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Short Report

Methylation and chromatin conformation in the *U2af1-rs1* imprinted gene in the male germ cell line

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ABSTRACT Genomic imprinting is an epigenetic mechanism in mammals by means of which the differential expression of certain gene alleles is governed by their parental origin from generation to generation. Epigenetic features such as allelic DNA methylation and chromosome-specific chromatin conformation have been related with imprinting. We have analyzed modifications of methylation in the maternally imprinted *U2af1-rs1* gene in three stages of male germ cell line development. Our results showed that restriction sequences analyzed were completely unmethylated in primordial germ cells, which are pluripotential cells that are the origin of germ cell line development, (PGCs) of the EG-1 cell line and the methylation status increased progressively in stem spermatogonia and mature sperm cells. We also studied the chromatin organization of the *U2af1-rs1* imprinted gene in EG cells and stem spermatogonia cells. This analysis revealed that the chromatin region analyzed was more sensitive to DNase-I in male germ cell nuclei than in Sertoli cell nuclei.

Objectives

The parental imprint that is present in all somatic cells of the embryo is erased in germ cells, and a new sex-specific imprint will be established to be inherited by the next generation. At present, it is not known when the imprint is established during germ cell line development. In an attempt to clarify this point, we have studied the chromatin conformation and methylation state of the imprinted *U2af1-rs1* gene in three different stages of male germ cell line development. This gene is located on mouse chromosome 11 and encodes a protein with significant similarity to U2 small nuclear ribonucleoprotein auxiliary factor, an essential mammalian splicing factor. The *U2af1-rs1* gene is methylated on the maternal allele and expressed from the paternal allele. We have studied this imprinted gene in PGCs, in stem spermatogonia and in mature sperm cells.

Materials and Methods

For the experiments with primordial germ cells we used cells of the EG-1 cell line. These embryonic germ cells were obtained from 8.5 dpc embryos and cultured on a mouse embryonic fibroblast feeder layer. The culture medium was supplemented with LIF (Leukemia Inhibitory Factor) and SF (Steel Factor) as growth factors. For our studies we isolated the stem spermatogonia cell population of 6-7 day old mice testes by recovering suspended cells from culture of seminiferous tubuli that were digested with Colagenase-II enzyme. Also, somatic cells adherent to culture flasks were included in our experiments. A Sertoli cell line, MSC-1, was used as control to somatic population. Completely differentiated male germ cells, sperm cells, were obtained from the deferent conducts and epididimus of 12-16 week old mice.

Chromatin organization was assayed by sensitivity to DNase-I in isolated nuclei. Nuclei from cells of the three differentiation stages were obtained as described in Feil *et al.,* 1997. Nuclease sensitivity assays were performed by adding DNase-I at 0, 10, 20, 30, 50, 100, 250 and 500 units/ml to aliquots of the nuclear suspension and incubating them for 10 min. at 25ºC. Proteinase K was added to 100 µg/ml and digestion was carried out overnight at 37ºC. The DNA was isolated by phenol:chloroform:isoamylalcohol (25:24:1) extraction. Genomic DNA was precipitated in isopropanol with 250 mM NaCl and dissolved in Tris:EDTA (10 mM:1 mM). For the methylation assays, cells were lysed with lysis buffer followed by digestion with Proteinase K at 37ºC overnight. The DNA isolation was performed as described for the nuclease sensitivity assays.

BglII, *NotI* and *HpaII* restriction enzymes were used in the *U2af1-rs1* methylation assays, as *NotI* and *HpaII* are sensitive to methylation. To perform the chromatin structure study, DNAs isolated after DNase-I digestion were digested only with BglII. Incubations with restriction endonucleases were developed for 5 h at 37ºC. Restricted DNAs were subjected to electrophoresis on 1% agarose gels in 1x TBE buffer, alka-

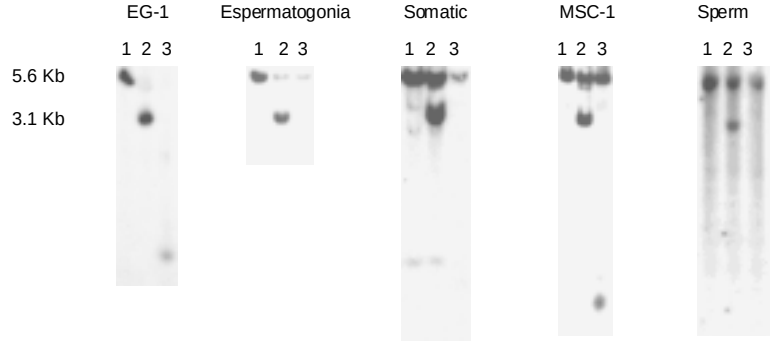

Fig. 1. *U2af1-rs1* methylation status. *Lanes 1-3 correspond to DNA digested with BglII, BglII+NotI and BglII+HpaII respectively.*

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Note: The authors mistakenly referred to "EG3" instead of "EG1" in the original printed version of this paper.

