT-4 cells at least in part through the regulation of the apoptosis involved in the BCL-2- and IL6st-

* Author for correspondence. e-mail: sshirasa@fukuoka-u.ac.jp; phone: +81-92-801-1011; fax: +81-92-864-3865

Abbreviations used: C2H2 – Cys2-His2; ZFAT – zinc-finger gene in autoimmune thyroid disease susceptibility region

mediated pathways. Further elucidation of the molecular functions for ZFAT might shed light on the cellular programs in the mesoderm-derived cells.

Key words: ZFAT, Transcription factor, MEFs, Apoptosis, Gene expression

INTRODUCTION

Autoimmune thyroid disease (AITD), including Graves' disease and Hashimoto thyroiditis, is caused by an immune response to self-thyroid antigens. It affects 2-5% of the general population [1]. Through linkage and association analyses on a large group of Japanese AITD patients [2, 3], we identified ZFAT (the zinc-finger gene in the AITD susceptibility region) as a susceptibility gene for AITD [3]. The human ZFAT gene encodes a 1,243-amino acid residue protein containing one AT-hook and 18 C2H2 zinc-finger domains. ZFAT is also highly conserved among species from fish to human [4]. The ZFAT protein is expressed in the B and T lymphocytes in mice, and ZFAT regulates the genes involved in immune responses [4]. Furthermore, we previously demonstrated that ZFAT is an anti-apoptotic molecule that is critical for cell survival in human leukemic MOLT-4 cells [5].

Recently, we demonstrated that *Zfat*-deficiency (*Zfat*$^{-/-}$) is lethal for mice by embryonic day (E) 8.5, with a reduction in the number of blood islands and hematopoietic progenitor cells. ZFAT directly regulates the transcription factors including *Tal1*, *Lmo2* and *Gata1* in the blood islands [6]. We also reported that ZFAT is essential for endothelial cell assembly and the branch point formation of capillary-like structures in an angiogenesis model, suggesting that ZFAT is a critical transcriptional regulator for endothelial differentiation [7].

Genetic variants of *ZFAT* were reported to be associated with adult height in the Japanese and Korean populations [8], and with interferon-beta responsiveness in multiple sclerosis in western populations [9]. These reports and our previous findings together suggest the possibility that ZFAT is as a critical regulator not only in immune responses but also in growth, differentiation and apoptosis for the mesoderm-derived and mesenchymal cells.

In this study, to characterize the biological functions for ZFAT in mouse embryonic fibroblasts (MEFs), we investigated the effects of ZFAT depletion through the use of siRNA targeting ZFAT in MEFs, showing that the depletion of ZFAT by siRNA knockdown significantly induced apoptosis in MEFs. Furthermore, we identified the *Bcl-2* and *Il6st* genes as the genes commonly regulated by ZFAT for both MEFs and MOLT-4 cells.

MATERIALS AND METHODS

Preparation of the mouse embryonic fibroblasts, and cell culture

Primary MEFs were isolated from E13-14 embryos of C57BL/6 mice and cultured at 37ºC in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 0.1 mmol/l non-essential amino acids,

50 μmol/l 2-mercaptoethanol and antibiotics. The experiments on the mice were approved by the University Committee on Animal Resources in the animal facility of Fukuoka University.

siRNAs and transfection

The siRNA for ZFAT and scrambled control siRNA were obtained from Invitrogen (Stealth RNAi). The sequences of the ZFAT stealth siRNAs were:

mZFAT-siRNA (for MEFs, mouse) 5'-AUC UGC UGC AGG AUA UUC ACA GCG G-3' and 5'-CCG CUG UGA AUA UCC UGC AGC AGA U-3';
control-siRNA 5'-AUC GAU GUC GGG ACU AUA CAC UCG G-3' and 5'-CCG AGU GUA UAG UCC CGA CAU CGA U-3';
hZFAT-siRNA (for MOLT-4, human) 5'-AAC AUU UAC ACA UAA AGA UGG CCG U-3' and 5'-ACG GCC AUC UUU AUG UGU AAA UGU U-3';
control-siRNA 5'-AAC CGA UUA UAC ACA UAA AGG UCG U-3' and 5'-ACG ACC UUU AUG UGU AUA AUC GGU U-3'.

