Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 91 (2004) 1–9
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Methylation analysis of the promoter F of estrogen receptor α gene: effects on the level of transcription on human osteoblastic cells

Letizia Penolazzi [a], Elisabetta Lambertini [a], Silvia Giordano [a], Vincenzo Sollazzo [b], Giancarlo Traina [b], Laura del Senno [a], Roberta Piva [a,*]

[a] *Dipartimento di Biochimica e Biologia Molecolare, Università degli Studi di Ferrara, Via L. Borsari, 46 44100 Ferrara, Italy*
[b] *Dipartimento di Scienze Biomediche e Terapie Avanzate Sezione di Clinica Ortopedica, Università degli Studi di Ferrara, Via L. Borsari, 46 44100 Ferrara, Italy*

Received 23 September 2003; accepted 2 February 2004

Abstract

In this study, the methylation status of the distal promoter F of estrogen receptor alfa (*ERα*) gene in human osteoblastic cells was investigated. The activity of this promoter is responsible for the *ERα* gene transcription in bone tissue. The methylation status of promoter F was here evaluated, for the first time, by direct sequencing of bisulfite-treated genomic DNA, at 10 CpG specific sites localized in a region of about 800 bp. An heterogeneous methylation pattern was observed. The most notable difference was found at four particular CpGs, distant from the exon F transcription start site, showing a methylation status that correlates with the expression level, being ERα mRNA transcription reduced in a partially methylated cells but preserved in demethylated cells. The other CpG sites, localized around the transcription start site, were always demethylated except for MG-63 cells showing the lowest level of ERα expression. By quantitative RT-PCR analysis we demonstrated that *ERα* gene expression was higher in primary osteoblasts than in bone-derived cells (MG-63 and SaOS-2) and in all cases the ERα mRNA is represented by the isoform F. The same 10 CpG sites were investigated in non-osseous cell lines and were found fully methylated in ERα-negative breast cancer cells (MDA-MB-231) and completely demethylated in ERα-positive breast cancer cells (MCF7). The overall results suggest that methylation of the CpG sites inside *ERα* gene promoter F here analyzed may contribute to ERα transcriptional control, directly or indirectly, influencing the tissue specific expression of the gene.

Keywords: DNA methylation; Estrogen receptor alfa; Osteoblasts

1. Introduction

Research in recent years has provided evidence for the involvement of the two isoforms of human estrogen receptor (hERα and hERβ) in the classical estrogen target tissues, such as breast and endometrium, and non-classical estrogen target tissues such as brain, bone, the cardiovascular system and adipose tissue [1]. It is well known that the estrogen receptors are members of the nuclear receptor superfamily and regulate the expression of target genes as ligand-inducible transcription factors [1,2].

Different tools to study the role of estrogen receptors in physiology and disease, and the regulation of their expression are described. In the case of *hERα* gene the issue is particularly intricate because the generation of hERα transcripts involved at least eight promoters and eight upstream exons whose utilization varies between different cell types [3]. The regulation at the level of the different promoters is certainly a key event in hERα mRNA formation that is controlled by both transcription factors and by epigenetic phenomena such as DNA methylation that is commonly associated with gene silencing [3–6]. Increasing evidences suggest that DNA methylation is associated with inhibition of hERα transcription in various forms of diseases including neoplastic and atherosclerotic lesions [7,8]. However, the transcriptional impact of the position and length of methylated zones relative to the single promoters and the coding region of the gene remains quite unclear.

This study investigates the methylation level of a number of CpG sites within the promoter F (PF) of the *hERα* gene and its relationship with regulation of *hERα* gene expression in osteoblasts. It is well known that estrogen has complex effects on the skeleton [9,10], including regulation of bone turnover, maintenance of bone mass and skeletal development, even if the complete mechanisms of its action

* Corresponding author. Tel.: +39 0532 291446; fax: +39 0532 202723.
E-mail address: piv@unife.it (R. Piva).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.02.005

are still unclear. Therefore, hERα being the main responsible for the effects of estrogen in osteoblasts, together with hERβ, may be considered an important factor in the maintenance of bone health, acting to conserve bone mass.

It was recently described that the promoter F of *hERα* gene plays a major role in the generation of hERα mRNA in bone [11], and we have demonstrated, by gene reporter assay, DNase footprinting and EMSA experiments, that the PF region from −117873 to −117140 belongs to a regulatory context that is crucial in the gene function [12].

Here we paid attention to the methylation of this regulatory region, that does not contain a typical CpG-rich island, to understand if this DNA modification may be involved in the process of loss of *hERα* gene expression in bone during, for example, osteoporosis and ageing process.

We determined which cytosine residues in the CpGs of promoter F are methylated in four human primary osteoblasts and two osteosarcoma cell lines, MG-63 and SaOS-2, in comparison with hERα-positive and -negative breast cancer cell lines. Then, we analyzed the functional consequences of methylation status of promoter F by measuring the hERα mRNA transcripts levels, both for canonic and F isoform, by quantitative RT-PCR, and the protein level by immunocytochemical analysis.

At present, several molecular biology methods are routinely used to determine the methylation status of a specific DNA sequence [13]. Among these, bisulfite nucleotide sequencing is a standard technique for detailed mapping of methylated cytosine residues within a gene promoter with several CpG sites or with a typical CpG-rich island [14]. This method, relies on the ability of sodium bisulfite to deaminate cytosine residues into uracil in genomic DNA, whereas the methylated cytosine residues are resistant to this modification. The target DNA is then amplified by PCR with specific primers to yield fragments in which all uracils are converted to thymine residues, whereas methylated cytosine residues are amplified as cytosine. The PCR products are sequenced and the methylation status of individual CpG sites is then analyzed by comparing it with the unmodified sequences. Using this method, we have investigated the status of promoter F CpGs methylation to correlate it with ERα expression in osteoblats.

