ABSTRACT

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The relationship between DNA methylation and nucleosome occupancy involving in a transcriptional activity of SOX2

(Under the Direction of Dr. Shaying Zhao)

Histone modifications, DNA methylation, and nucleosome occupancy are known to play an important role in regulation of transcription. The purpose of the study was to comprehend the relationship between DNA methylation and nucleosome occupancy on the promoter of SOX2. Since current studies showed that the more cells are differentiated, the more the promoter of SOX2 is methylated and occupied by nucleosomes, we sought to characterize the relationship between DNA methylation and nucleosome occupancy involved in transcriptional activity of SOX2 in H9 embryonic stem cells, H9 cardiac progenitor cells, H0 smooth muscle cells, and Hela cells. Our experimental data for Hela cells supports data from current studies that transcriptional silencing may include interactions between DNA methylation and nucleosomes.

INDEX WORDS: DNA methylation, nucleosome occupancy, SOX2, and transcriptional activity.

THE RELATIONSHIP BETWEEN DNA METHYLATION AND NUCLEOSOME OCCUPACY INVOLVING IN A TRANSCRIPTIONAL ACTIVITY OF SOX2

by

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DEDICATION

I would like to dedicate my thesis to my family, especially my father and mother, who have given me the strength for accomplishing this work.

“*成せば成る、成さねば成らぬ何事も、成らぬは人の成さぬなりけり*” 上杉鷹山

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CHAPTER 1.
INTRODUCTION

1.1 Overview

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that do not change the sequence of DNA. Two predominant epigenetic mechanisms are histone modification and DNA methylation. These chemical modifications and nucleosome occupancy play an important role in transcriptional regulation. Although some interactions among these have been revealed, further experiments are required to fully understand how interactions among them can influence transcriptional activity.

1.2 Literature Review:

1.2.1 Nucleosome Structure

The human genome , organized as 23 chromosome pairs, contains about 3 billion nucleotide pairs per haploid genome with a total length of DNA of 1.7 meter (Lodish et al., 2008). In order to allow 1.7 meter of DNA to fit into a nucleus 5 micrometer in diameter, the DNA must be properly organized and condensed. The nucleosome is a fundamental unit of eukaryotic chromatin organizing chromosomal DNA into a compact structure with the help of proteins called histones (Ho and Crabtree, 2010). Each nucleosome is about 10 nm in diameter, and its core is an octamer containing two copies each of histones H2A, H2B, H3, and H4 plus 147 base pairs of DNA wrapped one and two-thirds turns around these histones in a left-handed

superhelix. Nucleosomes are packed together with the aid of histone H1 in order to form the 30 nm fiber, which represents the first stage of higher-order packing of human chromosomes (Lodish et al., 2008).

Nucleosome occupancy, histone modifications, and DNA methylation of genes has been studied and is believed to have some influence on transcriptional activity. One study showed that in cancer cells, the promoter of methylated regions of MLH1 contained nucleosomes, which was not observed in normal cells (Lin et al., 2007).

1.2.2 Histone Modifications

Histone modifications are post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Vasquero, 2003), and they can either activate or repress transcription and influence transcription by affecting chromatin structure (Kondo, 2009). All core histones in the N-terminal domains are subject to chemical modifications. Consequences of these modifications depend on the chemical modification and the location where the chemical modification occurs in the histone protein (Cosgrove, 2004).

Generally, transcriptional activity is led by acetylation of lysine residues on histone H3 and H4, because this chemical modification allows a formation of an opened chromatin structure. On the other hand, methylation of lysine residues is associated with either activation or repression of transcription (Kondo, 2009). The monomethylated state of H3K9, H3K27, and H4K20 residues corresponds to actively transcribed genes, while dimethylation and trimethyaltion of these histone residues are associated with silent genes (Barski et al, 2007). Trimethylation of these residues is also associated with the formation of heterochromatin from

yeast to humans and regulates Hox gene expression and the early steps of X-inactivation, whereas dimethylation of these residues is involved in inactivating gene expression in euchromatic regions (Kondo, 2009; Wang et al., 2009).

