

# DNA methylation of the *U2af1-rs1* gene in embryonic stem cells and embryonal carcinoma cells after RA-induced differentiation and apoptosis

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## Introduction

Embryonic stem (ES) cells, derived from the inner cell mass of blastocysts, are totipotent and exhibit a large capacity for differentiation. AB1 ES cells were employed in our assays, based on previous studies where, using embryonal carcinoma (EC) cells, it was demonstrated that *in vitro* treatment with retinoic acid (RA) gave rise to cellular differentiation as well as apoptosis (Asumendi *et al.*, 2000; Atencia *et al.*, 1994). Apoptosis is a genetically controlled, selective cellular death mechanism, which occurs during biological processes of embryonic development, differentiation, tissue remodeling and regulation of cell populations (Vaux and Korsmeyer, 1999). Together with morphological changes, biochemical features such as the characteristic laddering of DNA after electrophoresis in agarose gels are usually observed. Furthermore, the role of methylation in the regulation of gene activity has become apparent in recent years. In this work, we have analyzed the final fragmentation of DNA, studying the *U2af1-rs1* gene that codes for a transcriptional factor. The possible relationship between chromatin fragmentation and DNA methylation has been studied in AB1 ES cells and F9 and P19 EC cells after retinoic acid (RA) exposure.

## Materials and Methods

### Cell culture

AB1 ES cells were cultured in gelatinized flasks, in ES medium containing 1000 units/ml LIF (Feil *et al.*, 1994). Both cell differentiation and apoptosis were induced by culture in the absence of LIF together with exposure to 1  $\mu$ M all-*trans* retinoic acid (Sigma) during 48 or 72 h, from a 1 mM stock solution dissolved in DMSO. F9 EC cells were cultured in gelatinized flasks and P19 EC cells in tissue culture treated plastic flasks. Both lines were grown as previously described (Atencia *et al.*, 1994). Cell differentiation and apoptosis were induced by 1  $\mu$ M all-*trans* retinoic acid during 48 or 72 h.

### Flow cytometry

AB1, F9 and P19 harvested cells from non-treated and 72 h 1  $\mu$ M RA-treated cultures together with their respective supernatants were centrifuged at 1740 g for 5 min, fixed in 70% ethanol in phosphate-buffered saline (PBS) during 1 h, centrifuged again and suspended into a solution containing 40 mM propidium iodide (Sigma) and 15 mM RNase A (Boehringer Mannheim) in PBS. After 30 min at 37°C, samples were analysed using a Coulter EPICS ELITE ESP flow cytometry.

## Methylation assays and Southern blotting

AB1, F9 and P19 cells from non-treated and 72 h 1  $\mu$ M RA-treated cultures as well as their supernatants were collected and lysed with lysis buffer followed by digestion with proteinase K (Boehringer Mannheim) at 37°C overnight. 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). DNAs were incubated with *Bgl*II (which delimited the studied *U2af1-rs1* gene) alone or in combination with *Not*I and *Hpa*II methylation dependent restriction enzymes and with *Msp*I and *Hind*III methylation independent enzymes (Amersham) during 5 h at 37°C. Restricted DNA electrophoresis and Southern blotting were performed. After prehybridization, hybridization was carried out in the presence of a mouse *U2af1-rs1* probe-1 labelled with [ $\alpha$ -<sup>32</sup>P]-dCTP by Random Primed DNA Labelling Kit (1004 760, Boehringer Mannheim).

## Results and Discussion

RA-induced AB1, F9 and P19 cell differentiation was measured as a decrease in the proliferative population (S-G<sub>2</sub>/M fraction), being more evident in AB1 cells (Table 1). In addition, the non-proliferative population (G<sub>0</sub>/G<sub>1</sub> fraction) increased after treatment, especially at 48 h in AB1 cells and at 72 h in F9 and P19 cells. RA treatment also induced apoptotic cell death in all cell lines, observed by sub-G<sub>0</sub>/G<sub>1</sub> fraction increments. An accumulation of cells in the sub-G<sub>0</sub>/G<sub>1</sub> fraction was mainly observed at 72 h of treatment. Moreover, AB1 cells were more sensitive to RA-induced cell death, showing nearly a 6-fold increment with respect to control.

