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Transcription of CLDND1 is Regulated Mainly by the Competitive Action of MZF1 and SP1 that Binds to the Enhancer of the Promoter Region

Akiho Shima, Hiroshi Matsuoka, Takahiro Hamashima, Alice Yamaoka, Yutaro Koga, and Akihiro Michihara*

Laboratory of Genomic Function and Pathophysiology, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan
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Increased permeability of vascular endothelial cells in the brain is an underlying cause of stroke, which is associated with high mortality rates worldwide. Vascular permeability is regulated by tight junctions (TJs) formed by claudin family and occludin proteins. In particular, increased vascular permeability is associated with decreased claudin domain-containing 1 (CLDND1) expression, which belongs to the TJs family. We previously reported that myeloid zinc finger 1 (MZF1) acts as an activator of *CLDND1* expression by binding to its first intron. Several transcription factors regulate transcription by acting on the promoter regions of target genes. However, transcription factors acting on the promoter of *CLDND1* are not completely elucidated. Thus, we focused on the promoter region of human *CLDND1* to identify factors that could regulate its transcription. Reporter analysis of *CLDND1* promoter region revealed an enhancer in the -742/-734 region with MZF1 and specificity protein 1 (SP1) binding sites. Chromatin immunoprecipitation assays confirmed that both MZF1 and SP1 could bind to *CLDND1* enhancer region. MZF1 overexpression significantly increased *CLDND1* expression, whereas overexpression of SP1 had no effect. Moreover, the identified enhancer region exhibited stronger transcriptional and binding capacity than the first intron. Thus, *CLDND1* expression is more strongly regulated by competitive action of MZF1 and SP1 binding to the promoter-enhancer region than the first intron silencer region. These results provide novel insights for the development of potential therapies and preventive strategies for stroke in the future.

Key words claudin, myeloid zinc finger 1, specificity protein 1, tight junctions, stroke, transcriptional regulation

INTRODUCTION

The claudin family includes major membrane proteins that form tight junctions (TJs) in the epithelium and endothelium.[1] Twenty-seven members of the claudin family have been identified in humans, and all contain four transmembrane regions with amino and carboxy termini facing the cytoplasm.[2,3] Each claudin member exhibits different tissue distribution and transcriptional regulation.[4,5] Claudin domain-containing 1 (CLDND1), also known as Claudin-25 (CLDN25), is homologous to this family and known to localize to TJs and the cytoplasm when exogenously expressed.[2] TJs regulate vascular permeability, ion homeostasis, and paracellular diffusion through the claudin family.[6] Deficiency or abnormal expression of some claudins has been reported to be associated with serious pathophysiological consequences.[6] Increased vascular permeability in vascular endothelial cells may cause angioedema and stroke.[7] Stroke accounts for most cerebrovascular diseases and is broadly divided into three types, namely, stroke, subarachnoid hemorrhage, and intracerebral hemorrhage. Hypertension exerts the strongest effect on the development of stroke.[8] In addition, degeneration, necrosis, and small aneurysm formation in the small arterioles of the brain are the major causes of vascular rupture and thrombosis.[7] Hypertension and increased vascular permeability are, thus, important risk factors for the onset of stroke. CLDND1 is strongly expressed in the brain and liver, and is particularly detected in the endothelial cells of the murine cerebellum and at TJs of human brain endothelial cells (HBECs).[9,10] *CLDND1* expression was downregulated in a mouse model of cerebellar hemorrhage, and the knockdown of *CLDND1* expression in HBECs increased vascular permeability.[10] Downregulated *CLDND1* expression increases vascular permeability and may be associated with an exacerbated pathological condition of cerebral hemorrhage. We have previously reported that cholesterol and the transcription factor myeloid zinc finger 1 (MZF1) are involved in the transcriptional regulation of human *CLDND1*.[11,12] MZF1 binds to the silencer region present in the first intron of human *CLDND1* and increases CLDND1 expression at the mRNA and protein level.[12] In general, many genes encoding eukaryotic proteins have transcriptional regulatory sequences that are located upstream of the transcription start site and are regulated by multiple transcription factors.[13,14]

Therefore, in this study, we focused on the promoter region of human *CLDND1* and attempted to elucidate the transcriptional regulation mechanism of human *CLDND1*.

