

Short communication

**ZFAT IS ESSENTIAL FOR ENDOTHELIAL CELL ASSEMBLY
AND THE BRANCH POINT FORMATION OF CAPILLARY-LIKE
STRUCTURES IN AN ANGIOGENESIS MODEL**YASUHIRO YOSHIDA^{1,2,§}, TOSHIYUKI TSUNODA^{1,3,§},
YASUO TAKASHIMA^{1,3}, TAKAHIRO FUJIMOTO^{1,3}, KEIKO DOI^{1,3},
TAKEHIKO SASAZUKI⁴, MASAHIDE KUROKI³, AKINORI IWASAKI²
and SENJI SHIRASAWA^{1,3,*}¹Department of Cell Biology, Faculty of Medicine, Fukuoka University,
Fukuoka 814-0180, Japan, ²Department of Thoracic, Endocrine and Pediatric
Surgery, Faculty of Medicine, Fukuoka University, Fukuoka 814-0180, Japan,
³Center for Advanced Molecular Medicine, Fukuoka University, Fukuoka
814-0180, Japan, ⁴Kyushu University, Fukuoka 812-8582, Japan

Abstract: *ZFAT*, originally identified as a susceptibility gene for autoimmune thyroid disease, encodes a transcriptional regulator with one AT-hook and 18 C₂H₂-type zinc-finger domains. It is highly conserved among species. Here, we demonstrate that *ZFAT* is clearly expressed in human umbilical vein endothelial cells (HUVECs). Furthermore, we show that endothelial cell assembly and the branch point formation of capillary-like structures in HUVECs is impaired by the reduction of *ZFAT* expression through the use of *ZFAT*-miRNAs, whereas differences in cell proliferation or apoptotic features were not observed after the reduction in *ZFAT* expression. These results suggest that *ZFAT* may have critical roles in the capillary-like network formation that is involved in vascular remodeling. Elucidating the *ZFAT*-mediated transcriptional network will lead to a better understanding of the molecular mechanisms of angiogenesis.

Key words: *ZFAT*, HUVECs, Endothelial cell assembly, Branch point formation, Vascular remodeling, Angiogenesis

[§]These authors contributed equally to this work.

* Author for correspondence. e-mail: sshirasa@fukuoka-u.ac.jp

Abbreviations used: C₂H₂ – Cys2-His2; HUVECs – human umbilical vein endothelial cells; miRNA – microRNA; *ZFAT* – zinc-finger gene in autoimmune thyroid disease susceptibility region

INTRODUCTION

The human *ZFAT* gene was originally identified as a susceptibility gene for autoimmune thyroid disease [1]. *ZFAT* encodes a transcriptional regulator containing one AT-hook and 18 C₂H₂-type zinc-finger domains, and it is highly conserved across species from fish to human [2]. We previously found that human *ZFAT* mRNA was strongly expressed in the placenta, kidneys, spleen, testes and peripheral blood mononuclear cells [1], and that mouse *ZFAT* protein was abundantly expressed in the thymus, spleen and lymph nodes, while its expression was restricted to the B and T lymphocytes in the peripheral blood [2]. Furthermore, cell-based approaches revealed that *ZFAT* specifically regulates immune-related genes in lymphocytes and functions as an anti-apoptotic molecule in the human acute T-lymphoblastic leukemia cell line, MOLT-4 [2, 3]. *ZFAT* mRNA was ubiquitously expressed in the tissues, but with lower expression levels in the non-immune-related tissues than in the immune-related tissues [1]. This suggests that *ZFAT* might also play physiological roles in non-immune-related cells. Recently, genetic variants in *ZFAT* were reported to be associated with the interferon-beta responsiveness in multiple sclerosis, an inflammatory autoimmune disease of the central nervous system [4], and with adult height in the Japanese and Korean populations [5, 6]. These findings suggest that *ZFAT* might have critical roles in non-immune-related cells involved in human diseases or altered physiological phenotypes.

Angiogenesis is the cellular mechanism by which the primitive vasculature is remodeled into a mature vascular bed comprising arteries, capillary networks and veins [7]. Vascular remodeling is an active process of structural alteration that involves changes in cellular processes, including cell growth, cell death and cell penetration into the extracellular matrix [8]. The mechanisms of vascular remodeling, in which endothelial cells play pivotal roles, include penetration via the sprouting and branching of vessels into avascular regions. This is observed under physiological and/or pathological conditions, such as wound healing, neovascularization in tumors, inflammation, autoimmune diseases and obesity [9-18]. In this study, we found evident expression of *ZFAT* in human umbilical vein endothelial cells (HUVECs), and evaluated the physiological roles for *ZFAT* in the angiogenic responses of HUVECs.