For each experiment, early passages (2 to 4) MEFs were plated at a density of 5 x 10^5 cells per 6-cm dish. The MEFs were transfected with either mZFAT-siRNA or control-siRNA using the Lipofectamine RNAi MAX reagent (Invitrogen) following the manufacturer's suggested protocol. The MOLT-4 cells were transfected with either hZFAT-siRNA or control-siRNA, as described previously [5].

Western blot analysis

Cells were lysed with a buffer containing 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and a protease inhibitor cocktail (Roche). The total cell lysates were subjected to SDS-PAGE and transferred to Nitrocellulose Transfer Membrane (Whatman). Western blot was done as described previously [6] using anti-ZFAT monoclonal antbody [6] or anti-ERK1 antibody (K-23; Santa Cruz Biotechnology). The signals were detected via enhanced chemiluminescence following the manufacturer's instructions (ECL detection system, Amersham).

Proliferation analysis

The proliferation activity was measured with the tetrazolium compound WST-8 (Cell Counting Kit-8; Dojindo Laboratories). The cells were plated in 48-well tissue culture plates at a density of 1 × 10^4 cells per well in 200 μl of medium, and were transfected with siRNA. After 24, 48 and 72 h, 10 μl of WST-8 solution was added to each well, followed by incubation at 37ºC for 30 min. Absorbance at 450 nm was measured using a microplate reader.

Apoptosis assay

Cells were plated in 48-well tissue culture plates at a density of 1 × 10^4 cells per well in 200 μl of medium and were transfected with siRNA. Caspase-3 and -7 activities were determined at 24 and 48 h using the Caspase-Glo 3/7 assay (Promega) by measuring luminescence as relative light units (RLUs) using a GLOMAX luminometer (Promega).

Expression array analyses and quantitative RT-PCR

Total RNA was extracted from the siRNA-transfected cells using a Qiagen RNeasy kit. The preparation of the biotinylated antisense cRNA, the hybridization to the GeneChip Human Genome U133 Plus 2.0 array (for MOLT-4 cells) or to the GeneChip Mouse Genome 430 2.0 array (for MEFs) (Affymetrix), and the washing, scanning and data processing were performed according to the manufacturers' instructions and as described previously [4]. Output files were then loaded into GeneSpring v11.0 (Agilent Technologies) with per gene normalization to the expression level of the control siRNA-transfected cells. Quantitative RT-PCR was performed using ABI PRISM 7900HT (Applied Biosystems) and Perfect Real Time Support System (TAKARA) [6]. The PCR primer IDs used for each of the 22 genes are listed in Supplementary Tab. 1 at http://dx.doi.org/10.2478/s11658-010-0041-1. Relative quantification for each gene expression was normalized by the expression level of *β-actin* or *Gapdh*. The data is presented as the means ± standard deviations of the means of triplicate samples.

Statistical analysis

The data is presented as the means ± standard deviations of the means of triplicate samples. Statistical analyses were performed with an unpaired Student's t-test. Differences at $P < 0.05$ are considered to be statistically significant.

RESULTS AND DISCUSSION

Apoptosis is induced by the depletion of ZFAT in MEFs

To characterize the biological functions for ZFAT in C57BL/6 MEFs, MEFs were transfected with mZFAT-siRNA or control-siRNA. ZFAT protein expression in MEFs transfected with mZFAT-siRNA was evidently suppressed compared with that in MEFs transfected with control-siRNA (Fig. 1A). As shown in Fig. 1B, depletion of ZFAT by siRNA knockdown significantly induced the reduction in cell proliferation at 48 and 72 h. To determine whether or not apoptosis is involved in the inhibition of cell growth, the activation of caspase-3 and -7 was analyzed. The activities of caspase-3 and -7 in MEFs transfected with ZFAT-siRNA were increased 1.74-fold at 24 h and 1.89-fold at 48 h, compared with those in MEFs transfected with control-siRNA (Fig. 1C), indicating that apoptosis was induced by ZFAT siRNA knockdown and that ZFAT is essential for cell survival for MEFs.

