***Zscan10* is dispensable for maintenance of pluripotency in mouse embryonic stem cells**

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Abstract

Zinc finger and SCAN domain-containing 10 (*Zscan10*, also known as *Zfp206*) encodes a transcription factor that has been reported to be involved in the maintenance of pluripotency in mouse embryonic stem (ES) cells. Here we generated inducible knockout ES cells for *Zscan10* using the Cre-*loxP* system and analyzed its function. We succeeded in establishing *Zscan10*-null ES cells and confirmed their pluripotency by the generation of chimeric embryos. Our results clearly indicate that *Zscan10* is dispensable for the ability of self-renewal and differentiation in ES cells.

Keywords

Zscan10, Pluripotency, Embryonic stem cells

Introduction

Embryonic stem (ES) cells are pluripotent stem cells derived from pre-implantation embryos[1,2]. ES cells possess a specific transcriptional factor network downstream of the integrated external signals, and the activity of this network enables to maintain their pluripotency and self-renewability[3]. Zinc finger and SCAN domain-containing 10 (*Zscan10*, also known as *Zfp206*) encodes a transcription factor and is regarded as an important member of the pluripotency-associated transcription factor network. *Zscan10* is highly expressed in ES cells and down-regulated upon differentiation[4,5]. The expression of *Zscan10* is directly regulated by the key pluripotency-associated transcription factors Oct3/4 and Sox2[6], and *Zscan10* binds to the enhancer region of *Oct3/4* with Oct3/4 and Sox2[7], suggesting its tight integration to the pluripotency-associated transcription factor network. Single cell analysis suggested that *Zscan10* is a component of the feedback regulation of *Oct3/4*[8]. Moreover, the overexpression of *Zscan10* stabilizes self-renewal and prevents differentiation whereas its knock-down by shRNA causes increase in spontaneous differentiation[4,5], suggesting its functional involvement in the maintenance of pluripotency. In addition, it was also reported that *Zscan10* might act as a transcriptional activator of the 2-cell-stage specific transcripts, such as *Zscan4* and *Tcstv1*, because their expression levels are decreased in *Zscan10* knocked-down ES cells[5]. In contrast, it was recently reported that *Zscan10* knockout mice can develop to adult organisms although they have some defects such as weight reduce or eye defect[9], suggesting that its function for the maintenance of the pluripotent cell population in early developmental process is either dispensable or masked by the maternal transcripts. In the previous reports that applied shRNA-mediated knock-down strategy for the loss of function experiments, 10-20% of total *Zscan10* transcripts still remained[4,5]. Thus, even though they indicated that *Zscan10* supports pluripotency in ES cells, it is still unclear whether the function of *Zscan10* is necessary for the maintenance of pluripotency in ES cells. To settle the argument, especially whether *Zscan10* is the key regulator of 2-cell-stage specific genes and pluripotency-associated genes, we established *Zscan10* inducible-knockout ES cell lines using the Cre-*loxP* system. By this strategy, we found that *Zscan10*-null ES cells continue self-renewal while keeping the expression of pluripotency-associated transcription factors. We also confirmed the pluripotency of the *Zscan10*-null ES cells by the generation of chimeric embryos. These

results suggest that the function of *Zscan10* is dispensable for the pluripotency-associated transcription factor network in ES cells.

Materials and methods

Cell culture

EB5 ES cells (derived from E14tg2a) and their descendants were cultured without feeder cells in Glasgow minimal essential medium (GMEM; Sigma) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol, $1\times$ nonessential amino acids, and 1,000 U/ml of leukemia inhibitory factor (LIF) on gelatin-coated dishes.

