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An invited review following *the Soujinkai Award*: Tissue-specific Expression of Estrogen Receptor1 is Regulated by DNA Methylation in a T-DMR

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Abstract *ESR1* expression was tissue-specific, being high in the endometrium and mammary gland and low/nil in the placenta and skin. A long-standing question was how this tissue-specific expression of *ESR1* was regulated. In other genes, DNA methylation of a region called the T-DMR (tissue-dependent and differentially methylated region) has been associated with tissue-specific gene expression. We recently found that human *ESR1* has a T-DMR and DNA methylation of the T-DMR, but not the promoter region, regulates its tissue-specific expression. Furthermore, we revealed that EGR1 is a possible transcription factor to bind the T-DMR and upregulate *ESR1* expression under DNA hypomethylation of the T-DMR.

Key words: estrogen receptor 1 (*ESR1*), tissue-specific expression, DNA methylation, tissue-dependent and differentially methylated region, breast cancer

Introduction

The estrogen receptor is a transcription factor that mediates estrogen hormone action in many physiological and pathological processes. Expression of human estrogen receptor 1 (*ESR1*), which codes ER-alpha, is tissue-specific (Fig. 1).[1-2] For example, *ESR1* expression is high in the endometrium and mammary gland and low in the placenta and skin. In addition, *ESR1* has several TSSs corresponding to upstream Exon-A to upstream Exon-E1 (Fig. 2A).[3] The transcription of *ESR1* starts from any of these upstream exons (Fig. 2B), and the upstream exons are used in a tissue-dependent manner.[4] A long-standing question was how this tissue-specific expression and the selection of upstream exons of *ESR1* was regulated.

DNA methylation is one of the most characterized epigenetic marks, and occurs at CpG sites. CpG islands, which are CpG site-rich regions, are located in the gene promoter near the transcription start site (TSS) and are hypomethylated in normal tissues (Fig. 3). It is thought that DNA methylation of the gene promoter plays a central role in gene silencing. In addition, DNA methylation of a specific region of the gene has an important role in determining tissue- and cell-specific gene expression.[5-11] The region regulating cell-specific gene expression is called the

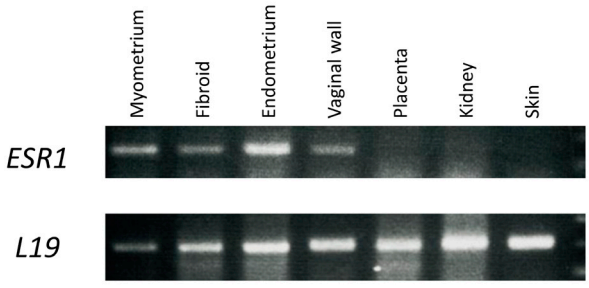

Fig. 1 Tissue-specific expression of *ESR1*.
Expression status of *ESR1* in several tissues was examined by RT-PCR. L19 was used as an internal control.

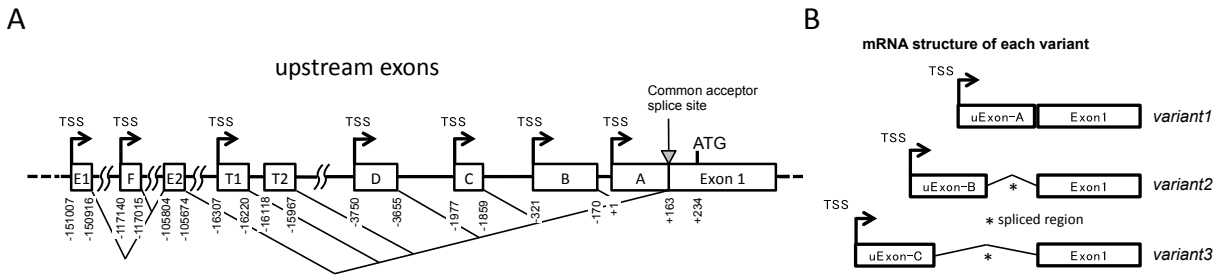

Fig. 2 Genomic organization of upstream exons and corresponding transcription start sites (TSSs) of *ESR1*.

A: The upstream exons are shown by boxes and the corresponding TSSs are indicated by arrows. The numbers below the upstream exon boxes indicate 5' start sites, splice donor site and acceptor sites, which are involved in generating mature *ESR1* mRNA with the distance from the originally described transcription start site at +1. All 5' upstream exons are spliced at the common acceptor splice site (+163 bp). B: The primer design to investigate the transcribed mRNAs of variant1, variant2 and variant3, separately.