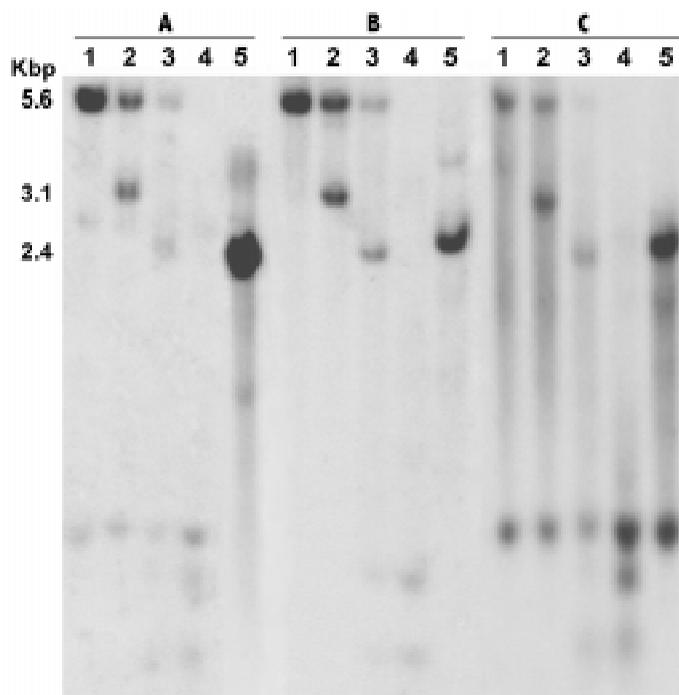
We analyzed the methylation status of the *U2af1-rs1* gene in AB1, F9 and P19 undifferentiated cells, RA-differentiated cells and apoptotic bodies from supernatants, using several restriction enzymes such

TABLE 1

APOPTOSIS AND CELL CYCLE PHASE DISTRIBUTION OF UNTREATED AND 1  $\mu$ M RA-TREATED AB1 ES CELLS AND F9 AND P19 EC CELLS

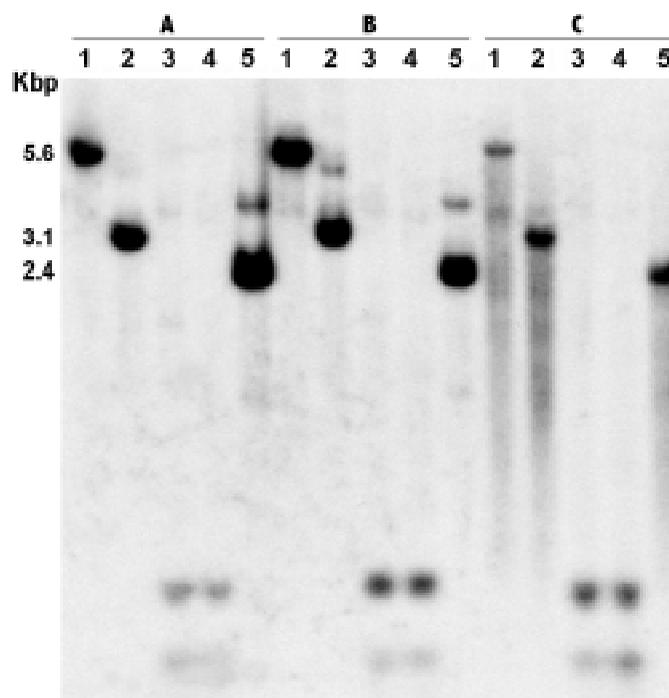
Time of treatment with RA (h)	Cell cycle distribution (%)								
	AB1 ES cells			F9 EC cells			P19 EC cells		
	S-G <sub>2</sub> /M	G <sub>0</sub> /G <sub>1</sub>	Sub-G <sub>0</sub> /G <sub>1</sub>	S-G <sub>2</sub> /M	G <sub>0</sub> /G <sub>1</sub>	Sub-G <sub>0</sub> /G <sub>1</sub>	S-G <sub>2</sub> /M	G <sub>0</sub> /G <sub>1</sub>	Sub-G <sub>0</sub> /G <sub>1</sub>
0	62	31	7	61	33	7	49	39	12
48	34	47	19	62	30	9	34	39	27
72	25	34	41	37	36	27	31	44	26

Data are expressed as percentage of cells in three separate experiments.