*To whom correspondence should be addressed. e-mail: mitihara@fukuyama-u.ac.jp

Table 1. Primers Used in This Study

Primer	Sequence
Luciferase reporter cloning	
pCLDND1-FW(-1017)	5′- GCATACGCGTATCAGTTTCACCCACTCAGG -3′
pCLDND1-FW(-827)	5′- GTCACGCGTTCAGCTTTCTCTGTCGGTTAC -3′
pCLDND1-FW(-778)	5′- CATACGCGTGTCCCAGGCCACGTTAATAG -3′
pCLDND1-FW(-734)	5′- GTCACGCGTTCCTGTCCATGAATTTCTGTG -3′
pCLDND1-FW(-678)	5′- GAAACGCGTTTGCCCCTGTGCCACACATC -3′
pCLDND1-FW(-660)	5′- ATCTGCGAGCTCCAATGCTGTCCATGACCTTATCTGCTTG -3′
pCLDND1-FW(-762)	5′- GCGACGCGTCTCATGTTATTGTTAAGATG -3′
pCLDND1-FW(-742)	5′- GCGACGCGTTCCCTGCTTCCTGTCCATG-3′
pCLDND1-RV(+192)	5′- AGCTGTCGACTCTAGCTCAGACCACAGCAC -3′
Promoter-sense	5′- TAAGATGTCCCTGCTTCCTGTC-3′
Promoter-antisense	5′- GACAGGAAGCAGGGACATCTTA -3′
Intron-sense	5′- GGATGAAGGGGGGATTTTTT -3′
Intron-antisense	5′- AAAAAATCCCCCCTTCATCC -3′
ChIP	
promoter - ChIP-FW	5′- CCTCTGGTCCTTGAGTCAGC-3′
promoter - ChIP-RV	5′- CAGATAAGGTCATGGACAGC-3′
Intron - ChIP-FW	5′- CAGTGCTGGGACCCTTTAAG -3′
Intron -ChIP-RV	5′- GACCGCACCAAACACCTTAC -3′
qRT-PCR	
rtCLDND1-FW	5′- CTAACTGAGCAGTTCATGGAG -3′
rtCLDND1-RV	5′- TAAGCTTCGGCAAATGCAAG -3′
rtMZF1-FW	5′- CCCGGAGATGGGTCACAGTC -3′
rtMZF1-RV	5′- GCATAGTCCTAGGAGGTGTC -3′
rtSP1-FW	5′- CAGCACAGGCAGTAGCAGCAG -3′
rtSP1-RV	5′- GGAGTTGTTGCTGTTCTCATTGG -3′
rt18SrRNA-FW	5′- CGATAACGAACGAGACTCTGG -3′
rt18SrRNA-RV	5′- TAGGGTAGGCACACGCTGAGC -3′

MATERIALS AND METHODS

Cell Culture Immortalized HBECs were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit HaEmek, Israel) and 100 μg/mL penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 5% CO_2 and 37°C.

Luciferase Reporter Constructs The reporter plasmids were engineered to contain a fragment of the human *CLDND1* promoter region from -1017 to +192 encompassing the transcription start site (GenBank accession no. NM_001040181). Deletion fragments were amplified by PCR (primers shown in Table 1) and cloned into the MluI/XhoI sites of luciferase-expressing vector pGVB2 (Nippon Gene, Tokyo, Japan). The luciferase reporter plasmids of promoter site (×3) and intron site (×3) were constructed as follows. Synthetic oligonucleotides corresponding to the sense and antisense MZF1 binding site (Promoter site-sense and Promoter site-antisense, Intron site-sense and Intron site-antisense, Table 1) were phosphorylated with T4 DNA polynucleotide kinase (Takara Bio, Shiga, Japan), mixed and annealed. The resulting double-stranded oligonucleotide was cloned into the SmaI site of the luciferase-expressing reporter vector pGVP2 (Nippon Gene) containing an SV40 promoter. Then, the plasmid clone containing the three tandem copies of the MZF1 binding site was selected by sequencing of the resulting recombinant DNA clones. The plasmids were purified using the QIAGEN Plasmid Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

Transfection and Luciferase Activity Assay Transfection was performed according to methods described previously.[12] Briefly, cells were seeded at 0.5×10^5 cells/well in 24-well plates containing DMEM with 10% FBS on the day prior to transfection. The cells in each well were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) for 15 h with a mixture of 200 ng of luciferase reporter constructs and 200 ng of β-galactosidase reporter plasmid, which was used to normalize the luciferase activity. For overexpression of *MZF1* and *SP1*, 100 ng of a MZF1-expressing vector (pMZF1 or pCl-MZF1), 100 ng of a SP1-expressing vector (pSP1), or an empty vector (pSG5 or pCl-neo) for control were co-transfected with the luciferase reporter constructs. The cells were grown for additional 31 h in fresh media. Luciferase activity was determined using the PicaGene kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's protocol, and measured with a Luminescencer-PSN AB-2200 (Atto Co., Tokyo, Japan).