Plasmid construction

For the generation of *Zscan10* KO vector, genomic DNA fragments for the 5' and 3' homology arms (Chr17: 23606039+23608324 and 23610845+23613905 in GRCm38/mm10) and the floxed region containing exon 6 (Chr17: 23608325+23610844 in GRCm38/mm10) were amplified from EB5 genomic DNA using the primer pairs shown in Table1 with KOD FX Neo (TOYOBO). The 5' arm was digested with *Cla*I and *Spe*I, 3' arm was digested with *Spe*I and *Not*I and cloned into *Cla*I-*Not*I of pBR-blue II to generate pBR-*Zscan10*-5'+3'. The floxed region was digested with *Spe*I and cloned into *Nhe*I of ploxP-BNA, resulting in ploxP-*Zscan10*. Next, *Spe*I fragment of ploxP-*Zscan10* was inserted into *Spe*I of pBR-*Zscan10*-5'+3'. Finally, to generate p*Zscan10*-floxKO plasmid, the Frt-SAIREsneopA-PGKpactkpA-Frt cassette was introduced into the *Bam*HI site of pBR-*Zscan10*-5'+flox+3' using adaptor. The PiggyBac vector pPBCAG-MerCreMer-IH was constructed based on pGG131[10] and pCAG-EGFP-IZ is described in elsewhere [11].

Generation of inducible *Zscan10* knockout ES cells

p*Zscan10*-floxKO plasmid was linearized by *Cla*I at the end of the 5' arm and introduced into EB5 ES cells by electroporation. After the selection with 200 μ g/ml of G418 and 1.5 μ g/ml of puromycin for 9 days, the colonies were picked, expanded and genotyped by PCR for the homologous recombination events at the 5' and 3' arms. Subsequent transfection with the *FLPe* expression vector followed by the selection with 1.5 μ g/ml of gancyclovir removed the cassette flanked by the Frt sites to generate *Zscan10*^{fl/+} ES cells. This step was repeated for the second allele to create homozygous *Zscan10*^{fl/fl} ES cells. To enable efficient conditional deletion, we stably transfected cells with an expression vector for the tamoxifen-inducible form of *Cre* (*MerCreMer*). Null

ES cell lines were obtained by clonal expansion of *Zscan10*^{fl/fl} ES cells with *MerCreMer* pre-treated with 200 ng/ml of Tamoxifen for 4 days followed by isolation of clonal colonies.

Western blotting

Western blotting was performed with anti-ZNF206 mouse monoclonal antibody (AF4020, R&D systems) and anti-Cdk2 (sc-163, Santa Cruz Biotechnology). The signal was detected with LAS-4000 (Fujifilm).

Real-time RT-PCR

Total RNAs were prepared by QuickGene RNA cultured cell HC kit (KURABO) and synthesized in 20 µR of the reaction mixture containing oligo-dT primers using a ReverTra Ace first strand synthesis kit (TOYOBO). Real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO) using CFX384 Real-Time System (Bio-Rad). Sequences of primer pairs are listed in Table2.

RNA-seq

Total RNAs were prepared by QuickGene RNA cultured cell HC kit (KURABO) following the manufacturer's instruction and treated with Ribo-Zero Gold Sample Prep Kit (Illumina) for deplete ribosomal RNA. Quality of total RNA was confirmed by BioAnalyzer 2100 (RIN>9). Library DNA were prepared according the Illumina Truseq protocol and sequenced by HiSeq 1500. Resulting reads were aligned to the mouse genome (mm10) using TopHat after trimming of adaptor sequences with trim_galore and quality check with FastQC. Gene expression level measured as FPKM was determined with Cuffdiff and differential expression was analysed with EdgeR.

Production of chimeric embryos

To visualize the in vivo contribution of *Zscan10*-null ES cells, the constitutive Egfp expression vector (pCAG-Egfp-IZ) was transfected by electroporation. ES cells with stable expression of EGFP were dissociated and injected into a C57BL6 blastocyst by microinjection. To evaluate chimeric contribution ability, embryos were collected at 13.5 dpc and fluorescent signals were detected by using fluorescence microscopy. All

animal experiments confirmed to our Guidelines for the Care and Use of Laboratory animals and were approved by the Institutional Committee for Laboratory Animal Experimentation (RIKEN Kobe Institute).