MATERIALS AND METHODS

Cell culture

The HEK293FT cells were cultured according to the supplier's instructions (Invitrogen), and the MOLT-4 cells were cultured as described previously [3]. The HUVECs (HUVEC-2, BD Biosciences) were cultured using Medium 200S (KURABO) supplemented with 2% (v/v) FBS, 10 ng/ml hEGF, 1 µg/ml hydrocortisone, 3 ng/ml hFGF-B and 10 µg/ml heparin. A three-dimensional culture with HUVECs was conducted with the cells from passage 3. For the

western blot, HUVECs and HUVEC-transfectants grown in a two-dimensional culture were expanded to a 75-cm² dish and then harvested in RIPA buffer containing a protein inhibitor cocktail. The culture medium was exchanged every 48 h.

Western blotting

Western blotting using anti-ZFAT or anti-actin antibodies was performed as previously described [2, 3]. The quantitative analysis of the western blot for ZFAT was done using the integration value of each blot (n = 3) through the measurement module (BZ-H1M, Keyence). The actin intensity was used as a control standard, and the relative intensity of the signal (ZFAT/Actin) was normalized by the signal-intensity in HUVECs as 1.0.

HUVEC-transfectants, immunofluorescence labeling and confocal microscopy

HUVEC-transfectants expressing the control miRNA and ZFAT-miRNAs were established using Gateway Technology with Clonase II (Invitrogen) and the BLOCK-iT HiPerform Lentiviral PolIII miR RNAi Expression System with EmGFP (Invitrogen). ZFAT-specific pre-miRNAs were designed using the BLOCK-iT RNAi Designer (Invitrogen), available at <https://rnaidesigner.invitrogen.com/rnaiexpress/>. The oligonucleotides used were: control miRNA top, 5'-TGC TGA ATT CAG CAC GAT AAT GCA GAG TTT TGG CCA CTG ACT GAC TCT GCA TTC GTG CTG AAT T-3' and control miRNA bottom, 5'-CCT GAA TTC AGC ACG AAT GCA GAG TCA GTC AGT GGC CAA AAC TCT GCA TTA TCG TGC TGA ATT C-3'; ZFAT-miRNA-1 top, 5'-TGC TGA ATA GTC GCA GGC GAA CTT CTG TTT TGG CCA CTG ACT GAC AGA AGT TCC TGC GAC TATT-3' and ZFAT-miRNA-1 bottom, 5'-CCT GAA TAG TCG CAG GAA CTT CTG TCA GTC AGT GGC CAA AAC AGA AGT TCG CCT GCG ACT ATTC-3'; ZFAT-miRNA-2 top, 5'-TGC TGA ACT GGT GAA GAC CAA GGC CTG TTT TGG CCA CTG ACT GAC AGG CCT TGC TTC ACC AGT T-3' and ZFAT-miRNA-2 bottom, 5'-CCT GAA CTG GTG AAG CAA GGC CTG TCA GTC AGT GGC CAA AAC AGG CCT TGG TCT TCA CCA GTT C-3'. Double-stranded oligonucleotides were subcloned into the pDONR221 vector and were re-subcloned into the pLenti6.4/R4R2/V5-DEST vector with pENTR5'/CMVp, which was designated as pLV. The pLV vectors were transfected into HEK293FT cells with Lipofectamin LTX according to the supplier's instructions (Invitrogen), and the lentiviruses expressing the ZFAT-specific miRNAs or the control miRNA were produced. After removing the cellular debris via filtration, the lentiviruses were infected into HUVECs for 6 h. The medium was replaced with Medium 200S, and the cells were cultured for 42 h. The HUVECs infected with lentiviruses were further selected using blasticidin, and the expression of green fluorescent protein (GFP) was confirmed using the Biorvo BZ-9000 fluorescence microscope system (Keyence). Nearly 100%

transfection efficiency was achieved in the HUVECs with the control miRNA, ZFAT-miRNA-1 and ZFAT-miRNA-2.

The immunofluorescence experiment was done as described previously [20].

Cell growth assay

1×10^4 HUVECs were cultured in a 96-well microplate (NUNC) at 37°C for 6, 12, 24 and 36 h in Medium 200S, and the absorbance for formazan solutions was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay-based Cell Counting kit-8 (Dojindo) according to the supplier's instructions. The relative growth rate was calculated from a comparison of the absorbance at a given time with the absorbance at 6 h (used as a control).