**Fig. 1. Methylation pattern analysis of the *U2af1-rs1* gene in AB1 ES cells by Southern blotting.** The blot represents DNA fragments following restriction endonuclease digestions with *Bgl*II alone (lane 1) or in combination with *Not*I, *Hpa*II, *Msp*I or *Hind*III (lane 2, 3, 4 or 5, respectively). (A) undifferentiated cells, (B) differentiated cells and (C) apoptotic bodies.

as *Bgl*II, *Not*I, *Hpa*II, *Msp*I and *Hind*III. Concerning AB1 control cells, they presented a partially methylated pattern with respect to GCGGCCG and CCGG sites, as illustrated the digestion performed by *Not*I and *Hpa*II/*Msp*I enzymes respectively (Fig. 1A, lanes 2,3,4). On the other hand, F9 (data not shown) and P19 control cells presented a fully unmethylated pattern with respect to those sites (Fig. 2A, lanes 2,3,4). In several works, the activity of *Not*I and other nucleases that specifically cleave CpG islands in euchromatin has been associated with the fragmentation cascade down to the 200 bp ladder formation (Chem *et al.*, 1998; Qui and Sit, 1998). Due to the fact that *Not*I and *Hpa*II are methylation dependent restriction enzymes, it is conceivable that changes in DNA methylation status may be associated with chromatin fragmentation during the apoptotic process. Our results revealed that the general pattern of endonuclease digestion observed in undifferentiated cells was similar for the corresponding differentiated cells and apoptotic bodies in each of the three cell lines studied (Figs. 1 and 2; B and C). In apoptotic bodies, the integrity of *U2af1-rs1* gene was demonstrated by the presence of a 5.6 kbp band delimited by the *Bgl*II sites, in spite of the characteristic apoptotic partial fragmentation of the gene indicated by a smear below the 5.6 kbp band observed in the same lane (Figs. 1 and 2C; lane 1). Similarly, the single *Not*I restriction site and the *Hpa*II/*Msp*I multiples sites placed into the *Bgl*II delimited sequence were not specifically targeted by endonuclease activity during chromatin fragmentation. This can be observed especially in the partially methylated AB1 cell line, where the corresponding 3.1 and 2.4 kbp bands of restricted sequences appeared (Fig. 1C; lanes 2,3,4).



**Fig. 2. Methylation pattern analysis of the *U2af1-rs1* gene in P19 EC cells by Southern blotting.** The blot represents DNA fragments following restriction endonuclease digestions with *Bgl*II alone (lane 1) or in combination with *Not*I, *Hpa*II, *Msp*I or *Hind*III (lane 2, 3, 4 or 5, respectively). (A) undifferentiated cells, (B) differentiated cells and (C) apoptotic bodies.

Similar results are shown for the *Hind*III restriction site (Fig. 1 and 2; A,B and C; lane 5).

In summary, apoptotic cell death associated with RA-induced differentiation was detected in the ES AB1 cell line as well as in the EC F9 and P19 cell lines. The apoptotic fraction obtained was noticeably higher in AB1 cells. The methylation status of the *U2af1-rs1* gene in undifferentiated embryonic stem cells and teratocarcinoma cells remained unaltered following cell differentiation or apoptosis. Moreover, analysis of the restriction pattern of the *U2af1-rs1* gene showed that the fragmentation observed during apoptosis did not specifically affect the restriction sites analyzed.

## References

- ASUMENDI, A., ANDOLLO, N., BOYANO, M.D., HILARIO, E., PEREZ-YARZA, G., ATENCIA, R., ARECHAGA, J. and GARCIA-SANZ, M. (2000). The role of cleavage of cell structures during apoptosis. *Cell Mol. Biol.* 46(1): 1-11.
- ATENCIA, R., GARCIA-SANZ, M., UNDA, F. and ARECHAGA, J. (1994). Apoptosis during retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *Exp. Cell Res.* 214(2): 663-667.
- CHEM, D.L., SWE, M. and SIT, K.H. (1998). G-band expression and megabase fragmentation in apoptosis. *Exp. Cell Res.* 240: 293-304.
- FEIL, R., WALTER, J., ALLEN, N.D. and REIK, W. (1994). Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes. *Development* 120: 2933-2943.
- QUI, L. and SIT, K.H. (1998). Euchromatin megabase cleavages and conjoint apoptotic-autophagic death expression with nucleolar ball-and-socket joint dislocations in human Chang liver cells arrested in S-phase by etoposide. *Eur. J. Cell. Biol.* 77: 239-246.
- VAUX, D.L. and KORSMEYER, S.J. (1999). Cell death in development. *Cell* 96: 245-254.