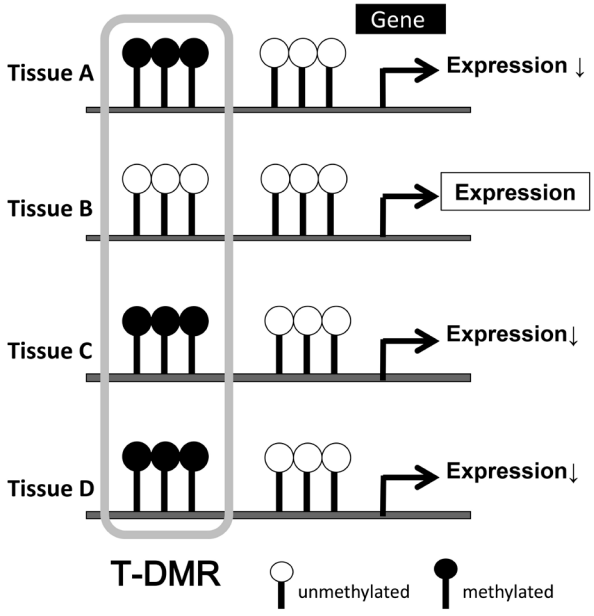

Fig. 3 T-DMRs (tissue-dependent and differentially methylated region)

DNA methylation of a specific region of genes has an important role in determining tissue- and cell-specific gene expression. The region regulating cell-specific gene expression is called the tissue-dependent and differentially methylated region (T-DMR). Open and filled circles indicate unmethylated and methylated CpG status, respectively.

In this review, we focus on the questions whether human *ESR1* has a T-DMR and whether DNA methylation of the T-DMR regulates its expression.

T-DMRs of *ESR1*

We previously found a possible link between the mRNA expression of *ESR1* and the DNA methylation status of a region distant from the TSS of *ESR1* (-1188 bp to -790 bp).[12] In human uterine leiomyomas, *ESR1* expression was elevated and the region from -1188 bp to -790 bp was less methylated in comparison with normal myometrium.[12] These findings, together with the finding that the DNA methylation status of the promoter region including the CpG island around TSS (-566 bp to +229 bp) was hypomethylated in both leiomyoma tissues and normal myometrium,[12] suggest that the region from -1188 bp to -790 bp distant from TSS is a T-DMR regulating *ESR1* expression via DNA methylation. We then tested the DNA methylation status of the promoter region (-566 bp to +229 bp) and the distal region (-1188 bp to -790 bp) from TSS in the tissues with high expression (endometrium and mammary gland) and low or negligible expression (placenta and skin) of *ESR1*. In all of the tissues, the promoter region from -566 bp to +229 bp was unmethylated, whereas in the distal region

from -1188 bp to -790 bp, endometrium and mammary gland showed unmethylation and hypomethylation statuses while placenta and skin showed moderate methylation and hypermethylation statuses, respectively.[13] The DNA methylation status in the distal region was strongly associated with *ESR1* expression, which suggests that the distal region is a T-DMR, which is designated as T-DMR1 (Fig. 4).

T-DMR-methylated reporter assay

The next question was whether the DNA methylation of T-DMR1 alone while keeping the promoter region hypomethylated has a suppressive effect on *ESR1* expression. For this purpose, we performed T-DMR-methylated/unmethylated reporter assay using two types of constructs; T-DMR-unmethylated/promoter unmethylated construct (TDMR-U, control) and T-DMR-methylated/promoter unmethylated construct (TDMR-M). The reporter assay indicated that the TDMR-U construct (control) had 5-fold higher reporter activities compared with the empty vector, whereas the TDMR-M construct reporter activity was significantly reduced by 47.5% when compared to the TDMR-U construct ($p < 0.05$).[13] These results indicate that DNA methylation of T-DMR1 suppresses *ESR1* expression, and T-DMR1 (-1188 bp to -790 bp) was determined as the T-DMR that regulates *ESR1* gene expression.