2. Materials and methods

2.1. Patient samples and cell lines

Recruitment of subjects donating osteoblasts was in accordance with approved procedures and the informed consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used. Bone samples were collected during surgery following Hardinge's surgical approach to the hip and the bone was cultured as previously described [15]. Briefly, bone was cut into small pieces that were rinsed and then cultured in Eagle's minimum essential medium (Sigma Aldrich, Chemical Co., St. Louis, MO, USA) supplemented with 20% fetal bovine serum (Invitrogen Corporation, CA, USA), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbate, at 37 °C in an humidified atmosphere of 5% CO_2. After about 5–7 days, outgrowth of bone cells started from the bone chips and confluency in 9 cm^2 dishes was usually reached after 4–6 weeks. For these studies, only first passage cells were used.

MG-63 and SaOS-2 osteosarcoma cell lines were grown in the same culture conditions.

2.2. Bisulfite treatment

Genomic DNA was modified by bisulfite treatment as detailed [16]. Briefly, 2–10 μg of genomic DNA was digest with *Eco*RI restriction endonuclease, then denatured by adding freshly prepared NaOH (final concentration 0.3 M) for 15 min at 37 °C. The urea/bisulfite solution and hydroquinone are added to the denatured DNA to final concentration of 5.36 M, 3.44 M and 0.5 mM, respectively. The samples were then incubated under mineral oil and subjected to 20 cycles of 15 min at 55 °C followed by denaturation at 95 °C for 30 s using a Violet Thermal Cycler. The Wizard™ DNA Clean-Up kit (Promega Corporation, WI, USA) was used to purify the modified DNA. After purification, the samples were treated with NaOH at a final concentration of 0.3 M in a volume of 100 μl for 15 min at 37 °C, then neutralized with 10 M NH_4 OAc (pH 7.0). The DNA was ethanol-precipitated and resuspended in water for methylation-specific PCR analysis.

Bisulfite-converted DNA was PCR amplified using the methylation-independent primers shown in Table 1. Each PCR mixture contained 100 μM deoxynucleotide triphosphates, 1 μM sense and antisense primers and 1.25 U of Platinum™ High Fidelity Taq DNA polymerase (Invitrogen Corporation, CA, USA). Each PCR program was as follows: 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at the specific temperature

Table 1
Summary of the primers and annealing temperatures for PF methylation analysis

Primers	Sequence	Annealing (°C)
F1m	5′-A*T*TG*T*ATAAA*T**T*A*T*A*T**T*AGA*T*AT-3′	48
F2m	5′-TAGTGTTAAA*T*AAA*T*AAT-3′	38
F3m	5′-GTAG*T*TAA*T*TTTGGATTAA*T*AA-3′	48
F4m	5′-*T*AGG*T*AGATTAA*T*A*T*ATGAT-3′	44
F5m	5′-TGGTGGTG*T*TAGTT*T*TTT-3′	42
R1m	5′-ACT*A*TCTTCTTAT*A*CTATA*A*AAT-3′	50
R2m	5′-TATCTTCT*A*T*A*A*A*T*A*TTT-3′	38
R3m	5′-TCTAACTTTCTTA*A*CACTTCCA-3′	51
R4m	5′-AT*A*ACCAT*A*AAATTA*A**A*AAAT*A*CA*A*T-3′	55
F1	5′-AGACATGATGAGATATTAAAATGT-3′	54
R2	5′-TATCTTCTGTGAGTGTTT-3′	42

listed in Table 1 for 45 s, and finally, a 45 s extension at 72 °C. A final 10-min extension at 72 °C completed each PCR program. A second round of PCR with nested primers reported in Table 1 was performed to obtain PCR products for sequencing. PCR products were then fractionated on 1% agarose gels, excised, and purified with the CONCERT™ Rapid Gel Extraction System (Invitrogen Corporation, CA, USA), according to manufacturer's recommendations. The amplified fragments were then sequenced by the dideoxynucleotide chain-termination method.

2.3. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in sub-confluent osteoblastic cells by the hydrolisis of *p*-nitrophenylphosphate (PNPP) according to Ibbotson [17]. Enzyme activity was expressed as U/μg protein. One unit was defined as the amount of enzyme which hydrolyses 1 μmol of PNPP/min. Cell protein was determined according to Lowry method. Effect of 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) on ALP activity was verified after incubation in medium containing 10 nM 1,25-$(OH)_2D_3$ for 48 h.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen Corporation, CA, USA), and quantitative real-time PCR analysis was performed essentially as described [18].

Reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland, CH). The PCR 2× Master Mix was based on AmpliTaq Gold DNA polymerase (Applied Biosystems, Rotkreuz, Switzerland, CH). In the same reaction, cDNA samples were analyzed both for canonical ERα or isoform F and the reference gene (*GAPDH*), using a multiplex approach as described in the Perkin-Elmer User Bulletin N. 2. The probe for GAPDH was fluorescently labeled with VIC, whereas probes for the genes of interest were labeled with FAM. Cycle temperature and times were as previously described [18]. Expression levels were calculated by normalizing the mRNA amount to the GAPDH RNA using the procedure with the $2^{\Delta\Delta Ct}$ formula as previously described [18].