Current studies have shown that histone modifications are also an important feature during cellular differentiation. For instance, trimethylated H3K27 is associated with crucial genes for cellular differentiation in human embryonic stem cells, such as the ES cell regulators OCT4 and SOX2; specifically, it represses the expression of these crucial genes before differentiation (Lee et al., 2006). Neuron-specific developmental genes lose trimethylated H3K27 during differentiation of murine ES cells to terminal neuronal cells. After the loss of trimethylated H3K27, these genes are changed into an activated form (Mohn et al., 2008).

1.2.3 DNA methylation

1.2.3.1 General

DNA methylation is universal in bacteria, plants, and animals. It is a type of chemical modification of DNA in which the 5 position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with a methyl group donor, S-adenosyl-methionine in eukaryotic organisms. In prokaryotic organisms, DNA methylation occurs at the 5 position of cytosine and 6 position of nitrogen in adenine. In mammalian cells, DNA methylation occurs at the 5 position of cytosine of a CpG dinucleotide, and this reaction is catalyzed by three different enzymes, DNMT1, DNMT3a, and DNMT3b (their functions discussed later). DNA methylation plays an important role in the long-term silencing of transcription including silencing of

transposons, X-inactivation, imprinting, and development, and also plays a role in heterochromatin formation (Miranda and Jones, 2007).

In terms of DNA methylation, there are two groups of sequences in the genome: CpG-poor regions and CpG islands. CpG islands contain a sequence longer than 500 bp and a CG content over 55%. They are often seen in promoter regions, and about 40% of CpG islands associated with genes are located in the end of 5' region, including promoter, untranslated region, and exon 1. CpG-poor regions are the intergenic and the intronic regions in the genome. CpG-poor regions are generally methylated in healthy cells, while CpG islands are usually hypomethylated with a few exceptions, such as regions of inactivated X chromosome. In cancer cells, CpG-poor regions become hypomethylated whereas CpG islands get hypermethylated, and this alteration in DNA sequence brings about changes in chromatin structure, which silences tumor suppressor genes (Miranda and Jones, 2007).

DNA methylation has two separated types of methylation: de novo and maintenance methylation. De novo methylation is important for constituting patterns of methylation in early embryos, during development, and during carcinogenesis. During cellular division DNMT1 has a role in establishing and regulating methylation patterns set by de novo methylation by copying DNA methylation patters to the daughter strands and carries on maintenance methylation. However, DNMT1 does not efficiently maintain the methylation in the CpG-condensed regions; therefore, DNMT3a and DNMT3b are recruited in somatic cells in order to compensate an inefficient activity of DNMT1 and prevent the loss of the methylation patterns (Miranda and Jones, 2007).

1.2.3.2 Role of DNA methylation in silencing

Silencing of genetic materials by histone modifications and chromatin structure may not be permanent because these modifications can be easily reversed. For long term silencing of genes, an additional mechanism is required in mammalian cells. DNA methylation can take this work, because its chemical modification is stable even during a cellular division. However, dilution of methylated C is expected to occur at every cell division. During replication, a methylated C:G base pair yields one methylated C:G daughter and one C:G daughter, thereby resulting in a 50% reduction in methylated C at this basepair. This hemimethyalted state is recogonized by DNMT1 and returned to the fully methylated form (Vilkaitis et al., 2005). Because to this, the daughter cells can continue to hold the same expression patterns that their precursor cells have. DNA methylation can make silenced genes remain silent even after histone modification is reversed. This characteristic contributes to silencing of transposons and imprinted genes, X chromosome inactivation, and development (Miranda and Jones, 2007; Reik, 2007).

1.2.3.2.1 Silencing transposons

Transposons are sequences of DNA that have an ability to move around to different locations in the genome of a single cell. They were discovered by Barbara McClintock (McClintock, 1950). Transposons or retroposons can embed themselves into functional genes, thereby disabling them. Since many of these genes contain promoters, they are able to transcribe their sequences. Current studies show that the expression of transposon sequences permits the movement of parasitic elements in the genome, which will eventually alter sequences of DNA.

In order to preserve genome, these elements have to be silenced by DNA methylation (Robertson and Wolffe, 2000; Miranda and Jones, 2007).