Genes regulated by ZFAT in the functions of apoptosis, cell growth and immune responses

To identify the genes commonly regulated by ZFAT in both MEFs and MOLT-4 cells, we compared the gene expression profiles of MEFs transfected with mZFAT-siRNA or control-siRNA and MOLT-4 cells transfected with hZFAT-siRNA or control-siRNA using microarray expression analysis. A gene was considered to be differentially expressed in ZFAT knockdown cells when the

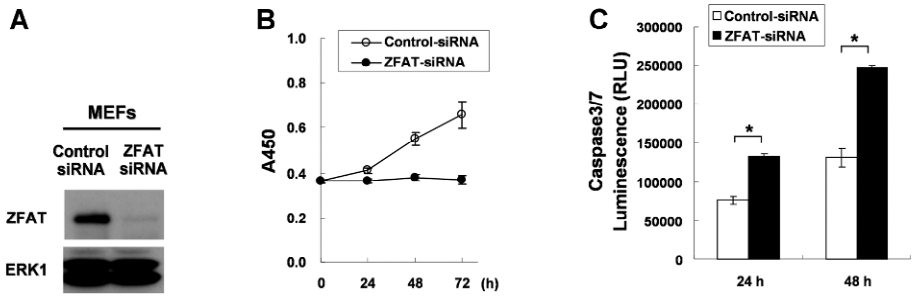

Fig. 1. The depletion of ZFAT by siRNA knockdown induces apoptosis in MEFs. Cells were transfected with mZFAT-siRNA or control-siRNA. A – The effects of siRNA on the levels of ZFAT protein expression. Western blot analysis for ZFAT expression at 24 h after transfection in MEFs transfected with mZFAT-siRNA or control-siRNA. ERK1 was used as a loading control. B – Suppression of cell growth in MEFs due to ZFAT knockdown. The cells were transfected with siRNA, and cell growth was determined at the indicated time points after transfection using the WST-8 colorimetric assay. The data is the average of three replicates ± S.D. C – Caspase-3 and -7 activity in MEFs with ZFAT knockdown. MEFs transfected with mZFAT-siRNA or control-siRNA were plated at 1×10^4 cells per well in 48-well plates. After 24 and 48 h, the caspase-3 and -7 activity was quantified. The activity was measured by cleavage of the fluorescent caspase-3 and -7 substrate Ac-DEVD-pNA. The data is the average of three replicates ± S.D. Statistically significant differences are indicated as $*P < 0.01$.

fold change was greater than or less than 1.25, compared with that of the cells transfected with control-siRNA. Based on these criteria, 131 genes were selected as the genes commonly down-regulated by depletion of ZFAT both in MEFs and in MOLT-4 cells. From the point of view of ZFAT functions in MEFs, the differentially expressed genes involved in cell death were *Bcl-2*, *Gfra1*, *Nek1* and *Perp*; in cellular growth and proliferation, they were *Adam17*, *Akap13*, *Cadm1*, *Depdc6*, *Jag1*, *Mxd1*, *Mid1*, *Nf2*, *Pmp22*, *Prrx1*, *Tmeff2*, *Vegfa* and *Zfhx3*; and in immune responses, they were *Bcl-6*, *Cxcl3*, *Flt1* and *Il6st* (Tabs 1 and 2).

To confirm the microarray gene expression data, the 21 detected differentially expressed genes were analyzed via quantitative RT-PCR. There was a concordant relationship between the data obtained from microarray expression analysis and quantitative RT-PCR (Supplementary Tab. 2). Quantitative RT-PCR analysis showed that the mRNA levels of six of the genes (*Bcl-2*, *Depdc6*, *Flt1*, *Il6st*, *Gfra1* and *Pmp22*) in the ZFAT-siRNA-transfected MEFs and MOLT-4 cells were significantly reduced compared with those in the control-siRNA-transfected MEFs and MOLT-4 cells (fold change compared with each control: *Bcl-2*, -2.3 (MEFs), -2.5 (MOLT-4); *Depdc6*, -2.2, -1.7; *Flt1*, -1.3, -1.4; *Il6st*, -1.4, -1.7; *Gfra1*, -2.0, -1.5; *Pmp22*, -3.0, -1.6, respectively; Fig. 2). Of note is that the *Bcl-2* mRNA levels in the ZFAT-siRNA-transfected MEFs and MOLT-4 cells were reduced to more than 50% of those in MEFs transfected with control-siRNA, suggesting that the reduction in BCL-2 expression may lead to an increase in caspase-3 activity, culminating in apoptosis in MEFs and MOLT-4 cells.