Quantitative analyses of cell assembly and branch point formation

Forty $\mu\text{l}/\text{cm}^2$ of Matrigel (BD Biosciences) was added to each well of an 8-well Lab-Tek Chamber Slide (NUNC) or to a 35-mm dish and was spread evenly. 2.4×10^4 cells/ cm^2 of HUVECs were spread on the Matrigel with Medium 200S containing 2% Matrigel and vascular endothelial growth factor (VEGF; 5 ng/ml, Humanzyme) as described [19]. They were incubated at 37°C for 24 h. Photographs were taken using Biorevo (Keyence). The total length of the capillary-like structures, the number of segments (where a segment was defined as that part of a capillary-like structure from one branch point to the next branch point), and the average segment length were measured in 3 areas in a duplicated chamber of each cell line using the measurement module in BZ-H1M (Keyence).

Statistical analysis

The data was presented as the means \pm standard deviation from triplicate western blot and endothelial cell differentiation assays and quadruplicate cell growth assays. The 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

ZFAT expression in HUVECs and HUVEC-derived transfectants expressing ZFAT-miRNA

To assess the ZFAT protein expression level in HUVECs, ZFAT expression in HUVECs was compared with that in MOLT-4 cells, in which ZFAT plays critical roles in anti-apoptotic function [3]. The level of ZFAT expression in HUVECs seemed to be greater than that in MOLT-4 cells, and ZFAT was detected as a 180-kDa protein, as expected (Fig. 1A). When all these findings are considered together, they suggest physiological roles for ZFAT in HUVECs. To reveal the physiological functions of ZFAT in HUVECs, HUVEC-transfectants expressing ZFAT-miRNA-1 or ZFAT-miRNA-2 were established through the use of lentiviruses. The ZFAT protein expression of the HUVEC-transfectants with ZFAT-miRNA-1 was decreased by 2.06-fold compared to that

of the HUVECs, with a statistical significance of $P = 0.013$ (Fig. 1B). The ZFAT expression of the HUVEC-transfectants with ZFAT-miRNA-2 was respectively decreased by 4.89- and 2.37-fold, compared to those in HUVECs and HUVEC-transfectants with ZFAT-miRNA-1 ($P = 0.006$ and 0.0031 , respectively; Fig. 1B). These results indicate that ZFAT expression was significantly inhibited by ZFAT-miRNA-1 or ZFAT-miRNA-2, and that the efficiency of ZFAT-knockdown in the HUVEC-transfectants with ZFAT-miRNA-2 was greater than that in the HUVEC-transfectants with ZFAT-miRNA-1.

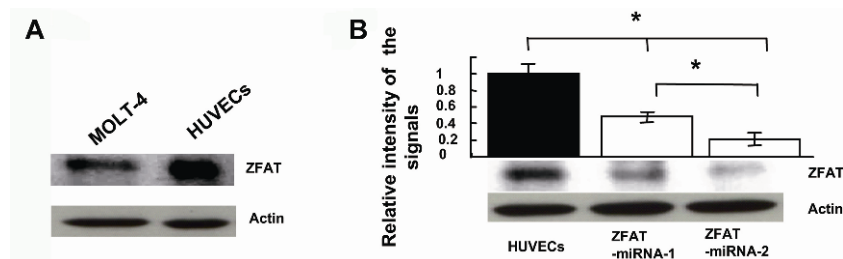


Fig. 1. ZFAT protein expression in HUVECs. A – ZFAT expression in HUVECs and MOLT-4 cells. B – Western blotting of ZFAT from HUVECs and the HUVEC-transfectants with ZFAT-miRNA-1 or ZFAT-miRNA-2, and the quantitative analyses of the ZFAT expression levels. The white bars are the relative intensities of the signals for each cell line with ZFAT-miRNAs, normalized by the signal for HUVECs (the black bar). $*P < 0.05$.

The reduction of ZFAT expression does not affect cell growth rate in HUVECs

To address whether ZFAT-knockdown affects the cell growth of HUVECs, a cell growth assay was done on HUVECs and the HUVEC-transfectants expressing ZFAT-miRNA-1 or ZFAT-miRNA-2. The relative growth rates of the cells grown for 12, 24 and 36 h compared to that for 6 h were as follows (Fig. 2): HUVECs (1.11-, 1.27- and 1.61-fold increases, respectively), HUVEC-transfectants with ZFAT-miRNA-1 (1.05-, 1.26- and 1.68-fold increases,