1.2.3.2.2 Inactivation of X chromosome and imprinted genes

DNA methyaltion plays a role in X-inactivation and controlling imprinted genes as well. X-inactivation is a process in which one of two copies of the X chromosome in female mammals is inactivated in order to avoid producing twice as much product of X-linked genes in the female as in the male (Takagi and Sasaki, 1975). Genomic imprinting is a genetic phenomenon that determines which parental allele will be expressed (Wilkinson et al., 2007). X-inactivation and imprinted genes do not follow Mendelian genetics in which both parental copies contribute equally to the outcome. For X-inactivation, DNA methylation of promoter regions inhibits gene expression, and is the inactivated X remains in a silent state through every cell division (Altun et al., 2010). For imprinted genes, essential sequence elements termed imprinting control regions (ICRs) are marked by DNA methylation, and they regulate mono-allelic, parent-of-origin-specific expression. These marks will be preserved even through cell division (Kacem and Feli, 2009).

1.2.3.2.3 Regulating transcription by DNA methylation

DNA methylation is believed to play an important role in the regulation of transcription as well. One of the most promising mechanisms proposed by current studies is that methylation of CpG sequences would alter chromatin structure associated with histone modifications and nuclesome occupancy within the promoter regions of genes (Miranda and Jones, 2007). CpG islands that have a permissive or open state for potential transcription may be kept nucleosome

free in order to allow transcriptional activators to bind to these regions and activate promoters. On the other hand, hypermethylated promoters with a closed state are not accessible for transcriptional activators, and therefore, transcription does not occur. One study found that the promoter CpG island of MLH1, which plays a role in the DNA mismatch repair system, undergoes a change in nucleosome occupancy in cancer cells. This region in cancer cells has three nucleosomes that are not present in normal cells, and these nucleosomes reside on the methylated and silenced promoter. This suggests that epigenic silencing is associated with the insertion of nucleosomes into a previously unoccupied place and furthermore that epigenetic silencing may include heritable changes in nucleosome occupancy allowed by DNA methylation (Lin et al, 2007).

1.2.3.2.4 Developmental regulation by DNA methylation

Current studies have shown that DNA methylation in some germ-line and tissue-specific promoters occurs; however, at the same time, there are many genes involving development that are not silenced by DNA methylation. One study showed that some genes, which are germ-line specific, are methylated in fibroblasts but not in sperm. This result indicates that DNA methylation in somatic cells contributes to cellular differentiation by inhibiting transcription of key genes in the germ-line and irreversibly forcing the somatic cells on a path to differentiation (Weber et al., 2007). On the other hand, another study showed that CpG dinucleotides in promoter and CpG island areas are hypomethylated in hESCs comparing to differentiated cells; for example, HOX gene clusters are the most significant hypomethylated regions in hESCs and become more methylated as they differentiate into fibroblasts derived from hESCs (Laurent et al., 2010).

1.3 Purpose of Study

The purpose of the study was to examine the relationship between nucleosome occupancy and DNA methylation on the promoter of SOX2, which is a crucial developmental gene. Since the study of Lin et al. (2007) showed that the more cells were differentiated, the more the promoter of SOX2 was methylated and occupied by nucleosomes, we sought to characterize the transcriptional activity of SOX2 in H9 embryonic stem cells, H9 cardiac progenitor cells, H9 smooth muscle cells, and Hela cells from the standpoint of DNA methylation and nucleosome occupancy and their relationship.

CHAPTER 2.
MATERIALS AND METHODS

2.1 Cell culture and DNA preparation

H9 human embryonic stem cells (ESCs), H9 cardiac progenitor cells (H9 CP), H9 smooth muscle cells (H9 SMC), and Hela cells were chosen for the experiment. ESCs, H9 CP, and H9 SMC, acquired from the lab of Dr. Stephen Dalton, were grown to near confluence and harvested. H9 ESCs are pluripotent stem cells, while H9 ISL-1 cardiac progenitor cells were derived from stem cells by 6 days of differentiation, and H9 SMCs were derived from stem cells by 22 days of differentiation. Hela cells were grown as a monolayer and cultured in DMEM (Gibco) medium containing 10% FBS (Atlanta Biologicals), and 1% Penicillin, Streptomycin, and Glutamate (Sigma) in a 37 °C incubator containing a 5% CO_2 atmosphere. Before trypsinization, cells except H9 SMC were detached from the flask by treating with 4 ml 0.05% trypsin/EDTA (Gibco) for 4 min at 37°C. H9 SMC was treated with 5 ml collagenase (acquired from Dr. Dalton's lab). Genomic DNA was purified with DNeasy Blood and Tissue Kit (QIAGEN).