Tab. 1. Down-regulated genes involved in the functions of cell death and cell proliferation by ZFAT-siRNA both in MEFs and in MOLT-4 cells.

Gene symbol (NetAffx)	Gene title (NetAffx)	MEFs_siZFAT		MOLT-4_siZFAT	
		Probe ID	Fold change mZFAT-siRNA/control-siRNA	Probe ID	Fold change hZFAT-siRNA/control-siRNA
Cell death					
Bcl2	B-cell leukemia/lymphoma 2	1437122_at	-2.22	203685_at	-2.12
		1457687_at	-1.74		
		1422938_at	-1.44		
Gfra1	glial cell line derived neurotrophic factor family receptor alpha 1	1450440_at	-3.97	227550_at	-1.62
		1439015_at	-1.81		
Nek1	NIMA (never in mitosis gene a)-related expressed kinase 1, mRNA (cDNA clone MGC:189932 IMAGE:9088119)	1438592_at	-1.94	216213_at	-1.65
		1434267_at	-1.78		
Perp	PERP, TP53 apoptosis effector	1416271_at	-3.00	217744_s_at	-1.50
Cellular growth and proliferation					
Adam17	a disintegrin and metallopeptidase domain 17	1421857_at	-1.97	237897_at	-1.43
		1421858_at	-1.97		
		1421859_at	-2.19		
Akap13	A kinase (PRKA) anchor protein 13	1430185_at	-1.30	232188_at	-1.39
Cadm1	cell adhesion molecule 1	1417377_at	-1.54	209030_s_at	-1.39
		1417376_a_at	-1.35		
Depdc6	DEP domain containing 6	1451348_at	-2.29	218858_at	-2.05
		1428622_at	-2.26		
		1443579_s_at	-1.84		
		1453571_at	-1.35		
Jag1	jagged 1	1421106_at	-1.47	209099_x_at	-1.53
		1434070_at	-1.46		
Mxd1	MAX dimerization protein 1	1434830_at	-2.60	228846_at	-1.36
Mid1	midline 1	1438239_at	-1.30	210694_s_at	-1.39
Nf2	neurofibromatosis 2	1450382_at	-1.58	238618_at	-1.35
		1421820_a_at	-1.47		
Pmp22	peripheral myelin protein 22	1417133_at	-2.91	210139_s_at	-1.32
Prrx1	paired related homeobox 1	1439774_at	-1.66	205991_s_at	-1.33
		1425528_at	-1.41		
		1425526_a_at	-1.33		
		1425527_at	-1.26		

Gene symbol (NetAffx)	Gene title (NetAffx)	MEFs_siZFAT		MOLT-4_siZFAT	
		Probe ID	Fold change mZFAT - siRNA/ control-siRNA	Probe ID	Fold change hZFAT-siRNA/ control-siRNA
Tmeff2	transmembrane protein with EGF-like and two follistatin-like domains 2	1441598_at	-1.74	224321_at	-1.46
Vegfa	vascular endothelial growth factor A	1420909_at	-1.27	210512_s_at	-1.31
				210513_s_at	-1.76
Zfhx3	zinc finger homeobox 3	1453267_at	-1.52	226137_at	-1.47
		1429725_at	-1.45		
		1449947_s_at	-1.39		
		1420650_at	-1.38		
		1420649_at	-1.38		

The mRNA profiling was carried out in MEFs and MOLT-4 cells transfected with ZFAT-siRNA or control-siRNA.

Tab. 2. Down-regulated genes involved in the function of the immune responses by ZFAT-siRNA both in MEFs and in MOLT-4 cells.

Gene symbol (NetAffx)	Gene title (NetAffx)	MEFs_siZFAT		MOLT-4_siZFAT	
		Probe ID	Fold change mZFAT-siRNA/ control-siRNA	Probe ID	Fold change hZFAT-siRNA/ control-siRNA
Immunological disease					
Bcl6	B-cell leukemia/lymphoma 6	1421818_at	-1.25	203140_at	-1.85
Cxcl3	chemokine (C-X-C motif) ligand 3	1438148_at	-1.56	207850_at	-1.61
Flt1	FMS-like tyrosine kinase 1	1451756_at	-1.62	232809_s_at	-1.31
		1454037_a_at	-1.33		
		1440926_at	-1.31		
Il6st	interleukin 6 signal transducer	1421239_at	-1.50	204864_s_at	-1.69
		1460295_s_at	-1.37		
		1452843_at	-1.37		

The mRNA profiling was carried out in MEFs and MOLT-4 cells transfected with ZFAT-siRNA or control-siRNA.