Chromatin Immunoprecipitation (ChIP) Assay HBECs were seeded at 4×10^5 cells/well in 6-well plates containing DMEM with 10% FBS. After 9 h at 37°C and 5% CO2, the cells were transfected for 15 h with a mixture of luciferase reporter construct (–1017/+192; 500 ng) and pMZF1

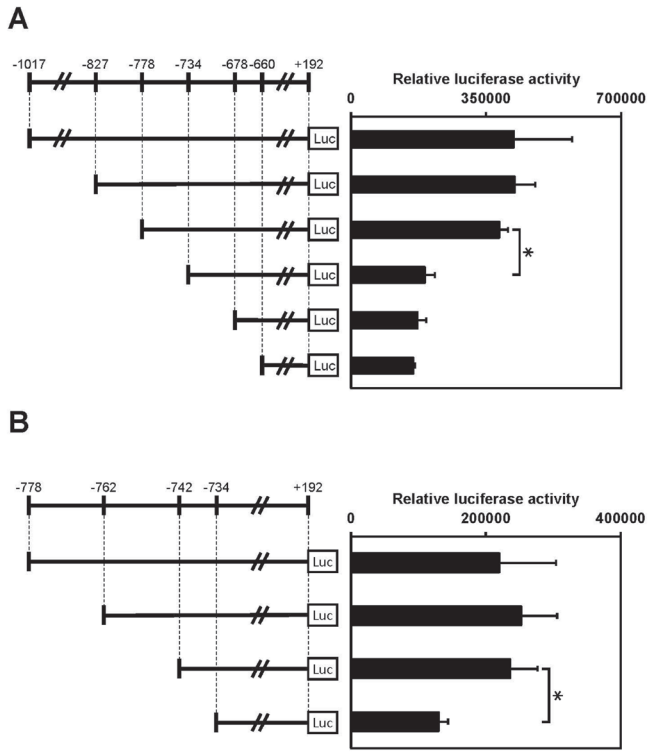

Fig. 1. Transcriptional Activity of the Promoter Region of Human *CLDND1*

Human brain endothelial cells (HBECs) were transfected with luciferase vectors carrying stepwise deletion mutants of *CLDND1* promoter region and evaluated for reporter activity. (**A**) Deletion mutants of -1017 to -660 regions from the region spanning-1017 to +192. (**B**) Deletion mutants of -778 to -734 regions from the region spanning -778 to +192. Data are expressed as mean ± standard error (n = 3), *$p < 0.05$.