Results

Generation of inducible knockout ES cells of *Zscan10*

Zscan10 consists of 6 exons and encodes the protein containing 782 amino acids[12,13]. In order to analyze the precise function of *Zscan10* in the self-renewal and differentiation of ES cells, we designed an inducible knockout vector that removes exon 6 by the Cre-lox system because the entire Zinc-finger domain for DNA binding is coded by this exon. The targeting vector containing 2.3 kb of 5' arm and 3.1 kb of 3' arm flanking the loxP sites as well as the SA-IRES-neo- pA: PGK-pacΔtk-pA cassette flanked by the *Frt* sites was linearized by *ClaI* at the end of the 5' arm and introduced into EB5 ES cells (Fig.1). After the selection with G418 and puromycin for 9 days, the colonies were picked, expanded and genotyped by PCR for the homologous recombination events at the 5' and 3' arms (Fig.1 and Table 1). Among the 73 clones screened, 30 clones were correctly targeted at the 5' ends and 12 of 30 possessed the integration of the 3' loxP site. One targeted clone was selected and transfected with the expression vector of *FLPe* recombinase followed by the selection with gancyclovir, resulting in the isolation of the clones in which the *Frt* cassette was removed. One clone was chosen for the second round targeting followed by the removal of the *Frt* cassette. As a result, we obtained ES cells in which both *Zscan10* alleles were modified to carry the loxP sites at intron 5 and exon 6 (Fig. 1 and Fig. 2A,B). Finally, the expression vector for a hormone-inducible Cre recombinase (*MerCreMer*) was introduced into the *Zscan10*^{fl/fl} ES cells using the Piggy-bac transposon system, and a constitutively active *Egfp* expression vector was also transfected by electroporation, resulting in the establishment of the inducible *Zscan10* knockout ES cells.

Zscan10 knockout shows no impact on self-renewal of ES cells

For isolation of *Zscan10*-null ES cells, the *Zscan10*^{fl/fl} ES cells carrying *MerCreMer* and *Egfp* were treated with Tamoxifen and then were seeded at a clonal density after dissociation. When the resulting clones were isolated, expanded and analyzed for the genotype with genomic DNA PCR, we found 35.9% of the ES cells

carried the deletion of both floxed *Zscan10* allele. The QPCR with the primers that specifically detect exon 6 showed the loss of *Zscan10*-transcription (Fig. 3A). Finally we succeeded in confirming the proper loss of *Zscan10* protein in multiple ES cell lines by western blot analysis using the antibody against the N-terminal region (Fig. 2C). The recognition site of this antibody is remained in the targeted allele and the truncated protein product with the expected size (89kD) from the knockout allele was not detected, suggesting that the truncated protein was unstable and should not give residual function. These *Zscan10*-null cells could be maintained in the conventional culture condition for long term with normal growth speed and morphology as the parental *Zscan10*^{fl/fl} ES cells (Fig. 2D). These data suggested that the deletion of *Zscan10* function has no effect on the self-renewal of ES cells.

Expression levels of pluripotency-associated genes in *Zscan10*-null ES cells are intact

We next ascertained the expression levels of the pluripotency-associated genes *Oct3/4*, *Sox2* and *Nanog* by QPCR analysis. As a result, there were no differences in the expression levels of these pluripotency-associated genes (Fig. 3A). We also investigated the global gene expression profile by RNA-seq analysis and found that 53 genes were up-regulated and 26 genes were down-regulated in *Zscan10*-null ES cells compared to *Zscan10*^{fl/fl} ES cells (Fig. 3B). However if we focused on pluripotency-associated genes, only *Tbx3* was slightly up-regulated in *Zscan10*-null ES cells and the expression of other pluripotency-associated genes were equivalent between *Zscan10*^{fl/fl} and *Zscan10*-null ES cells (Fig. 3C), indicating that the deletion of *Zscan10* gave very faint impact on the gene expression profile in ES cells.

Previous report indicated that *Zscan10* acts as a transcriptional activator of the 2-cell-stage specific transcripts such as *Zscan4* and *Tcstv1* since their expressions were down-regulated in *Zscan10* knocked-down ES cells [5]. However, in our investigation by both QPCR and RNA-seq, both *Zscan4c* and *Tcstv1* were not reduced in *Zscan10*-null ES cells (Fig 3 B.C). This result clearly indicates that *Zscan10* is not a critical regulator of these genes or its function is dispensable for their regulation in constitutive knockout ES cells.