2.5. Immunocytochemistry

Immunocytochemistry analysis for hERα, hERβ and OPN was performed employing the streptavidin–biotin method using Ultraystain Polyvalent-HRP Immunostaining Kit (Ylem, Rome, Italy). Cells grown in chamber slides were fixed in cold 100% methanol, and permeabilized with 0.2% (v/v) Triton X-100 (Sigma Aldrich, Chemical Co., St. Louis, MO, USA) in Tris-buffered saline (TBS). Cells were incubated in 3% H_2O_2 and the endogenous peroxidase was blocked with Super Block reagent (Ultraystain Polyvalent-HRP Immunostaining Kit, Ylem). Afterwards, the primary antibody, a monoclonal antibody for hERα (rat anti-human H222, 1:60 dilution—a generous gift from Prof. G. Green, Chicago, USA) or polyclonal antibody for hERβ (rabbit anti-human, 1:60 dilution—Santa Cruz Biotec., USA) or OPN (rabbit anti-human LF-123,1:120 dilution—a generous gift from Dr. L.W. Fisher, Bethesda, MD, USA) were applied and incubated at 4 °C overnight. Cells were then incubated at room temperature with anti-polyvalent biotinylated antibody (Ultraystain Polyvalent-HRP Immunostaining Kit, Ylem). After rinsing in TBS, streptavidin HRP (Ultraystain Polyvalent-HRP Immunostaining Kit, Ylem) was applied, and followed by the addition of substrate-chromogen mix (AEC Cromogeno kit, Ylem). After washing, cells were mounted in glycerol/PBS (9:1) and observed using a Leitz microscope.

To avoid the variance in the intensity of immunostaining among experimental groups, the control and experimental cells were immunostained at the same time.

3. Results

3.1. CpG sites in the promoter F of ERα gene

In Fig. 1 a schematic representation of the *hERα* gene promoter F is reported. We examined the methylation status of the 10 CpG sites localized in the region from −117873 to −117140, belonging to distal exon F promoter which is used in bone tissue to generate the so-called mRNA isoform F. The sequence of the promoter F was previously characterized for typical promoter elements and possible *cis*-regulatory elements. In particular, we found binding sites for transcription factors that are critical for osteoblast differentiation, establishment of the skeleton and organ development, such as TAAT elements, Runx2/Cbfa1, Sry and Sox consensus sequences [12].

3.2. Mapping of methylation status of PF promoter of ERα gene by bisulfite modification

A methylation semi-quantitative analysis of single CpG sites was performed using the sodium bisulfite conversion of genomic DNA with the specific primers summarized in Table 1.

We compared MG-63 and SaOS-2 osteosarcoma cell lines, and four primary cultures of osteoblasts (hOB) obtained from surgical procedures on patients of different age. Before methylation analysis, all samples were tested for the level of bone differentiation, analyzing the activity of alkaline phosphatase, which is induced early in the osteoblast differentiation process. As reported in the *Panel A* of Fig. 2, both MG-63 and SaOS-2 cells and all primary hOBs analyzed were ALP positive confirming their osteoblastic phenotype.

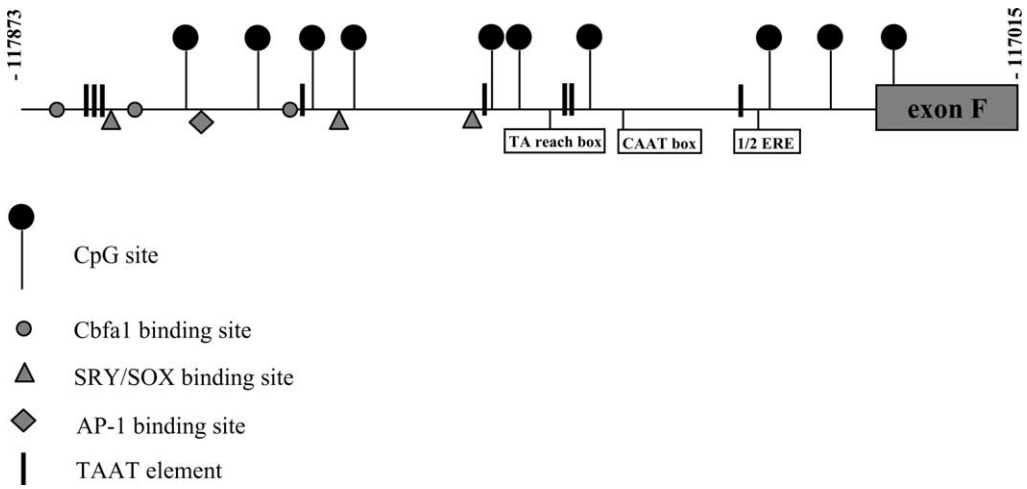

Fig. 1. A schematic representation of the *hERα* gene promoter F region from −117873 is shown. The nomenclature of upstream exon F is in accordance with the recently described genomic organization of the *hERα* gene as suggested by Kos et al. [32]. The 10 CpG sites in the region analyzed are reported. The TA reach box, CAAT box, 1/2 ERE and some *cis*-regulatory elements (the symbols are reported in the panel) are also indicated.

PCR products, obtained as reported in Section 2, were then subjected to DNA sequencing. Examples of methylation analysis performed on the reverse strand of the DNA fragment −117611/−117641 are reported in the *Panel B* of Fig. 2: CpG-methylated cytosines remained as cytosines (guanine in the reverse strand), whereas unmethylated cytosines changed to thymidines (adenine in the reverse strand) in the PCR products. When the residues were partially methylated comigrating bands corresponding to cytosines and thymidines were observed. The efficiency of the method was tested comparing the sequencing of the samples before and after bisulfite treatment: in the example reported in the figure the conversion of guanines to adenines in the reverse strand of DNA from MDA-MB-231 cells is clearly evident.