DNA methylation statuses around upstream Exons and mRNA expression of *ESR1* variants in normal tissues

ESR1 has several upstream exons (upstream Exon-A to upstream Exon-E1) in which transcription starts from the corresponding TSSs (Fig. 2A).[3] Three upstream Exons, Exon-A, -B and -C, are often used in the tissues with high *ESR1* expression.[3] The transcription products from upstream Exons-A, -B and -C are spliced to generate mature *ESR1* mRNAs, called variant1, variant2 and variant3, respectively (Fig. 2B). All three variants were highly expressed in the endometrium and mammary gland, whereas none of the variants were expressed in the placenta and skin, indicating that the transcription from each upstream Exon is tissue-specific.[13]

Around upstream Exon-A, -B and -C (-2953 bp to +229 bp), CpG sites located from -566 bp to +229 bp were unmethylated in all the tissues (AB-promoter in Figure 4).[13] CpG sites of T-DMR1 were unmethylated in the endometrium, hypermethylated in the mammary gland, moderately methylated in the placenta and hypermethylated in the skin. CpG sites in the region from -2099 bp to -1876 bp, corresponding to upstream Exon-C, were unmethylated or hypomethylated in all the tissues (C-promoter in Figure 4).[13] The region from -2953 bp to -2302 bp was unmethylated in the endometrium, hypomethylated in the mammary gland, and moderately methylated in the placenta and skin, indicating that the region from -2953 bp to -2302 bp is another T-DMR that regulates variant3 expression (T-DMR2 in Figure 4).[13] These findings suggested that each upstream exon has its own T-DMR.

DNA methylation statuses around upstream Exons and mRNA expression of *ESR1* variants

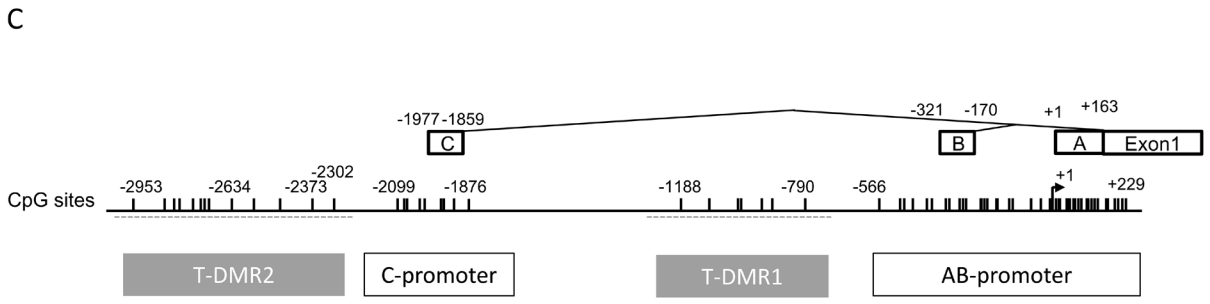

Fig. 4 The definition of T-DMRs in *ESR1*

The location of AB-promoter (-556 bp to +229 bp), T-DMR1 (-1188 bp to -790 bp), C-promoter (-2099 bp to -1876 bp), and T-DMR2 (-2953 bp to -2302 bp) are shown.

in breast cancer

Regarding the *ESR1* expression in breast cancer, downregulation of *ESR1* expression has been associated with a poor prognosis[14] and DNA methylation of the *ESR1* promoter down-regulates *ESR1* transcription.[15-19] However, it is unclear why some cases of breast cancer show various levels of *ESR1* expression despite DNA hypomethylation in the promoter region.[17,19] Only 25 % of ER-alpha-negative breast cancer tissues show DNA methylation in the promoter region.[17] In addition, upstream exons used for *ESR1* expression are different among individuals and different upstream exons are associated with clinicopathological variations.[20] These findings raise the question whether DNA methylation of T-DMR contributes to the regulation of *ESR1* expression in transcription levels in breast cancer.