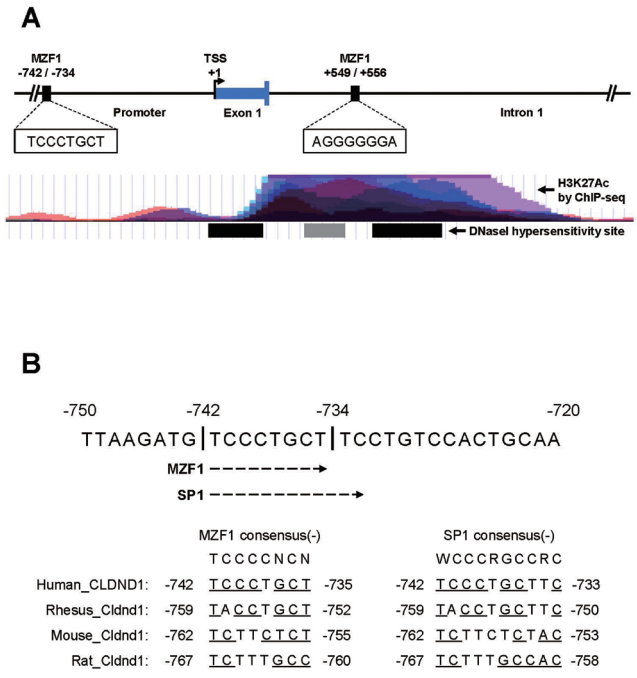

Fig. 2. MZF1- and SP1-binding Sites in the Human *CLDND1* Promoter Region

(**A**) The results of the DNaseI susceptibility test (sensitivity: black boxes > grey box) and the chromatin immunoprecipitation sequencing (ChIP-seq) assay for H3K27Ac (each color represents a different cell type; the stronger the binding to the transcription factor, darker is the color) are shown integrated in the ENCODE project data in the UCSC Genome Browser. The transcription start point is +1 and the estimated MZF1-binding sites in the first intron and promoter region are shown. (**B**) Sequence of human *CLDND1* promoter region -750 to -720. Alignment of the putative MZF1- and SP1-binding sites on *CLDND1* in humans, rhesus monkeys, mice, and rats. The sequences of the putative response elements are shown, and the conserved region of the binding consensus sequence is underlined.

or pSP1 (500 ng). The cells were then cultured for additional 48 h in fresh media. The ChIP assay was conducted following the manufacturer's instructions for the OneDay ChIP kit (Diagenode, Liège, Belgium). The reactions were carried out overnight at 4°C, with mixing, upon addition of 1 µg of either anti-MZF1 or anti-SP1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), or normal nonimmunized IgG as a negative control. The antibody-protein-DNA complex was enriched by incubation with unblocked protein A beads (Diagenode) for 1 h at 4°C, with mixing, and the bound DNA was purified following the manufacturer's instructions. Sample DNA was amplified by PCR (primers shown in Table 1). The relative binding intensity was calculated by fixed quantitative PCR using a SYBR Green Real-Time PCR master-mix (Toyobo, Osaka, Japan) in a LightCycler real-time PCR system (Roche, Penzberg, Germany).

Overexpression and quantitative RT-PCR (qRT-PCR) Transfection was performed according to methods described previously.[12] Briefly, HBECs were seeded at 2×10^5 cells/well in 12-well plates containing DMEM and transfected for 15 h with a MZF1-expressing vector (pCl-MZF1; 500 ng) or 500 ng of pSP1 or an empty vector (pCl-neo or pSG5). The cells were then grown for another 72 h in fresh media and lysed. Total RNA was prepared using ISOGEN (Nippon Gene) according to the manufacturer's protocol and reverse-transcribed for 90 min at 37°C in using 200 U Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific). The cDNA was directly amplified using a Roche Light Cycler in 12 µL reaction volume containing SYBR Green Real-Time PCR master mix (Toyobo) and 1 µM primers. The primers used to amplify the selected gene are shown in Table 1. Gene expression was quantified using the Light Cycler software (Roche) as the second derivative maximum of the curve.

Statistical Analysis All experiments were performed at least three times. Data is expressed as the mean ± standard deviation unless otherwise specified. Error bars represent the mean ± standard error. Comparisons of two groups were performed using the Student's *t*-test. Values were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

To assess the responsiveness of the *CLDND1* promoter region, a luciferase reporter assay was performed with plasmids carrying various deletions. The reporter assay based on the -1017 to +192 sequence revealed that deletion of the -778 to -734 region resulted in a significant decrease of approximately 0.5-fold ($p < 0.05$) in promoter responsiveness. Thus, this specific region was found to serve as an enhancer (Fig. 1A). To further investigate it, we sequentially deleted a small portion within the -778/-734 region and found a significant decrease of approximately 0.6-fold ($p < 0.05$) in pro-