As negative and positive references, the CpG methylation level of the PF promoter in two non-osseous cell lines, the human breast cancer MDA-MB-231 which does not express the *ERα* gene, and the MCF-7 breast cancer cells which express *ERα* gene at very high level, was also analyzed. A summary of overall data obtained from the different samples analyzed with this experimental approach is reported in the Fig. 3. The sequence analysis of the specific PCR products obtained revealed an heterogeneous methylation status of the promoter F. MG-63 cells were partially methylated, SaOS-2 partially demethylated, while all CpG sites analyzed were methylated in MDA-MB-231 cells, and completely unmethylated in MCF7. In all samples, except MDA-MB-231 cells, the CpG site at position 8, which is proximal to the transcription initiation site, is always completely unmethylated also in the primary osteoblasts. In the osteoblastic cells, the major methylation targets are cytosine residues at positions 2 and 3: MG-63 showed 100% methylation, SaOS-2 showed approximately 50% and three of the four patients showed a level of methylation between 50 and 75%. Methylation is more variable at n.1 and n.4 CpG sites. Only the osteoblasts obtained from the patient p.1 that is the oldest one showed all CpG completely unmethylated, whereas the DNA from the other primary osteoblast cultures showed a methylation pattern that is intermediate between that observed in the osteosarcoma cell lines DNA.

3.3. Methylation of the promoter F versus transcriptional activity and ERα protein expression

To assess the functional consequences of promoter F methylation, the ERα mRNA expression was quantified by quantitative RT-PCR in the cell lines and in the osteoblasts from the four patients.

ERα mRNA expression was measured both at canonical and at ERα F isoform content using primers flanking exons 5–6 and upstream exons F–E of *ERα* gene, respectively. As shown in the Fig. 4, the samples analyzed presented different levels of ERα mRNA. The levels of both ERα transcripts in SaOS-2 cells, that are significantly more demethylated in comparison with MG-63 cells, were about 10 fold higher than the levels found in MG-63 cells. The osteoblasts obtained from the patients, also when partially methylated, expressed ERα mRNA at appreciable levels.

As expected, no ERα expression was found in MDA-MB-231 cells whereas both transcripts are present at high level in MCF7 cells.

mRNA expression data were correlated with protein level by immunocytochemical analysis as reported in Fig. 5. The presence of ERα protein was demonstrated both in MG-63 and SaOS-2 cells. The hOBs having a number of cells suitable for this analysis were those from the patients p.3 and p.4. Osteoblasts derived from the female patient p.3 revealed a more intense ERα nuclear immunoreactivity in comparison with that observed in the osteoblasts from the male patient p.4, presenting an immunoreactivity restricted to the cytoplasm. High level of ERα protein was also observed in

(A)

	Sex	Age (years)	ALP (U/μg protein)
MG-63	male		±2.8 ± 0.7
SaOS-2	female		± 18 ± 0.5
p.1	female	76	± 42 ± 1.2
p.2	female	21	± 39 ± 0.5
p.3	female	58	± 53 ± 1
p.4	male	74	± 37 ± 0.7

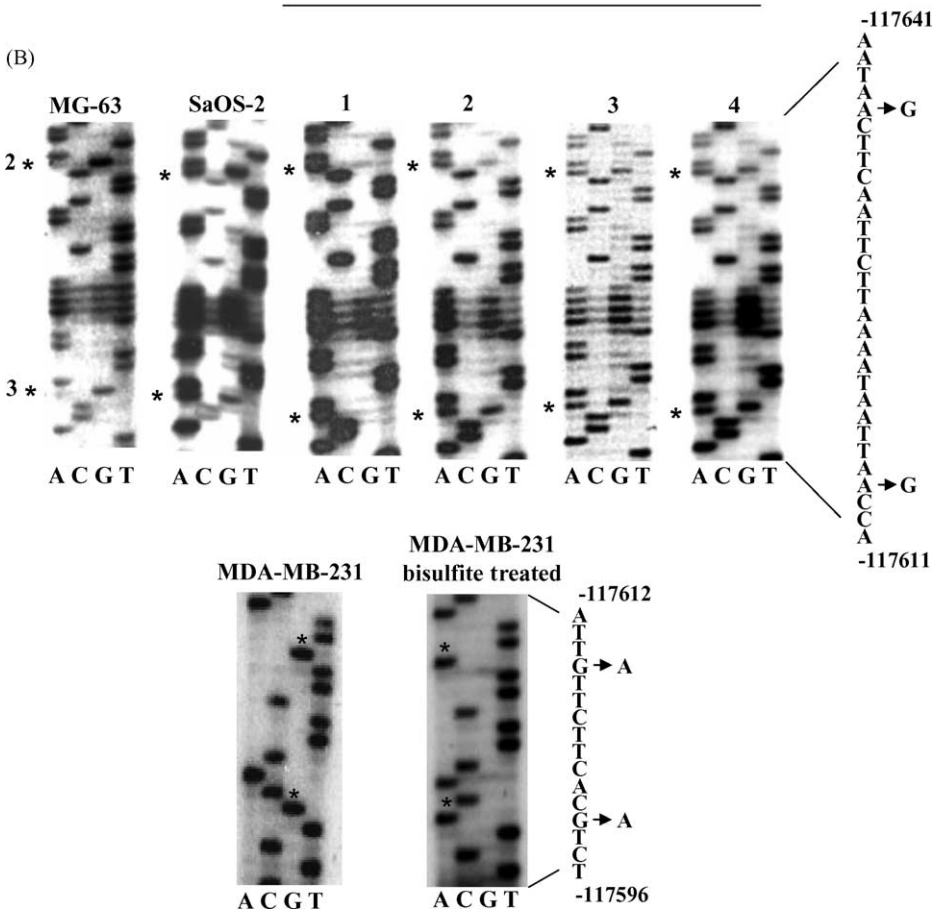

Fig. 2. The alkaline phosphatase activity (ALP) was valuated in MG-63 and SaOS-2 osteosarcoma cell lines and in four primary osteoblasts (p.1–4) whose sex and age are also reported (*Panel A*). ALP activity is expressed as U/μg protein after 48 h of 1,25-dihydroxyviamin D3 treatment. Values are the mean ± S.D. of three independent experiments. DNA from MG-63 and SaOS-2 cell lines and from four primary osteoblasts (1–4) was modified by bisulfite conversion. Converted DNA was then amplified, and PCR products were sequenced. In the non-coding strand, the sequence is from the original strand (cytosine converted to thymines); in the coding strand the sequence is from the complementary strand (guanines are converted to adenines). In the *Panel B*, a representative sequencing of a region from −117641 to −117611 (reverse strand) is reported. The symbol (*) indicates the location of two CpG sites belonging to the sequence reported. In the lower part of the panel, an example of bisulfite treatment efficiency assay, on DNA from MDA-MB-231 breast cancer cells, is reported. The sequencing of the sample before and after the chemical treatment was compared: the conversion of G to A in the reverse strand is clearly evident.