2.2 Bisulfite-DNA treatment

Bisulfite DNA treatment using the EpiTect Bisulfite Kit (QIAGEN) was performed in order to differentiate methylated and unmethylated sequences during Polymerase Chain Reaction (PCR). This treatment can convert cytosine residues into uracil residues in DNA.

2.3 Primer design for SOX2 gene

The sequence of the SOX2 gene was obtained from the UCSC genome browser, and primers complementary to the gene were designed. Although many primers pairs were designed for this gene, only one pair (named SOX BSP3) successfully produced a PCR product. The forward and reverse primers of this pair and other pairs used for experiments can be found inTable 1.

2.4 PCR and gel extraction

In order to carry out PCR in a thermal cycler, the mixture of the bisulfate-treated DNA and the primer pair complementary to the sequence of the SOX2 gene were mixed with uracil-insensitive iTaq DNA polymerase (BIO-RAD). Cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 60 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min; extension at 68°C for 10 min. During this reaction, the uracil residues produced by bisulfite conversion of unmethylated cytosines were amplified as thymines. Products made from PCR were obtained by electrophoresis through a 1% agarose gel and extraction by Gel Extraction Kit (QIAGEN).

2.5 Cloning, inoculation, and purification of plasmid DNA

In order to prepare plasmids, previously obtained PCR products and vector DNA were introduced into Escherichia coli bacterial cultures by pGEM-T and p-GEM-T Easy Vector Systems (Promega). Colonies were grown on the culture dish for 13-14 hours. Colonies from each dish were picked up for inoculation. Average time for inoculation was 15-16 hours. In order to purify plasmid DNA, the FastPlasmid Mini Kit (5 PRIME) was utilized. In order to

confirm the insertion of the insert into the vector, digestion with enzyme EcoR1 was performed in a 37 °C incubator for an hour, followed byagarose gel electrophoresis for detection. Bands having the predicted size were sent for sequencing toMWG/Operon, Huntsiville, AL.

CHAPTER 3.
RESULTS

3.1 Size of the PCR products on agarose gel

After PCR was carried out, an agrose gel was run in order to confirm that the expected product was obtained. The expected size of the PCR product was 372 bp. Bands of the expected size were utilized for gel extraction (Figure 1).

3.2 Confirmation of vector insertion on agarose gel

After digestion with enzyme EcoR1, bands should be detected at 400bp for the PCR product. Although the size of the product was 372bp, the size of the product after digestion became slightly bigger because part of the vector stayed attached to the product during Eco R1 digestion. Bands for the inserted vector should be detected at 3 Kbp (Figure 2). EcoR1 is located in 52 and 70. A restriction map is shown in Figure 3.

3.3 DNA methylation on the promoter of SOX2

DNA methylation analysis was conducted for Hela cells. Sequencing results showed that the promoter region of SOX2 in Hela cells was hypermethylated: 12-15 out of 19 CpG dinucleotides were methylated. Hela-B17 is a plasmid obtained from colony number 17 on the transformation plate, which had a SOX2 PCR amplification product of the expected size. Hela-B18 is the same except that the plasmid was obtained from colony number 18 (Figure 4).

CHAPTER 4.
DISCUSSION

4.1 DNA methylation

Through DNA methylation analysis, hypermethylation on the promoter of SOX2 in Hela cells became clear. Data from previous and ongoing studies in our lab has shown that this region is occupied by nucleosomes in Hela cells. According to the study of Lin et al. (2007), in H9 hESCs only a few methylated CpG dinucleotides were detected on the promoter of SOX2 within a free nucleosome area, and the more cells became differentiated, the more the promoter of SOX2 became methylated and occupied by nucleosomes (Lin et al., 2007). Our experimental data for Hela cells provides support for their hypothesis that the promoter of SOX2 in differentiated cells, such as Hela cells, becomes more methylated and also occupied by nucleosomes. However, the study from our lab showed that SOX2 in H9 cardiac progenitor and H9 smooth muscle is poised for active transcription based on the nucleosome positioning, although these cell lines are more differentiated than H9 hESCs. DNA methylation analysis for H9 cardiac progenitor cells and H9 smooth muscle cells is currently underway.

