Methylation of CpG Islands in the Promoter Regions of SOX9 Gene: Detection by Pyrosequencing

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Introduction
Mesenchymal stem cells have lower chondrogenic potential compared with chondrocytes. The authors have previously reported a lower gene expression of SOX9 in MSCs than that in Chondrocytes. In this study, we investigated whether this decreased expression of SOX9 gene is associated with increased methylation of CpG sites of MSCs compared with those of chondrocytes. In this study, we investigated SOX9 promoter methylation in mesenchymal stem cells (MSCs) and chondrocytes.

Materials and Methods
DNA samples and sodium bisulfite modification
Genomic DNA was extracted from the chondrocyte, MSC at passage 3, and MSC at passage 3 which was further cultured in a pellet for 21 days. Bisulfite modification of genomic DNA was performed using EZ DNA Methylation-Gold kit (Zymo Research, USA). The bisulfite reaction was carried out on 400ng gDNA: the reaction volume was adjusted to 20μl with sterile water and 130μl of CT conversion reagent were added. The sample tubes were placed in a thermal cycler and performed in the following steps: 10min at 98℃, 2 hours 30min at 64℃, and stored at 4℃. The DNA was purified using reagent in the kit.

Pyrosequencing analysis
Pyrosequencing was used for the methylation analyses of the SOX9 promoter. PCR reaction was carried out in a volume of 50μl with 20ng of converted gDNA, 5μl of 10X Taq buffer, 5unit Hot/Start Taq polymerase (Enzymics, Korea), 4μl of each 2.5mM dNTP mixture, 2μl of 10pmole/μl Primer-S, and 2μl of 10pmole/μl biotinylated-Primer-A. The amplification was carried by denaturing at 95℃ for 15min, followed by 45 cycles at 95℃ for 30sec, at 60℃ for 30sec, at 72℃ for 30sec and a final extension at 72℃ for 5min. The PCR reaction (5μl) was confirmed by electrophoresis in a 3% agarose gel and visualized by ethidium bromide staining. ssDNA template was prepared from 20-25μl biotinylated PCR product using streptavidin Sepharose® HP beads (Amersham Biosciences, Sweden). Fifteen picomoles of the respective sequencing primer were added for analysis. Sequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Biotage, USA). The methylation percentage was calculated by the average of the CpG sites analyzed.

Results
Several CpG sites of the SOX9 promoter were shown to be methylated in MSCs while few were so in chondrocytes (Figure 3). The ratio of methylated sites was 0% in chondrocytes and 7.3% in MSCs. After 3 weeks of pellet culture, the ratio of methylation decreased to 2.1%.

Discussion and Conclusions
The differences in the methylation of CpG sites in SOX9 promoter as demonstrated in this study may explain the lower level of SOX9 gene expression and lower chondrogenic potential of MSCs when compared with chondrocytes. Culturing MSCs in pellet may change the methylation profiles of MSCs.

References
1. Robertson KD. (2005) Nat Genet, 6, 597

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Fig 1. The principle of the pyrosequencing reaction. DNA polymerase elongates the single-stranded template DNA from the sequencing primer, which anneals near the mutation to be genotyped. dNTPs are sequentially added, and their incorporation results in the release of pyrophosphate molecules that are converted to ATP by the sulfurylase. These ATP molecules are converted into a detectable light by the luciferase, while the remaining nucleotides are degraded by the apyrase.

Fig 2. Example of a program The upper part indicates the SOX9 promoter sequences of the CpG island region. The percentage of CpG methylation is shown above and was directly taken from program evaluation. Analysis of CpG island methylation status of the promoter of SOX9 by pyrosequencing in MSCs and chondrocytes.

Fig 3. Methylation of SOX9 promoter Comparison of SOX9 methylation with chondrocytes, MSCs and pellet-cultured MSCs.