MCF7 control cells, but not in MDA-MB-231 cells in according with the literature data. hOBs samples resulted also positive for the immunodetection of osteopontin that is a typical osteoblastic marker involved in normal tissue remodeling processes such as bone resorption [19].

As positive control, we also analyzed ERβ protein expression that was detected at different levels in all samples analyzed.

4. Discussion

Research in the recent years has provided evidence for the involvement of DNA methylation of nuclear receptor genes mainly in the development and progression of human diseases in particular malignancies [7,20,21]. Several reports clearly demonstrated that selective hypermethylation can selectively silence multiple promoters of steroid receptors in

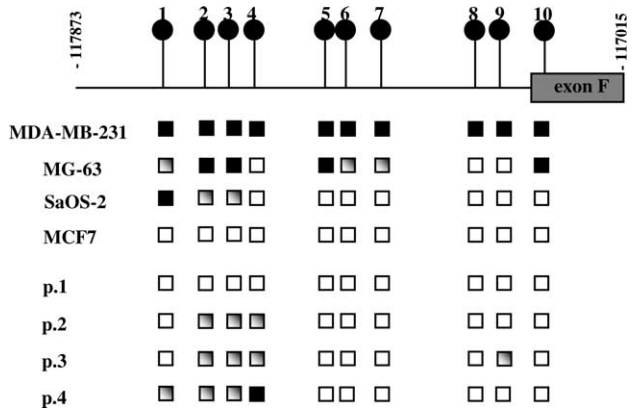

Fig. 3. Summary of bisulfite sequencing data of 10 single CpG sites. (■), 100% methylated; (❒), 50–75% methylated; (□), unmethylated.

carcinogenesis [22,23]. Nevertheless, it is well known that altered DNA methylation patterns play an important role also during physiological processes like senescence [5,24–26]. In respect of this issue, it is important to be considered that the normal bone aging is associated with a loss of bone mass which may be related to a decrease of ERα expression. Only in some tissues, such as breast, endometrium, colon, prostate and cardiovascular muscle cells an association between loss of ERα expression and promoter methylation was detected [20,27–31], while no correlation was observed in other cases. This may be due to the different regulation that the several tissue specific *ERα* gene promoters may have [32]. The methylation status of promoter regions as mechanism that potentially regulates *hERα* gene expression was up to now investigated mainly at A, B and C promoters that are clustered in a restricted region of the large and complex 5′ sequence of the gene. Several studies, performed both in tissues and cell lines, have demonstrated that selective hypermethylation can selectively silence these multiple promoters in breast, endometrial and prostate carcinogenesis, or in aortic smooth muscle cells [20,27–31]. Here we analyzed the possibility that the DNA methylation of the promoter F, that is the prevalent promoter used in osteoblastic cells may interfere with the process of promoter F activity, and, therefore, with the ERα transcription in bone.

To date only a few studies addressed the question regarding epigenetic alteration of crucial genes associated specifically with osteoblastic differentiation. For example, Villagra et al. [33] described methylation analysis of critical regions of the osteocalcin gene, coding for a bone specific calcium-binding protein.

To investigate whether the expression of mRNAs for *ERα* gene is inactivated in osteoblasts by specific methylation status of the CpG inside the promoter F, in the present study we analyzed the region from −117873 to −117140, which was by us previously characterized as determinant for transcriptional regulation [12]. Using the highly sensitive quantitative RT-PCR and immunocytochemical analysis, we found that ERα expression was higher in primary osteoblasts than in bone-derived cells (MG-63 and SaOS-2) and in all cases the ERα mRNA was prevalently represented by the isoform F. MG-63 cells with a low level of ERα mRNA showed a heavy methylated level of promoter F, whereas SaOS-2 cells expressing a major level of ERα mRNA showed a low methylation pattern comparable with that found in primary osteoblasts. The most notable difference was found at four particular CpGs (n.1–4), distant from the exon F transcription start site, showing a methylation status that correlates with the expression level, being