***Zscan10*-null ES cells retain pluripotency**

To fully evaluate the differentiation ability of the *Zscan10*-null ES cells, the dissociated *Zscan10*^{fl/fl} and *Zscan10*-null ES cells expressing *Egfp* were injected into the blastocysts and their contribution in embryos dissected at E13.5 was evaluated (Fig. 4). As a result, we found that *Zscan10*-null ES cells contributed to chimera embryos with the same efficiency as *Zscan10*^{fl/fl} ES cells (Table. 3). These data indicated that the function of *Zscan10* is dispensable for the maintenance of pluripotency in ES cells.

Discussion

Cell type specificities are defined by specific networks of transcription factors. ES cells also have their own transcription factor network that confers their pluripotency. Many genes are recently reported as putative components of the pluripotency-associated transcription factor network in ES cells, which play the roles in the maintenance of pluripotency and continuous self-renewal. To confirm the function of these transcription factors, loss-of-function experiments are required.

For loss-of-function analysis, shRNA-mediated knock-down strategy has often been applied. In the case of *Zscan10*, previous reports applied this strategy and one of them found that the knock-down of *Zscan10* caused up-regulation of the pluripotency-associated transcription factors such as *Esrrb*, *Klf2* and *Klf4*. [5]. However, our RNA-seq result clearly indicated that the expressions of these transcription factors are intact in *Zscan10*-null cells compared to *Zscan10*^{fl/fl} cells. The discrepancy could be due to the difference in the genetic backgrounds of the ES cells as well as the difference in the culture conditions, but our data indicated that at least in our condition the function of *Zscan10* on transcriptional regulation of target genes is quite faint, suggesting that the context-independent function is minimal. Moreover, the knock-down studies do not clarify whether *Zscan10* is necessary or not for the maintenance of pluripotency because the knocked-down ES cells expressed 10-20% of total *Zscan10* transcripts, retaining the possibility that the residual expression may be sufficient. Here we achieved the complete loss of *Zscan10* by inducible knockout strategy and demonstrated that the *Zscan10*-null ES cells are able to continue self-renewal while keeping pluripotency. These data clearly indicated that the function of *Zscan10* is dispensable for the maintenance of pluripotency in mouse ES cells.

Generally, loss of a single transcription factor does not cause loss of pluripotency in many cases. The most obvious was the case of *Nanog* which is known

as an important component of the pluripotency-associated transcription factor network because of the co-localization with *Oct3/4* and *Sox2* in the target sites of the genome[14]: despite the significance of *Nanog*, the *Nanog*-null ES cells could be established while keeping pluripotency[15]. Such phenomenon might be due to the relative importance of the factor in the network, functional redundancy among similar factors, or the robustness of the transcription factor network. In the case of *Zscan10*, it was reported that it functions as a homodimer as well as a heterodimer with the related protein *Zfp110*[11], suggesting their overlapped function. To dissect these possibilities, in-depth analyses of the stable *Zscan10*-null ES cells as well as the inducible knockout process of *Zscan10* will be required in future.

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Figure legends

Figure 1. Generation of *Zscan10* inducible knockout ES cells.

(A) *Zscan10* knockout vector carrying the *loxP* sites in which drug-resistant gene cassette flanked by the *Frt* sites in the middle were introduced into ES cells followed by the selection with G418 and puromycin. Correctly targeted clones were identified by genomic PCR. (B) The drug-resistant gene cassette was removed by the transient expression of *FLPe* followed by the selection with Gancyclovir. (C) Second round targeting for another allele. (D) The expression vector for hormone-inducible form of Cre (*MerCreMer*) was introduced into the *Zscan10^{fl/fl}* ES cells to establish the *Zscan10* inducible knockout ES cells.

Figure 2. The confirmations of *Zscan10* inducible knock out ES cells.

(A) The position of primer for genotyping PCR. (B) The wild type allele (wt) gives 1051bp, floxed allele gives 1150bp and deleted allele gives 2603bp band. (C) Western blot analysis of ZSCAN10 in *Zscan10^{fl/fl}* ES cells and *Zscan10*-null cells. (D) The morphology of the colonies of *Zscan10^{fl/fl}* and *Zscan10*-null ES cells.

Figure 3. Gene expression profiles in *Zscan10^{fl/fl}* and *Zscan10*-null ES cells.