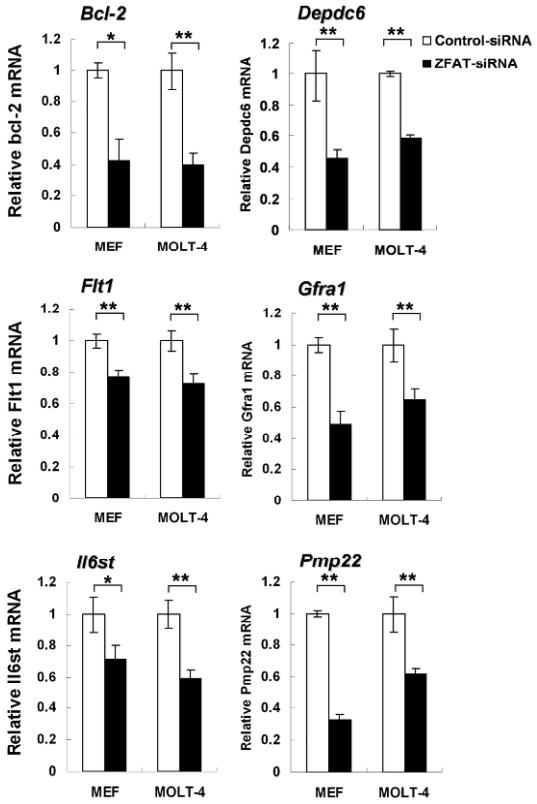

Fig. 2. A reduction in the expression of apoptotic and immune-related genes through the depletion of ZFAT in MEFs and MOLT-4 cells. The mRNA levels of the six genes (*Bcl-2*, *Depdc6*, *Flt1*, *Il6st*, *Gfra1* and *Pmp22*) in MEFs and MOLT-4 cells transfected with ZFAT-siRNA or control-siRNA are shown. Quantitative RT-PCR was performed using RNA from MEFs and MOLT-4 cells transfected with ZFAT-siRNA or control-siRNA. The data is the average of three replicates ± S.D. Statistically significant differences are indicated as $*P < 0.05$, $**P < 0.01$.

In this article, we demonstrated that ZFAT knockdown by small interfering RNA in MEFs also induced apoptosis as with MOLT-4 cells [5]. Furthermore, we showed that particular apoptosis-related genes including *Bcl-2* and *Il6st* were down-regulated by the depletion of ZFAT both in MEFs and in MOLT-4 cells. Together, this suggests that ZFAT critically regulates the expressions of *Bcl-2 and Il6st* (Fig. 3).

ZFAT is highly conserved among ZFAT orthologues from fish to mammalian species [4], and *Zfat*$^{-/-}$ mice die by embryonic day (E) 8.5 [6], indicating that ZFAT plays pivotal roles in mouse development. ZFAT knockdown by small interfering RNA in MEFs induced apoptosis, supporting the hypothesis that ZFAT is essential for cell survival and plays a critical role in mesoderm-derived cells and mesenchymal cells.

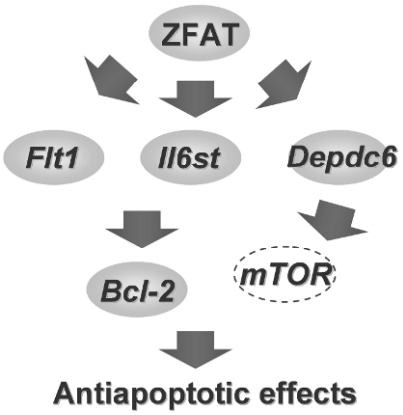

Fig. 3. The molecular mechanisms underlying the anti-apoptotic activity of ZFAT.