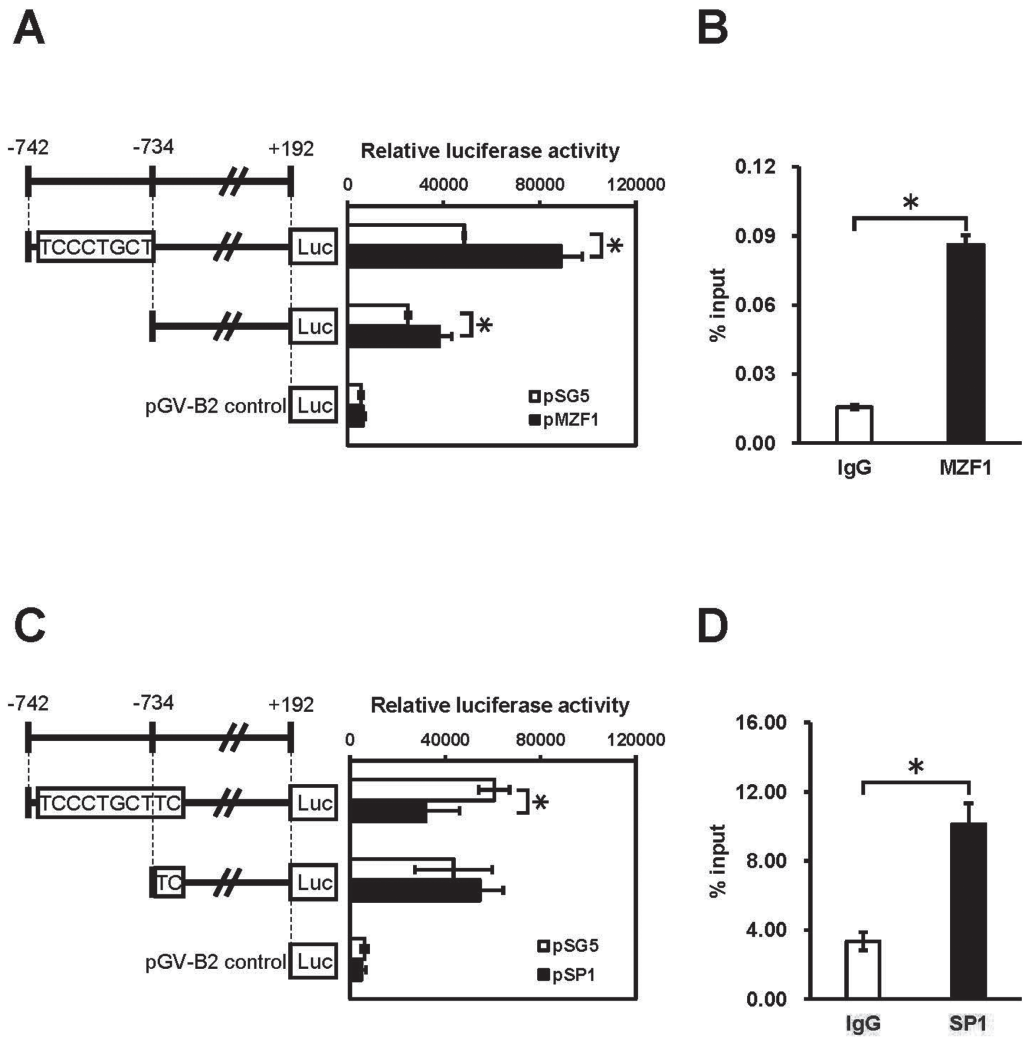

Fig. 3. Evaluation of MZF1 and SP1 Responsiveness to Enhancer Sequences in the *CLDND1* Promoter Region

(**A**,**C**) Assessment of MZF1 and SP1 responsiveness to enhancer regions. Human brain endothelial cells (HBECs) were co-transfected with a luciferase reporter vector ligated from -742 to +192 regions or -734 to +192 regions with a MZF1-expressing vector (pMZF1) (**A**) or a SP1-expressing vector (pSP1) (**C**). Empty vectors (pGV-B2 and pSG5) were used as controls. The responsiveness of each transcription factor was evaluated by a reporter assay. Data are expressed as mean ± standard error (n = 3), *$p < 0.05$. (**B**,**D**) Assessment of MZF1 and SP1 binding to enhancer regions. HBECs were co-transfected with a plasmid carrying the human *CLDND1* promoter region (-1017 to +192) containing the enhancer with a pMZF1 (**B**) or pSP1 (**D**) vector. After 48 h, the binding intensity of each transcription factor was evaluated by a chromatin immunoprecipitation (ChIP) assay. Anti-MZF1, anti-SP1, and nonimmune IgG antibodies were used for immunoprecipitation. DNA occupancy levels are indicated as percentage (%) of input. Data are expressed as mean ± standard error (n = 6), *$p < 0.05$.

moter responsiveness compared with that observed before the deletion (Fig. 1B). Data from the ENCODE project in the University of California Santa Cruz (http://genome.ucsc.edu/ENCODE/) were used to analyze the enhancer-740/-734 region in detail. DNase I sensitivity test revealed low sensitivity. However, results of ChIP-seq for acetylated histones (H3K27Ac) in multiple cultured human cells showed low histone peaks in human lymphocytes (Fig. 2A). The chromatin structure formed by the binding of histones to DNA loosed by histone acetylation, making it easier for transcription factors to bind to DNA.[15] Furthermore, acetylation of histone H3K27 was detected by the enhancer of the active gene, suggesting that the -742 to -734 region is an enhancer and is involved in the transcription of *CLDND1*.