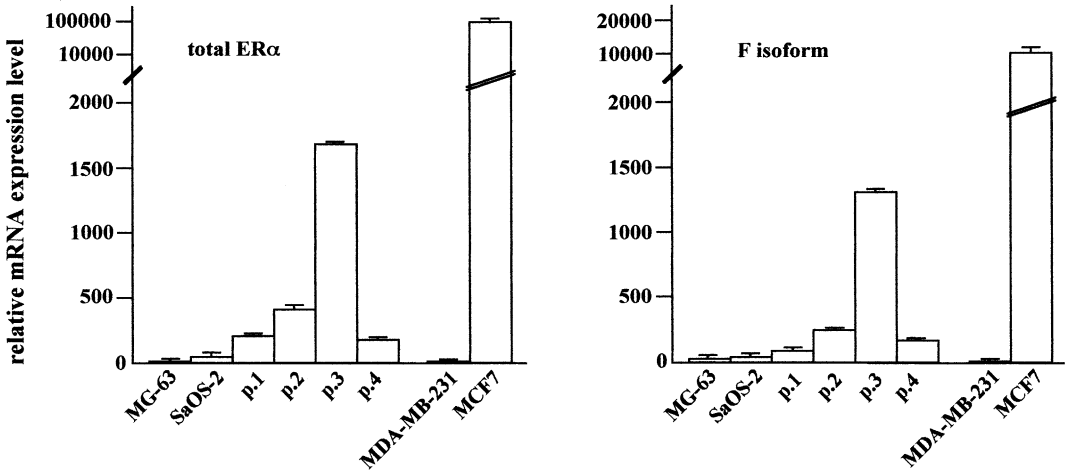

Fig. 4. Analysis of *ERα* gene expression by quantitative RT-PCR. The cDNA obtained from MG-63, SaOS-2, MCF7 and MDA-MB-231 cell lines and from primary osteoblasts (p.1, p.2, p.3, p.4) was subjected to quantitative TaqMan RT-PCR for total (ERα) and upstream (F) transcripts analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate; ΔΔCt method was used to compare gene expression data, standard error of the mean (S.E.M.) was calculated.

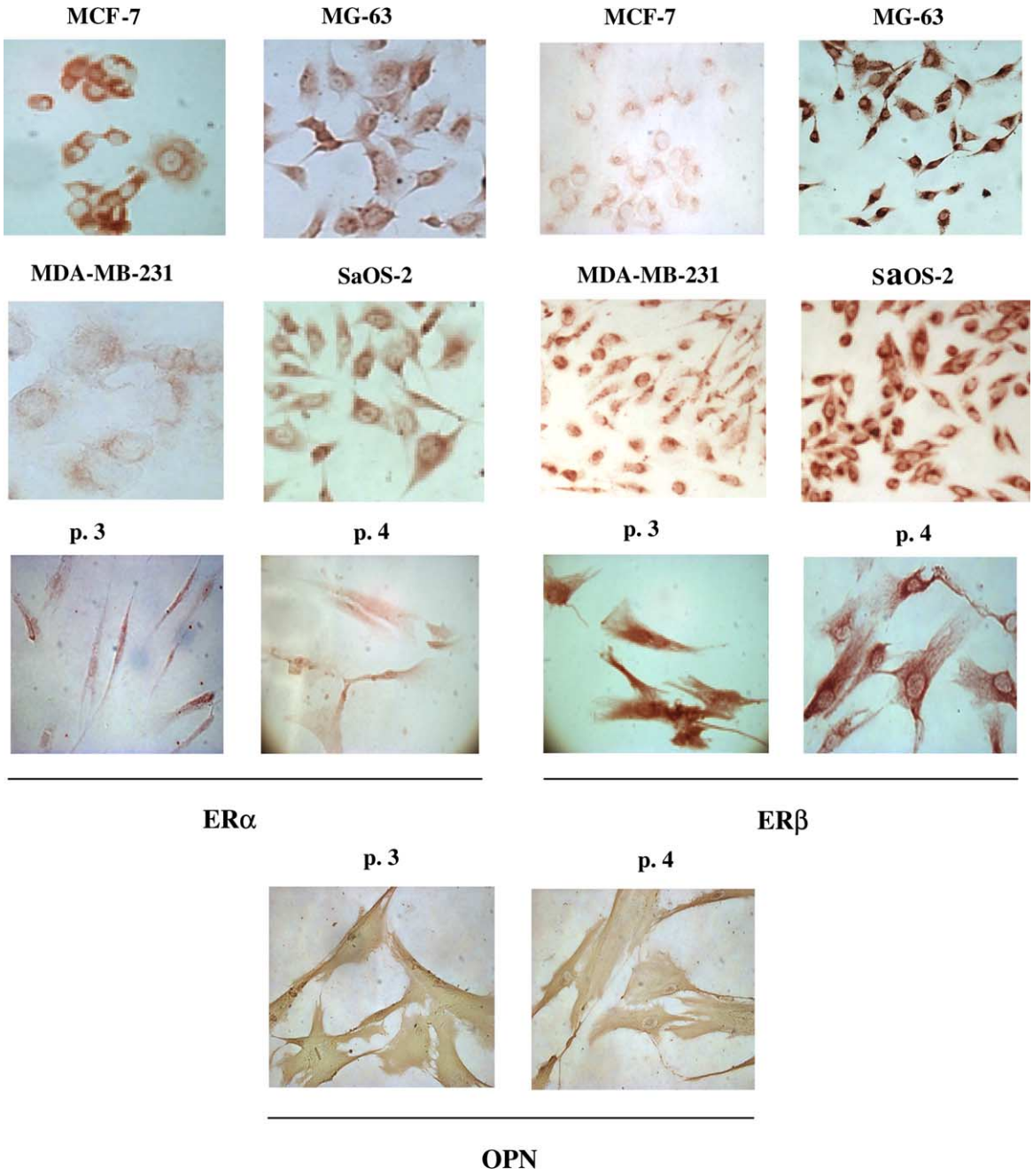

Fig. 5. Analysis of gene expression by immunocytochemistry. The levels of ERα, OPN and ERβ were determined by testing the immunoreactivity of MCF7, MG-63, MDA-MB-231, SaOS-2 cell lines and primary osteoblasts (p.3 and p.4) with the specific antibodies. Original magnification is 40×.

ERα mRNA transcription reduced in a partially methylated cells but preserved in demethylated cells. The other CpG sites, localized around the transcription start site, were always demethylated or hypomethylated except for MG-63 cells showing the lowest level of ERα expression.

Interestingly, the same 10 sites were found hypermethylated in ERα-negative breast cancer cells (MDA-MB-231) and completely demethylated in ERα-positive breast cancer cells (MCF7) that used in part the promoter F in addition to other promoters. Therefore, the data obtained in the cell lines analyzed suggest that the methylation status of the *ERα* gene, at sites determined in this study, correlates with the expression of this gene.