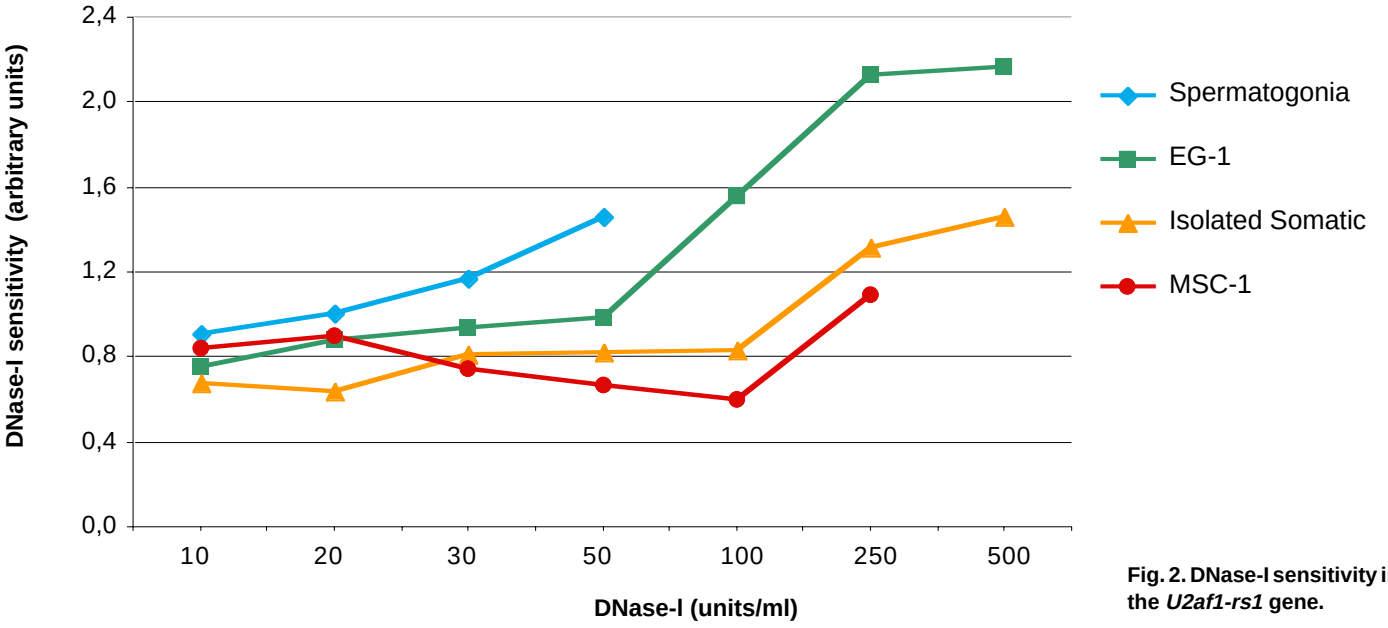

Fig. 2. DNase-I sensitivity in the *U2af1-rs1* gene.

line blotted onto nylon membrane and UV cross-linked. After prehybridization, hybridization was performed at 65ºC in the presence of *U2af1-rs1*-probe-1 labeled with [α^{32}P]-dCTP by Random Primed DNA Labeling Kit (Boehringer Mannheim). The probe corresponds to a 550-base pair EcoRI fragment of the *U2af1-rs1* upstream region.

Results and Conclusions

When DNA was digested with *BglII*, a 5.6 Kb band appeared in the autoradiogram, corresponding to the 5'-UTR region of the *U2af1-rs1* gene. The 3.1 Kb band corresponds to the unmethylated NotI restriction site. The methylation assays results showed that NotI and HpaII restriction sites were completely unmethylated in PGCs of the EG-1 cell line. The analysis of autoradiograms by densitometry revealed that the *NotI* and *HpaII* restriction sequences were partially unmethylated in stem spermatogonia cells (69% and 72% respectively). However, in isolated somatic cells, 50% and 80% of the NotI and HpaII restriction sites respectively were unmethylated. Sertoly cells of the MSC-1 cell line exhibited the same status of NotI restriction site methylation (50%) as isolated somatic cells, whereas in these cells also half of the HpaII restriction sites were unmethylated. Finally, mature sperm cells showed partial methylation in both of the restriction sequences analyzed.

The DNase-I assays showed a pronounced difference between nuclei from germ cells (stem spermatogonia and PGCs) and Sertoli cells' (somatic cells of primary cultures and MSC-1 cells) in the genetic *U2af1-rs1* region limited by *BglII* restriction sites.

The methylation assays of the 5'-UTR region analyzed in the maternally imprinted *U2af1-rs1* gene showed that NotI and HpaII restriction sites were completely unmethylated in PGCs and partially methylated in stem spermatogonia cells. In mature sperm cells the grade of methylation in these genetic sequences rose to 50%.

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