We investigated the transcription factors that could bind to the enhancer region (-742 to -734) by performing an *in silico* analysis using TFBIND software,[16] and found a highly homologous SP1-binding region in the SP1-binding sequence. The SP1 consensus sequence is similar to the binding sequence of the MZF1 transcription factor, suggesting that MZF1 may also bind to this region. Comparison of each consensus sequence with the binding sites in humans, rhesus monkeys, mice, and rats revealed their high homology, as these elements were highly conserved in multiple species (Fig. 2B).

To evaluate the responsiveness of MZF1 and SP1 to the enhancer regions, a luciferase assay was performed. HBECs were co-transfected with a luciferase reporter vector (-742 to +192 and -734 to +192), an empty vector (pSG5), MZF1-expressing vector (pMZF1), or a SP1-expressing vector (pSP1). The responsiveness of -742 to +192 during MZF1 overexpression significantly increased by approximately 2-fold ($p < 0.05$) compared with that of pSG5. Furthermore, the responsiveness of -734 to +192 during MZF1 overexpression was also significantly higher ($p < 0.05$) than the control

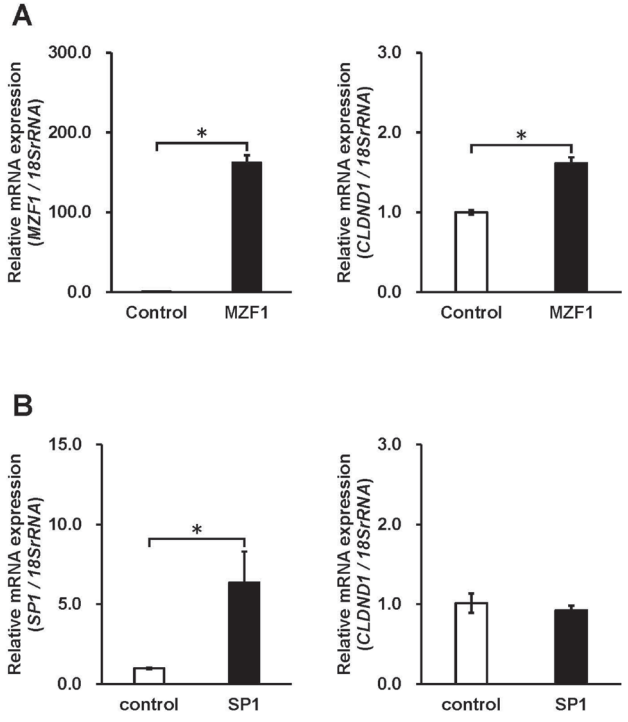

Fig. 4. Human *CLDND1* Expression Following Overexpression of MZF1 and SP1

(**A**) Human brain endothelial cells (HBECs) were transfected with an empty vector (pCI-neo) or an MZF1-expressing vector (pCI-MZF1) for 72 h. (**B**) HBECs were transfected with an empty vector (pSG5) or an SP1-expressing vector (pSP1) for 72 h. The expression of each mRNA was analyzed by quantitative RT-PCR and normalized to that of *18S* rRNA. Data are expressed as mean ±standard error (n = 5), *$p < 0.05$.

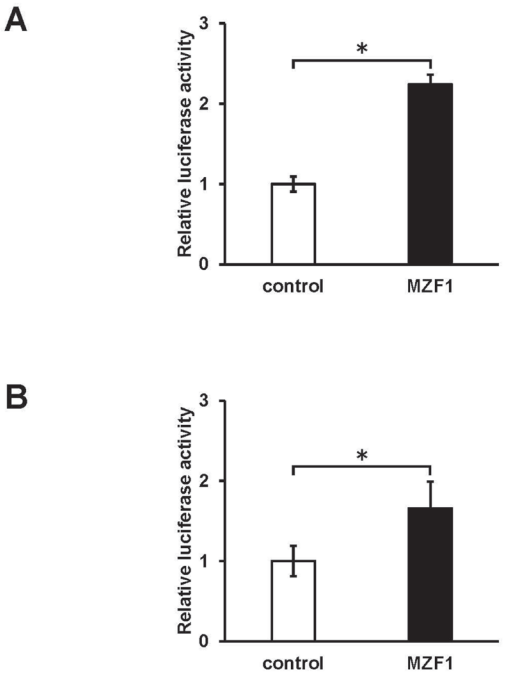

Fig. 5. Comparison of MZF1-binding Capacity to the Promoter and First Intron Sites