The heterogeneous pattern of methylation of the single CpG sites observed in the osteoblasts primary cultures, may be, in part, explained with the fact that the osteoblasts are obtained from bone tissues with a peculiar differentiation and proliferating program. In addition, primary osteoblasts analyzed exibit a higher degree of ERα mRNA in comparison with MG-63 and SaOS-2 osteosarcoma cell lines,

representing specific differentiation stages that are particularly useful in a study of this kind.

In addition, the observation that a specific CpG site was only partially methylated suggests that may be, within the osteoblastic population, a small number of cells with altered methylation, and therefore, possessing a selective growth advantage over the other cells. Another aspect to be considered is the ERα promoter methylation as a function of age. In the experiments here presented ERα methylation seems to decrease with increasing age because the complete demethylation pattern was observed in the hOB from the oldest patient, and this is in agreement with the data obtained in other conditions such as colorectal cancers [34] and adulte acute myeloid leukemia [35].

Although future studies will be aimed at increasing the number of cases, our results suggest that methylation of certain critical CpGs inside *ERα* gene promoter F may contribute indirectly to ERα transcriptional control in osteoblasts influencing binding of specific nuclear factors or recruitment of methyl-CpG-binding proteins (MBPs), and, consequently, the expression of the gene. This is in agreement with several observations regarding the selective promoter usage based on gene-specific methylation, the different promoter methylated profile showed by different cancers and the recruitment of specific transcriptional coregulators on the ERα promoter that might control chromatin organization by inducing different methylation levels [36–40], and could be important for the silencing or transcriptional regulation of the *ERα* gene.

Although the regulation of *ERα* gene expression is only partially known, it is well accepted that the promoter methylation is not the only mechanism involved in the repression of *ERα* gene expression, but other mechanisms involving histone methylation, acetylation, and deacetylation, recruitment of corepressor complexes, and overall changes in chromatin configuration contribute to the regulation of the expression. Since the alteration of these mechanisms may render the cells susceptible to pathological conditions, a preliminary analysis as that here reported on promoter F methylation and its impact on *ERα* gene transcriptional regulation in a specific context, such as osteoblastic cells, may have important implications. In particular, it is noteworthy that a number of drugs including tamoxifen and progestins, have been used to upregulate ERα, but proper formulation of therapeutic strategy will rely on a better understanding of the genomic signaling. Therefore, also the study of methylation of *ERα* gene promoters may contribute to improve diagnosis and therapy of bone specific disorders.

Acknowledgements

E.L. is supported by a fellowship from Cassa di Risparmio di Cento. This work was supported by FIRB-2000, CNR Agenzia 2000, AIRC and Ministero della Sanità, Ricerca Finalizzata 2001.

References

[1] S. Nilsson, S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, J.-A. Gustafsson, Mechanism of estrogen action, Physiol. Rev. 4 (2001) 1535–1565.
[2] J.G. Moggs, G. Orphanides, Estrogen receptors: orchestrators of pleiotropic cellular responses, EMBO Rep. 2 (2001) 775–781.
[3] G. Flouriot, C. Griffin, M. Kenealy, V. Sonntag-Buck, F. Gannon, Differentially expressed mRNA isoforms of the human estrogen receptor-α gene are generated by alternative splicing and promoter usage, Mol. Endocrinol. 12 (1998) 1938–1954.
[4] J.M. Ordway, T. Curran, Methylation matters: modeling a manageable genome, Cell Growth Differ. 13 (2002) 149–162.
[5] P.A. Jones, D. Takai, The role of DNA methylation in mammalian epigenetics, Science 293 (2001) 1068–1070.
[6] P.A. Wade, Methyl CpG-binding proteins and transcriptional repression, Bioessays 23 (2001) 1131–1137.
[7] J. Berger, G. Daxenbichler, DNA methylation of nuclear receptor genes—possible role in malignancy, J. Steroid Biochem. Mol. Biol. 80 (2002) 1–11.
[8] W.S. Post, P.J. Goldschmidt-Clermont, C.C. Wilhide, A.W. Heldman, M.S. Sussman, P. Ouyang, E.E. Milliken, J.P. Issa, Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system, Cardiovasc. Res. 43 (1999) 985–991.
[9] J.E. Compston, Sex steroids and bone, Physiol. Rev. 81 (2001) 419–447.
[10] J. Arts, G.G.J.M. Kuiper, J.M.M.F. Janssen, J.-A. Gustafsson, C.W.G.M. Lowik, H.A.P. Pols, J.P.T.M. Van Leeuwen, Differential expression of estrogen receptor α and β mRNA during differentiation of human osteoblast SV-HFO cells, Endocrinology 138 (1997) 5067–5070.
[11] S. Denger, G. Reid, M. Kos, G. Flouriot, D. Parsch, H. Brand, K.S. Korach, V. Sonntag-Buck, F. Gannon, *ERα* gene expression in human primary osteoblasts: evidence for the expression of two receptor proteins, Mol. Endocrinol. 15 (2001) 2064–2077.
[12] E. Lambertini, L. Penolazzi, S. Giordano, L. Del Senno, R. Piva, Expression of the human oestrogen receptor-alpha gene is regulated by promoter F in MG-63 osteoblastic cells, Biochem. J. 372 (2003) 831–839.
[13] K.D. Siegmund, P.W. Laird, Analysis of complex methylation data, Methods 27 (2002) 170–178.
[14] M. Frommer, L.E. McDonald, D.S. Millar, C.M. Collis, F. Watt, G.W. Grigg, P.L. Molloy, C.L. Paul, A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands, Proc. Natl. Acad. Sci. 89 (1992) 1827–1831.
[15] E. Lambertini, L. Penolazzi, V. Sollazzo, F. Pezzetti, M. de Mattei, L. del Senno, G.C. Traina, R. Piva, Modulation of gene expression in human osteoblasts by targeting a distal promoter region of human estrogen receptor-α gene, J. Endocrinol. 172 (2002) 683–693.
[16] R. Paulin, G.W. Grigg, M.W. Davey, A.A. Piper, Urea improves efficiency of bisulphite-mediated sequencing of 5′-methylcytosine in genomic DNA, Nucleic Acid Res. 21 (1998) 5009–5010.
[17] K.J. Ibbotson, J. Harrod, M. Gowen, S. D'Souza, D.D. Smith, M.E. Winkler, R. Derynck, G.R. Mundy, Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 2228–2232.
[18] E. Lambertini, L. Penolazzi, G. Aguiari, L. del Senno, F. Pezzetti, V. Sollazzo, R. Piva, Osteoblastic differentiation induced by transcription factor decoy against human estrogen receptor α gene, Biochem. Biophys. Res. Commun. 292 (2002) 761–770.
[19] D.T. Denhardt, X. Guo, Osteopontin: a protein with diverse functions, FASEB J. 7 (1993) 1475–1482.
[20] M. Sasaki, Y. Tanaka, G. Perinchery, A. Dharia, I. Kotcherguina, S. Fujimoto, R. Dahiya, Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer, J. Natl. Cancer Inst. 94 (2002) 384–390.