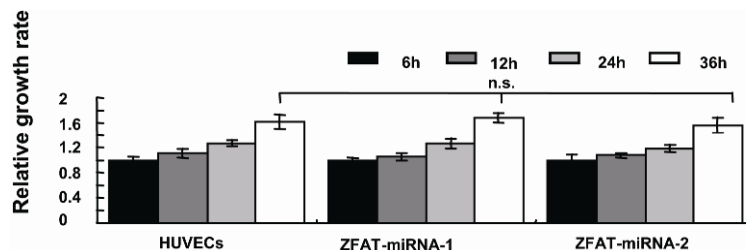


Fig. 2. The reduction of ZFAT expression does not affect the cell growth rate in HUVECs. The white, light gray and dark gray bars represent the relative growth rates at each time point compared to that at 6 h (black bar). n.s., no significant difference.

respectively) and HUVEC-transfectants with ZFAT-miRNA-2 (1.08-, 1.18- and 1.56-fold increases, respectively), indicating the relative growth rates at 36 h for HUVECs, HUVEC-transfectants with ZFAT-miRNA-1 and HUVEC-transfectants with ZFAT-miRNA-2 were not significantly different ($P > 0.05$). All these results indicated that the cell growth rate for HUVECs was not inhibited by the reduction in ZFAT expression, suggesting that the ZFAT in HUVECs is not involved in proliferation or apoptosis, which are essential components of vascular remodeling.

ZFAT is essential for endothelial cell assembly and branch point formation in HUVECs

To address the possibility that ZFAT plays critical roles in the vascular remodeling, morphological and quantitative analyses were performed on the formation of capillary-like structures in the HUVEC-transfectants. The HUVEC-transfectants with the control miRNA manifested assembly into capillary-like structures and the branch point formation of interconnected capillary-like structures, whereas the capillary-like network formation in the HUVEC-transfectants with ZFAT-miRNA-1 seemed to be impaired compared to that of the HUVEC-transfectants with the control miRNA at 12 h (Fig. 3A, upper panels) and 24 h (Fig. 3A, middle and lower panels). Of great interest was that the capillary-like network formations of the HUVEC-transfectants with ZFAT-miRNA-2 were dramatically impaired compared to those of the HUVEC-transfectants with the control miRNA or ZFAT-miRNA-1, suggesting that the capillary-like network formation for HUVECs can be impaired depending on the reduction level of ZFAT protein.

To more precisely determine the effect induced by the reduction of ZFAT expression in HUVECs, the total length of the capillary-like structures was quantified. The total length of the capillary-like structures in the HUVEC-transfectants with ZFAT-miRNA-1 did not change (1.00-fold) compared to that of the HUVEC-transfectants with the control miRNA (Fig. 3B). On the other hand, the total length of the capillary-like structures of the HUVECs with ZFAT-miRNA-2 was significantly decreased (2.84-fold) compared to that of the HUVEC-transfectants with the control miRNA ($P = 0.0005$). All these results suggest that endothelial cell assembly for HUVECs was impaired depending on the reduction level of ZFAT (Fig. 3B).

As no significant difference was observed in the total length of the capillary-like structures between the HUVEC-transfectants with control miRNA and ZFAT-miRNA-1 (Fig. 3B), we further quantified the number of the segments and the segment lengths between these transfectants. These parameters were not examined for the HUVEC-transfectants with ZFAT-miRNA-2, as the HUVEC-transfectants with ZFAT-miRNA-2 showed few capillary-like structures (Fig. 3A). The number of the segments of the HUVEC-transfectants with ZFAT-miRNA-1 decreased 1.50-fold compared to that of the HUVEC-transfectants