Human brain endothelial cells (HBECs) were co-transfected with an empty vector or MZF1-expressing vector with a luciferase reporter plasmid containing the MZF1-binding sequence of the promoter site (**A**) or the first intron site (**B**) of *CLDND1*. The luciferase reporter plasmid contained three MZF1-binding sites to directly increase the responsiveness. Data are expressed as mean ±standard error (n = 4-5), *$p < 0.05$.

vector. In contrast, the responsiveness of -742 to +192 following SP1 overexpression was significantly lower (0.5-fold) than that of pSG5, and the responsiveness of-734 to +192 during SP1 overexpression showed no significant change (Fig. 3A, C). Next, the binding ability of MZF1 and SP1 to the enhancer region was evaluated by a ChIP assay. HBECs overexpressing MZF1 or SP1 were cross-linked with transcription factor proteins and genomic DNA using formaldehyde, and immunoprecipitation was carried out using anti-MZF1 or anti-SP1 antibodies. The samples precipitated with MZF1 and SP1 antibodies showed a 5.5-fold and 3.0-fold higher binding potential, respectively, than the sample treated with the negative control IgG antibody (Fig. 3B, D). These results suggest that MZF1 binds to the enhancer region and activates transcription, whereas the binding of SP1 to the enhancer region suppresses the transcription.

A previous study reported that overexpression of MZF1 increases protein levels of MZF1 and CLDND1.[12] However, the effect of SP1 overexpression on expression of *CLDND1* has not been investigated. Therefore, we performed qRT-PCR according to previously described methods to evaluate the effect of MZF1 and SP1 on human *CLDND1* expression. After 72 h of HBEC transfection with MZF1- or SP1-expressing vector, the total RNA was adjusted and the mRNA expression of *MZF1*, *SP1*, and *CLDND1* was evaluated. The expression of *CLDND1* significantly increased (1.6-fold) with the increase in the expression of *MZF1* (160-fold) compared with that observed in the presence of the empty vector, as previously reported (Fig. 4A).[12] In contrast, no significant change was observed in *CLDND1* expression despite the significant increase (6.4-fold) in *SP1* expression (Fig. 4B). Thus, MZF1 can activate the transcription of *CLDND1* by binding to its enhancer region. In contrast to our expectation, although SP1 binds to the enhancer region, it may not affect *CLDND1* transcription. Therefore, it is possible that there is a part where transcription is promoted by SP1 and the inhibitory effect is offset.

A reporter assay was performed to determine whether MZF1 could bind more strongly to the previously reported intron site[12] or the newly identified promoter site. The reporter assay was performed using a luciferase vector carrying three direct repeats of each region. The results showed a significant increase of approximately 2.2-fold and 1.7-fold in responsiveness at the promoter and first intron sites, respectively, during MZF1 overexpression compared with that of an empty vector (pSG5; Fig. 5A and 5B). Overall, the promoter site showed a significant increase in responsiveness (1.4-fold) as compared with the intron site, suggesting that the promoter site is more strongly involved in transcriptional regulation (data not shown). Furthermore, the MZF1-binding ability at the intron and promoter site was 2.8-fold and 5.5-fold higher than that of the IgG,[12] respectively, suggesting that MZF1 strongly acts at the promoter site.