The gene expression levels of pluripotency markers (A) and 2-cell related markers (B) in wild-type EB5, *Zscan10^{fl/fl}* ES cells and *Zscan10*-null ES cells. Q-PCR was performed with biological triplicates and the relative expression levels to wild-type ES cells (set at 1.0) were shown with standard deviation. The primers are described in Table 2. (C) The volcano plot of RNA-seq data from *Zscan10^{fl/fl}* ES cells and *Zscan10*-null ES cells. Red dots indicate the genes that showed significant difference in the expression levels with LogFC absolute value ≥ 2 and q-value <0.01 . (D) Comparison of the expression levels of pluripotency-associated genes from RNA-seq data of *Zscan10^{fl/fl}* and *Zscan10*-null ES cells. Relative expression levels to *Zscan10^{fl/fl}* ES cells (set at 1.0) were shown with standard deviation.

Figure 4. Chimeric embryo derived from *Zscan10*-null ES cells.

Chimeric embryos obtained by the injection of *Zscan10^{fl/fl}* and *Zscan10*-null ES cells carrying the ubiquitous EGFP expression vector.

Table1

Primer Name	Sequence (5'-3')
Zscan10S10186ClaI	ATTATCGATGCAAATTCCACTGGCCTCCATCTGTG
Zscan10AS12469SpeI	ATTACTAGTAGGGAGATTCCTAGATTTGGCTATGGG
Zscan10S12470SpeI	ATTACTAGTTCACCCTTTCTGACAGCCTTTGTGCC
Zscan10AS14989SpeI	ATTACTAGTGGTGAAGCAGTTAGTACAGCGATTCCC
Zscan10S14990SpeI	ATTACTAGTCATGTTCCAGCAACCTTGTCCCATGTG
Zscan10AS18050PmeINotI	AATGCGGCCGCGTTTAACTACTGGGTCCTCAGCCTTCCTTGTG
Zscan10S10186ClaI	ATTATCGATGCAAATTCCACTGGCCTCCATCTGTG
Zscan10 5'ext-F	AGAGACATCAGCCACATGGGGCCACTG
IRES-AS	CCTTATTCCAAGCGGCTTCGGCCAG
Zscan10 3'ext-R	GGGGATTCTGTAGGCCTTTAGAGCCAG
IRES-S	GAACCACGGGGACGTGGTTTTTCCTTTG
Zscan10 5'arm-F	AATCCTCCTGCCTCTGCCTCCCAAGTG
Zscan10 Floxed-ex6-R	TGGGTCCAGAAGTGGAAGCAGCCCAG
Zscan10 3'arm-R	TCTCCTCACAAAGTCGCAGTGCTCCAG

Table2

Primer Name	Sequence (5'-3')
GapdhS	TGCCATCACTGCCACCCAGAAGACTG
GapdhAS	TGAGGTCCACCACCCTGTTGCTGTAG
Zscan10S	CCGGAGAAAGCAGTGAGGTGGA
Zscan10AS	CGAAAATGCTAACAGTTGAT
Oct3/4S	AAGCCCTCCCTACAGCAGAT
Oct3/4AS	CTGGGAAAGGTGTCCCTGTA
Sox2S	GAGTGGAACCTTTTGTCCGAGA
Sox2AS	GAAGCGTGTACTTATCCTTCTTCAT
NanogS	ACCTGAGCTATAAGCAGGTTAAGAC
NanogAS	GTGCTGAGCCCTTCTGAATCAGAC
Zscan4cS	CCGGAGAAAGCAGTGAGGTGGA
Zscan4cAS	CGAAAATGCTAACAGTTGAT

Tcstv1S	TCCCCAGGAGATGTGGAAAA
Tcstv1AS	CGTCACAGGTCACAAGATCCAGA

Table3

	GFP+ / collected / injected
flox clone1	3 / 16 / 40
flox clone2	1 / 9 / 40
KO clone1	3 / 12 / 40
KO clone2	5 / 14 / 40

Fig1

A

Zscan10: Ch 17

Selection of homologous recombinants

B

Removal of Frt cassette by transient expression of FLP recombinase

C

Second round targeting

D

Induction of knockout by activation of MerCreMer with tamoxifen

PB CAG MerCreMer IRES Hygro pA PB

Fig2

A

B

Fig.2

C

D

Fig.3

A

B

C

D

Fig4 A