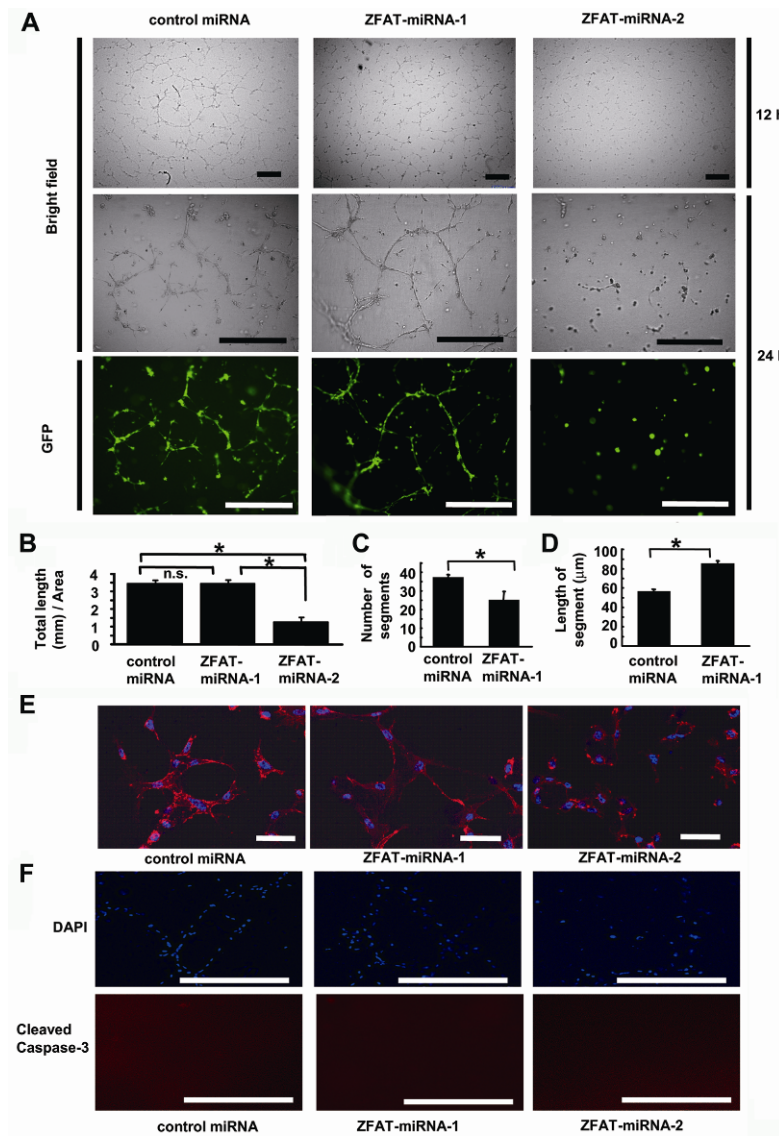


Fig. 3. ZFAT is essential for endothelial cell assembly and branch point formation in HUVECs. A – Bright field images (upper and middle panels) and GFP images (lower panels) of the HUVEC-transfectants expressing the control miRNA, ZFAT-miRNA-1 and ZFAT-miRNA-2 at 12 h (upper panels) and 24 h (middle and lower panels). Bar = 500 μm. B – The total length of capillary-like structures (μm) per area; C – the number of segments in the capillary-like structures; and D – the average segment length of the capillary-like structures in the HUVEC-transfectants with the control miRNA and ZFAT-miRNA-1. * $P < 0.05$. n.s., no significant difference. E – The staining patterns of DAPI (blue) and phalloidin (red) in the HUVEC-transfectants. Bar = 50 μm. F – The staining pattern of DAPI (upper) and antibodies to the cleaved caspase-3 (lower) in the HUVEC-transfectants. DAPI, blue; cleaved caspase-3, red. Bar = 500 μm.

with the control miRNA ($P = 0.008$), indicating impaired branch point formation in the capillary-like structures caused by the reduction in ZFAT expression (Fig. 3C). Furthermore, the average segment length of the capillary-like structures in the HUVEC-transfectants with ZFAT-miRNA-1 was significantly increased (1.52-fold) compared to that of the HUVEC-transfectants with the control miRNA ($P = 0.0002$), indicating that impaired branch point formation due to the reduction of ZFAT expression culminated in the increase in the length of each segment in the capillary-like structure (Fig. 3D).

To further elucidate the cell assembly and cell viability, cell-to-cell contact was examined. The HUVEC-transfectants with the control miRNA formed highly branched cell-to-cell networks via cellular protrusion with each other, whereas the HUVEC-transfectants with ZFAT-miRNA-1 formed a less branched network, with more elongated cellular protrusions, compared to that of the HUVEC-transfectants with the control miRNA (Fig. 3E). Notably, the HUVEC-transfectants with ZFAT-miRNA-2 did not contact with each other (Fig. 3E). On the other hand, apoptotic responses including DNA fragmentation or the signals for the cleaved caspase-3 were not detected in the HUVEC-transfectants with the control miRNA or ZFAT-miRNAs (Fig. 3E and F).

In conclusion, we found evident ZFAT expression in HUVECs and addressed the angiogenesis-related physiological functions for ZFAT, demonstrating that the cell assembly and branch point formation for HUVECs are impaired by the reduction of ZFAT expression. These results suggested that ZFAT may play various kinds of physiological roles, in addition to apoptosis [2, 3], depending on the cell types and environmental conditions. This study describes a novel and important aspect of ZFAT function in vascular remodeling that does not affect the viability of endothelial cells, and that might lead to a better understanding of the molecular mechanisms of angiogenesis.

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