4.2 Conclusion

Our experimental data for Hela cells supported an idea that epigenetic silencing may include interactions between nucleosomes and DNA methylation as previously suggested; however, the reason why SOX2 was poised in H9 cardiac progenitor and H9 smooth muscle for

an active transcription is still unknown. In order to provide details of DNA methylation level in these two lines, experiments are currently underway.

4.3 Future directions

DNA methylation, nucleosome occupancy, and histone modifications are known to play an important role in regulation of transcription. The interaction between DNA methylation and nucleosomes in different cell lines has been shown in our lab and another study (Lin et al., 2007), and the correlation between nucleosome occupancy and histone modification (trimethylated H9K3) has been studied in our lab as well. However, an interrelation between DNA methylation and histone modification has not been studied yet, and the study of the relationship of these would help us capture a wider image of chromatin structure and transcriptional activity in these cell lines.

Table 1: Sequences of Primers

The name of primers	Sequence 5' to 3' Forward	Sequence 5' to 3' Reverse
hSOX2 CL1	ATGAGCGGGAGAACAATGAC	CCCCTTTTGCAAACACTCTC
hSOX2 CL2	AACTCCTGCACTGGCTGTT	TCTGCCTTGACAACTCCTGA
hSOX2 CL3	GGCTGTTTCCAGAAATACGAG	TCTGCCTTGACAACTCCTGA
hSOX2 BSP1	GGTTTTTTAGTGGTTGGTAGGT	AAAACTCAAACTTCTCTCCCTTT
hSOX2 BSP2	TTAGTGGTTGGTAGGTTGGTTT	CCTAAAACTCAAACTTCTCTCCC
hSOX2 BSP3	GGTTTTTTAGTGGTTGGTAGG	AAAACTCAAACTTCTCTCCCTTT
hSOX2 BSP4	TGTGGTGTGATTTGTTGTTG	CCTAAAACTCAAACTTCTCTCCC

This is a list of sequences of primers utilized for experiments. hSOX2 BSP3 worked well as shown in figure 1 and it was used for experiments for different cell lines.

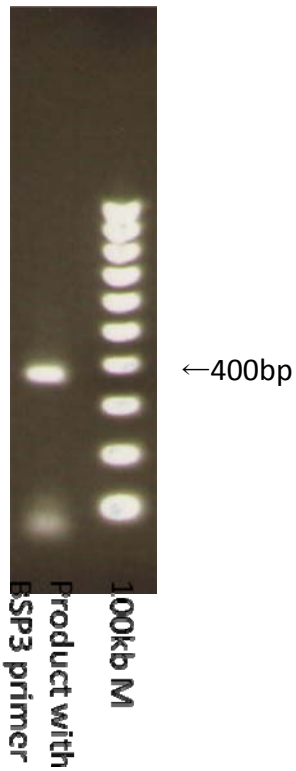

Figure 1. The Size of the Product on Agarose Gel.

Gel picture of a PCR product from Hela cells and primer SOX2-BS3. The expected size of the PCR product was 372 bp, and this figure indicated that the correct size of the product was obtained.