Epidemiological studies have reported that low cholester-

ol increases the risk of intracerebral hemorrhage[17,18] and high cholesterol increases the risk of developing cerebral infarction.[19,20] However, lowering cholesterol levels has a suppressive effect on the transcription of *CLDND1*, thereby increasing the risk of stroke.[11,12] Hence, a mechanism that can modulate CLDND1 expression regardless of cholesterol levels may pave the way for the development of therapeutic and prevention strategies for stroke. The previously reported MZF1 binding site in the first intron of *CLDND1* is present in the silencer region,[12] which is usually considered a region where repressors bind to suppresses transcription. However, as we previously reported, MZF1 can bind to the silencer region and act as an activator. In the present study, the experimental data suggested the presence of an enhancer region in the promoter of *CLDND1*, in which MZF1 acts as an activator and SP1 as a competitor. Based on the results of ChIP assay and luciferase assay, we consider that MZF1 acts strongly as an activator as a result of the competition between MZF1 and SP1 for the enhancer region in *CLDND1* promoter. Based on these results, it is reasonable to speculate that CLDND1 expression can be increased by lowering SP1 and increasing MZF1 expression, resulting in reduced vascular permeability, which may be useful for the treatment and prevention of stroke.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Lal-Nag M, Morin PJ. The claudins. *Genome Biol.*, **10**, 235 (2009).
2) Mineta K, Yamamoto Y, Yamazaki Y, Tanaka H, Tada Y, Saito K, Tamura A, Igarashi M, Endo T, Takeuchi K, Tsukita S. Predicted expansion of the claudin multigene family. *FEBS Lett.*, **585**, 606–612 (2011).
3) Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc. Natl. Acad. Sci. USA*, **96**, 511–516 (1999).
4) Günzel D, Yu ASL. Claudins and the modulation of tight junction permeability. *Physiol. Rev.*, **93**, 525–569 (2013).
5) Khan N, Asif AR. Transcriptional regulators of claudins in epithelial tight junctions. *Mediators Inflamm.* (2015).
6) Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.*, **2**, 285–293 (2001).
7) Hademenos GJ, Massoud TF. Biophysical mechanisms of stroke. *Stroke*, **28**, 2067–2077 (1997).
8) MacMahon S, Peto R, Collins R, Godwin J, MacMahon S, Cutler J, Sorlie P, Abbott R, Collins R, Neaton J, Abbott R, Dyer A, Stamler J. Blood pressure, stroke, and coronary heart disease. Part 1, prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. *Lancet*, **335**, 765–774 (1990).
9) Matsuoka H, Shima A, Uda A, Ezaki H, Michihara A. The retinoic acid receptor-related orphan receptor a positively regulates tight junction protein claudin domain-containing 1 mRNA expression in human brain endothelial cells. *J. Biochem.*, **161**, 441–450 (2017).
10) Ohnishi M, Ochiai H, Matsuoka K, Akagi M, Nakayama Y, Shima A, Uda A, Matsuoka H, Kamishikiryo J, Michihara A, Inoue A. Claudin domain containing 1 contributing to endothelial cell adhesion decreases in presence of cerebellar hemorrhage. *J. Neurosci. Res.*, **95**, 2051–2058 (2017).
11) Shima A, Matsuoka H, Miya K, Michihara A. Lovastatin Suppresses the Transcriptional Regulation of CLDND1 in Human Hepatoma Cells. *BPB Reports*, **3**, 113–118 (2020).
12) Shima A, Matsuoka H, Yamaoka A, Michihara A. Transcription of CLDND1 in human brain endothelial cells is regulated by the myeloid zinc finger 1. *Clin. Exp. Pharmacol. Physiol.* (2020). doi.org/10.1111/1440-1681.13416
13) Serfling E, Jasin M, Schaffner W. Enhancers and eukaryotic gene transcription. *Trends Genet.*, **1**, 224–230 (1985).
14) Dynan WS, Tjian R. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature*, **316**, 774–778 (1985).
15) Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.*, **12**, 599–606 (1998).
16) Tsunoda T, Takagi T. Estimating transcription factor bindability on DNA. *Bioinformatics*, **15**, 622–630 (1999).
17) Valappil AV, Chaudhary NV, Praveenkumar R, Gopalakrishnan B, Girija AS. Low cholesterol as a risk factor for primary intracerebral hemorrhage: A case-control study. *Ann. Indian Acad. Neurol.*, **15**, 19–22 (2012).
18) Kim MK, Han K, Kim HS, Park YM, Kwon HS, Yoon KH, Lee SH. Cholesterol variability and the risk of mortality, myocardial infarction, and stroke: A nationwide population-based study. *Eur. Heart J.*, **38**, 3560–3566 (2017).
19) Patel A. Cholesterol, coronary heart disease, and stroke in the Asia Pacific region. *Int. J. Epidemiol.*, **32**, 563–572 (2003).
20) Leppälä JM, Virtamo J, Fogelholm R, Albanes D, Heinonen OP. Different Risk Factors for Different Stroke Subtypes. *Stroke*, **30**, 2535–2540 (1999).