BCL-2 is a negative regulator for cellular apoptosis and a major regulator of the intrinsic apoptosis pathways [10]. Our data indicated that the reduced BCL-2 expression might result in an increase in caspase-3 activity in MEFs and MOLT-4 cells transfected with ZFAT-siRNA. On the other hand, *Il6st* (also known as gp130) is a common subunit for the interleukin-6 (IL-6) family of cytokines, which are involved in cell proliferation, differentiation and cell death in various tissues [11]. The mutation of tyrosine 767 in the membrane-proximal 133 amino acid residues of IL6st disrupts STAT3 activation, resulting in apoptosis and the loss of Bcl-2 induction [12]. Depletion of ZFAT in MEFs and MOLT-4 cells induced a reduction in the expressions of *Il6st* and *Bcl-2*, suggesting that IL6st and BCL-2 participate in the ZFAT-mediated anti-apoptosis function.

Depdc6 encodes DEPTOR, which is a novel binding partner for mTOR [13, 14]. Depleting multiple myeloma cells of DEPTOR activates mTORC1 and inhibits PKB phosphorylation, and inhibits proliferation and induces apoptosis [13]. Our data demonstrated that *Depdc6* mRNA was down-regulated by the depletion of ZFAT. The molecular mechanisms underlying the apoptosis induced by the depletion of ZFAT in MEFs and MOLT-4 cells might be involved in DEPTOR- and mTOR-mediated signaling pathways (Fig. 3).

Flt1 (also known as VEGFR1) plays an active role in vascular endothelial growth factor-mediated (VEGF-mediated) autocrine signaling of tumor growth and angiogenesis. Recent studies of Flt1 in several tumor cells suggest that VEGF/Flt1 signaling has autocrine survival activity [15-18]. Treatment with anti-VEGF and anti-Flt1 antibodies inhibits ERK1/2 activation and down-regulates *Bcl-2* expression in the highly malignant neuroblastoma cell line SK-N-BE(2) [19]. Furthermore, reduced endogenous VEGF or VEGFR1 expressions induce apoptosis in MDA-MB-231 and MCF-7 breast cancer cells [20]. Our studies showed that *Vegfa*, *Flt1* and *Bcl-2* were down-regulated by a depletion of ZFAT in MEFs and MOLT-4 cells. One possible mechanism of apoptosis through the depletion of ZFAT might be the reduction of BCL-2 via the VEGF/Flt1 signaling pathway.

In summary, our report demonstrated that ZFAT is a critical regulator for cell survival in MEFs, and that the apoptosis induced by a depletion of ZFAT may be involved in BCL-2-mediated pathways. Further studies are necessary to elucidate the functional roles of the ZFAT-regulated genes identified in this study, and to identify the signaling pathways responsible for the induction of apoptosis through the depletion of ZFAT. Evidence is accumulating that ZFAT is an important factor in contributing to cellular differentiation, development and a variety of immunological diseases. Elucidation of the precise molecular mechanisms for ZFAT will provide important information not only for cellular programs but also for understanding the pathogenic mechanisms of diseases, and might provide specific targets for therapeutic intervention.

Acknowledgements. The authors would like to thank Ms T. Danno for her technical assistance. This study was supported by a grant from the Genome Network Project; a Grant-in-Aid for Scientific Research on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology; and a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of the Science.

REFERENCES

1. Jacobson, E.M. and Tomer, Y. The CD40, CTLA-4, thyroglobulin, TSH receptor, and PTPN22 gene quintet and its contribution to thyroid autoimmunity: back to the future. **J. Autoimmun.** 28 (2007) 85-98.
2. Sakai, K., Shirasawa, S., Ishikawa, N., Ito, K., Tamai, H., Kuma, K., Akamizu, T., Tanimura, M., Furugaki, K., Yamamoto, K. and Sasazuki, T. Identification of susceptibility loci for autoimmune thyroid disease to 5q31-q33 and Hashimoto's thyroiditis to 8q23-q24 by multipoint affected sib-pair linkage analysis in Japanese. **Hum. Mol. Genet.** 10 (2001) 1379-1386.
3. Shirasawa, S., Harada, H., Furugaki, K., Akamizu, T., Ishikawa, N., Ito, K., Tamai, H., Kuma, K., Kubota, S., Hiratani, H., Tsuchiya, T., Baba, I., Ishikawa, M., Tanaka, M., Sakai, K., Aoki, M., Yamamoto, K. and Sasazuki, T. SNPs in the promoter of a B cell-specific antisense transcript, SAS-ZFAT, determine susceptibility to autoimmune thyroid disease. **Hum. Mol. Genet.** 13 (2004) 2221-2231.
4. Koyanagi, M., Nakabayashi, K., Fujimoto, T., Gu, N., Baba, I., Takashima, Y., Doi, K., Harada, H., Kato, N., Sasazuki, T. and Shirasawa, S. ZFAT expression in B and T lymphocytes and identification of ZFAT-regulated genes. **Genomics** 91 (2008) 451-457.
5. Fujimoto, T., Doi, K., Koyanagi, M., Tsunoda, T., Takashima, Y., Yoshida, Y., Sasazuki, T. and Shirasawa, S. ZFAT is an antiapoptotic molecule and critical for cell survival in MOLT-4 cells. **FEBS Lett.** 583 (2009) 568-572.
6. Tsunoda, T., Takashima, Y., Tanaka, Y., Fujimoto, T., Doi, K., Hirose, Y., Koyanagi, M., Yoshida, Y., Okamura, T., Kuroki, M., Sasazuki, T. and Shirasawa, S. Immune-related zinc finger gene ZFAT is an essential