To investigate whether DNA methylation of the T-DMRs is involved in the regulation of transcription of the *ESR1* mRNA variants in breast cancer, the mRNA expression levels of variant1, variant2 and variant3 in breast cancer tissues and DNA methylation statuses around upstream Exon-A, -B and -C were examined. We also examined the mRNA expression of the three variants in MCF7 and MDA-MB-231 cells, which are known as an ER-alpha-positive and an ER-alpha-negative breast cancer cell line, respectively. Examined breast cancer tissue samples and cell lines were classified into three groups; all three variants were expressed (Group X), none of the variants were expressed (Group Y), and variant1 and variant2 were expressed (Group Z).[13] MCF7 and MDA-MB-231 were classified into Group Z and Group Y, respectively.[13] In Group X, the AB-promoter and C-promoter were unmethylated and hypomethylated, and T-DMR1 and T-DMR2 were hypomethylated and moderately methylated, respectively, similar to what was observed in the DNA methylation profiles of the endometrium and mammary gland.[13] In Group Y, T-DMR1 and T-DMR2 were hypermethylated as was observed in the placenta and skin, but interestingly, the methylation statuses of the C-promoter varied among the cases (moderate methylation or unmethylation).[13] Furthermore, in MDA-MB-231 expressing none of the variants, even B-promoter and C-promoter in addition to T-DMR1 and T-DMR2 were moderately methylated and hypermethylated, respectively.[13] In Group Z, although the DNA methylation pattern was similar to that of Group X, only variant1 and variant2 were expressed.[13] In addition, in MCF7, although both T-DMR1 and T-DMR2 were moderately methylated, MCF7 expressed variant1 and variant2 but not variant3.[13] These findings indicate that transcriptional regulation of *ESR1* expression is abnormal in some breast cancers. In other words, some cases of breast cancer may have other regulation mechanisms in *ESR1* expression than DNA methylation at T-DMRs. Since the promoter regions were methylated in some cases of breast cancer samples, it is also suggested that DNA methylation aberrantly occurs in breast cancer.

Potential transcription factors and histone modifications at T-DMRs

By a motif analysis of T-DMR1 and T-DMR2, EGR1 was extracted as a potential transcription factor. EGR1 has the consensus DNA sequence (GCGTGGGCG) in both T-DMRs. EGR1, which belongs to the EGR family of C2H2-type zinc-finger proteins, is a nuclear protein and functions as a transcriptional regulator.[21] EGR1 knockdown significantly suppressed *ESR1* expression in human ESC, which express *ESR1*, indicating EGR1 is associated with the upregulation of *ESR1* expression.[13] Since DNA hypomethylation within the promoter region of the gene facilitates the EGR1 binding to its consensus motif,[22] we investigated whether the binding of EGR1 to the T-DMRs is affected by DNA methylation at the T-DMRs using a ChIP assay. The result showed that the EGR1 bindings to the T-DMR1 and T-DMR2 were significantly higher in ESC (TDMRs-hypomethylated) than in MDA-MB-231 (TDMRs-hypermethylated), suggesting that the binding of EGR1 to the T-DMRs was interrupted by DNA methylation (Fig. 5).[13] Furthermore, the levels of H3K27me3, which is well-known repressive histone modification and associated with DNA methylation,[23-25] in both T-DMR1 and T-DMR2 were significantly lower in ESC than in MDA-MB-231.[13]

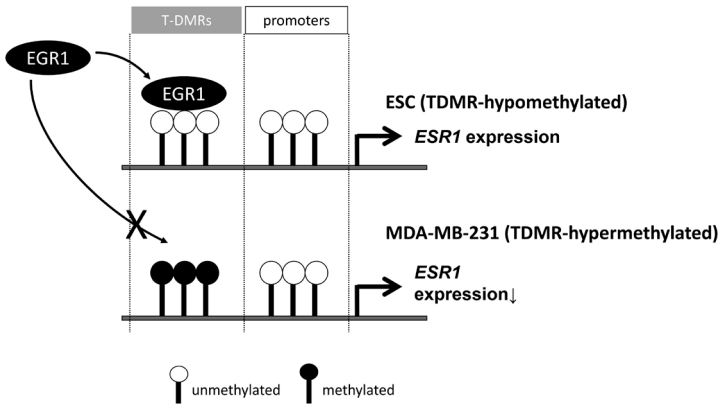

Fig. 5 Interruption of EGR1 binding to the T-DMRs by DNA hypermethylation.
The binding of EGR1 to the T-DMRs may be interrupted by DNA methylation, because EGR1 bound to T-DMRs in ESC in which T-DMRs are unmethylated, but the bindings of EGR1 were inhibited in MDA-MB-231 cells in which T-DMRs are hypermethylated. Open and filled circles indicate unmethylated and methylated CpG status, respectively.

Conclusion

ESR1 has T-DMRs and the T-DMRs regulate tissue-specific *ESR1* expression via DNA methylation. Each upstream Exon has a corresponding T-DMR, which regulates transcription from the upstream Exon.

Conflict of Interest

The authors declare no conflict of interest.

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