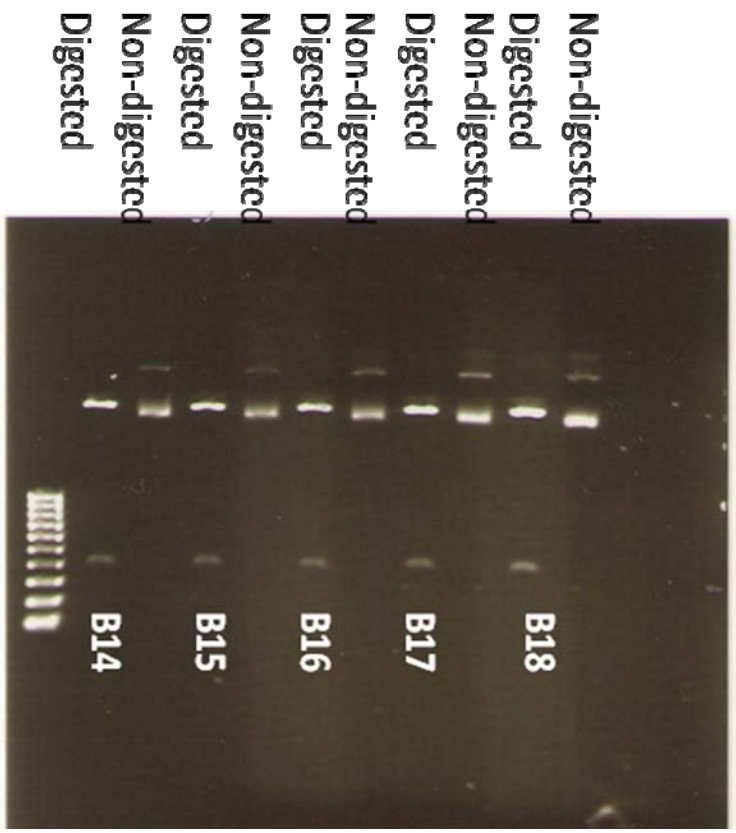

Figure 2. Confirmation of Vector Insertion on Agarose Gel.

Agarose-gel electrophoresis of undigested versus EcoR1-digested plasmid DNA samples. All of the EcoR1-digested samples show the expected band at ~400 bp, indicating that the PCR product was successfully inserted into the EcoR1 sites of the pGEM-T Easy vector (see Fig. 3). Those plasmids exhibiting the strongest signals (B17 and B18) were submitted for sequencing.

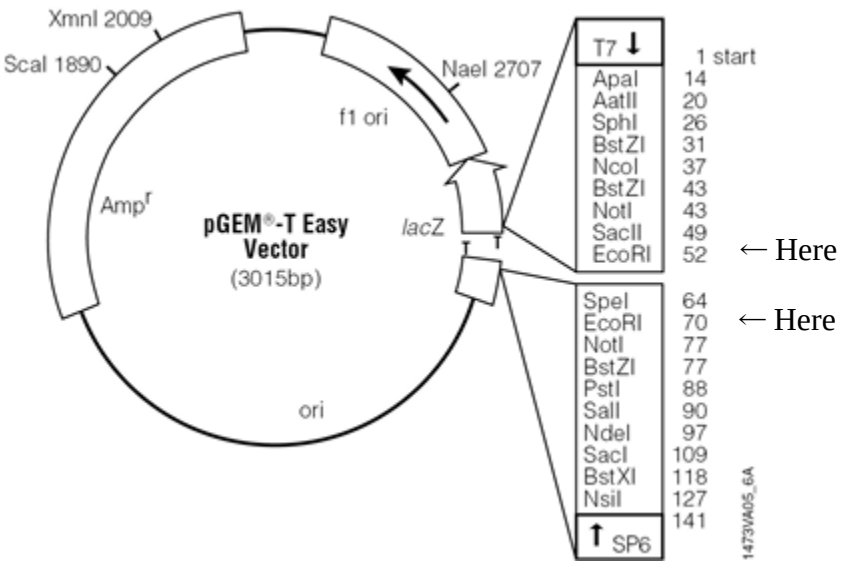

Figure 3. The Restriction Map of the Vector.

Map of pGEM-T Easy Vector (Promega). The vector was digested with EcoR1 (locations 52 and 70, indicated by arrows) in preparation for cloning PCR fragments containing EcoR1 sticky ends.

	CpG site																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Hela-B17	M	M	M	M	M	M	M	M	U	U	M	M	M	M	M	M	M	U	U
Hela-B18	U	M	M	M	U	M	M	U	U	U	M	M	M	M	M	M	M	U	U

Figure 4. SOX2 DNA Methylation in Hela Cells.

The promoter of the SOX2 gene is hypermethylated in Hela cells: 15 out of 19 CpG sites are hypermethylated for Hela-B17 and 12 out of 19 for Hela-B18. The SOX2 gene was PCR-amplified from bisulfite-treated genomic DNA, and the PCR amplification products were ligated into the EcoR1 sites of plasmid pGEM-T Easy and transformed into E. coli. Hela-B17 is a plasmid obtained from colony number 17 on the transformation plate, which had a SOX2 PCR amplification product of the expected size. Hela-B18 is the same except that the plasmid was obtained from colony number 18. "M" indicates a methylated cytosine; "U" indicates an unmethylated cytosine.

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