transcriptional regulator for hematopoietic differentiation in blood islands. **Proc. Natl. Acad. Sci. USA** 107 (2010) 14199-14204.
7. Yoshida, Y., Tsunoda, T., Takashima, Y., Fujimoto, T., Doi, K., Sasazuki, T., Kuroki, M., Iwasaki, A. and Shirasawa, S. ZFAT is essential for endothelial cell assembly and the branch point formation of capillary-like structures in an angiogenesis model. **Cell. Mol. Biol. Lett.** 15 (2010) 541-550.
8. Takeuchi, F., Nabika, T., Isono, M., Katsuya, T., Sugiyama, T., Yamaguchi, S., Kobayashi, S., Yamori, Y., Ogihara, T. and Kato, N. Evaluation of genetic loci influencing adult height in the Japanese population. **J. Hum. Genet.** 54 (2009) 749-752.
9. Comabella, M., Craig, D.W., Morcillo-Suarez, C., Rio, J., Navarro, A., Fernandez, M., Martin, R. and Montalban, X. Genome-wide scan of 500,000 single-nucleotide polymorphisms among responders and nonresponders to interferon beta therapy in multiple sclerosis. **Arch. Neurol.** 66 (2009) 972-978.
10. Chao, D.T. and Korsmeyer, S.J. BCL-2 family: regulators of cell death. **Annu. Rev. Immunol.** 16 (1998) 395-419.
11. Hirano, T., Nakajima, K. and Hibi, M. Signaling mechanisms through gp130: a model of the cytokine system. **Cytokine Growth Factor Rev.** 8 (1997) 241-252.
12. Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K. and Hirano, T. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. **Immunity** 5 (1996) 449-460.
13. Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S. and Sabatini, D.M. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. **Cell** 137 (2009) 873-886.
14. Proud, C.G. Dynamic balancing: DEPTOR tips the scales. **J. Mol. Cell. Biol.** 1(2009) 61-63.
15. Wang, E.S., Teruya-Feldstein, J., Wu, Y., Zhu, Z., Hicklin, D.J. and Moore, M.A. Targeting autocrine and paracrine VEGF receptor pathways inhibits human lymphoma xenografts in vivo. **Blood** 104 (2004) 2893-2902.
16. Santos, S.C. and Dias, S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. **Blood** 103 (2004) 3883-3889.
17. Lee, Y.K., Bone, N.D., Strege, A.K., Shanafelt, T.D., Jelinek, D.F. and Kay, N.E. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. **Blood** 104 (2004) 788-794.
18. Bellamy, W.T., Richter, L., Sirjani, D., Roxas, C., Glinsmann-Gibson, B., Frutiger, Y., Grogan, T.M. and List, A.F. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. **Blood** 97 (2001) 1427-1434.

19. Das, B., Yeger, H., Tsuchida, R., Torkin, R., Gee, M.F., Thorner, P.S., Shibuya, M., Malkin, D. and Baruchel, S. A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop interacts with hypoxia-inducible factor-1alpha through mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. **Cancer Res.** 65 (2005) 7267-7275.
20. Lee, T.H., Seng, S., Sekine, M., Hinton, C., Fu, Y., Avraham, H.K. and Avraham, S. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. **PLoS Med.** 4 (2007) e186.