[21] M. Sasaki, A. Dharia, B.R. Oh, Y. Tanaka, S. Fujimoto, R. Dahiya, Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer, Cancer Res. 61 (2001) 97–102.
[22] M. Sasaki, M. Kaneuchi, S. Fujimoto, Y. Tanaka, R. Dahiya, Hypermethylation can selectively silence multiple promoters of steroid receptors in cancers, Mol. Cell. Endocrinol. 202 (2003) 201–207.
[23] Z.J. Liu, M. Maekawa, Gene promoter methylation—a potential cancer marker, Adv. Clin. Pathol. 6 (2002) 45–49.
[24] R. Jaenisch, A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals, Nat. Genet. 33 (2003) 245–254.
[25] Y. Yuasa, DNA methylation in cancer and ageing, Mech. Ageing Dev. 123 (2002) 1649–1654.
[26] N. Ahuja, Q. Li, A.L. Mohan, S.B. Baylin, J.P. Issa, Aging and DNA methylation in colorectal mucosa and cancer, Cancer Res. 58 (1998) 5489–5494.
[27] T. Yoshid, H. Eguchi, K. Nakachi, K. Tanimoto, Y. Higashi, K. Suemasu, Y. Iino, Y. Morishita, S.I. Hayashi, Distinct mechanisms of loss of estrogen receptor α gene expression in human breast cancer: methylation of the gene and alteration of *trans*-acting factors, Carcinogenesis 21 (2000) 2193–2201.
[28] Y.L. Ottaviano, J.P. Issa, F.F. Parl, H.S. Smith, S.B. Baylin, N.E. Davidson, Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells, Cancer Res. 54 (1994) 2552–2555.
[29] M. Sasaki, L. Kotcherguina, A. Dharia, S. Fujimoto, R. Dahiya, Cytosine–phosphoguanine methylation of estrogen receptors in endometrial cancer, Cancer Res. 61 (2001) 3262–3266.
[30] J.P. Issa, Y.L. Ottaviano, P. Celano, S.R. Hamilton, N.E. Davidson, S.B. Baylin, Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon, Nat. Genet. 7 (1994) 536–540.
[31] A.K. Ying, H.H. Hassanain, C.M. Roos, D.J. Smiraglia, J.P.J. Issa, R.E. Michler, M. Caligiuri, C. Plass, P.J. Goldschmidt-Clermont, Methylation of the estrogen receptor-α gene promoter is selectively increased in proliferating human aortic smooth muscle cells, Cardiovasc. Res. 46 (2000) 172–179.
[32] M. Kos, G. Reid, S. Denger, F. Gannon, Minireview: genomic organization of the human *ERα* gene promoter region, Mol. Endocrinol. 15 (2001) 2057–2063.
[33] A. Villagra, J. Gutierrez, R. Paredes, J. Sierra, M. Puchi, M. Imschenetzky, A. Wijnen Av, J. Lian, G. Stein, J. Stein, M. Montecino, Reduced CpG methylation is associated with transcriptional activation of the bone-specific rat osteocalcin gene in osteoblasts, J. Cell. Biochem. 85 (2002) 112–122.
[34] F. al-Azzawi, M. Wahab, Estrogen and colon cancer: current issues, Climacteric 5 (2002) 3–14.
[35] Q. Li, K.J. Kopecky, A. Mohan, C.L. Willman, F.R. Appelbaum, J.K. Weick, J.P. Issa, Estrogen receptor methylation is associated with improved survival in adult acute myeloid leukemia, Clin. Cancer Res. 5 (1999) 1077–1084.
[36] Z.J. Liu, M. Maekawa, T. Horii, M. Morita, The multiple promoter methylation profile of PR gene and ER alpha gene in tumor cell lines, Life Sci. 73 (2003) 1963–1972.
[37] M. Esteller, P.G. Corn, S.B. Baylin, J.G. Herman, A gene hypermethylation profile of human cancer, Cancer Res. 61 (2001) 3225–3229.
[38] S.B. Baylin, J.G. Herman, DNA hypermethylation in tumorigenesis: epigenetics joins genetics, Trends Genet. 16 (2000) 168–174.
[39] M. Maekawa, K. Sugano, M. Ushiama, N. Fukayama, K. Nomoto, H. Kashiwabara, S. Fujita, T. Kakizoe, Heterogeneity of DNA methylation status analyzed by bisulfite-PCR-SSCP and correlation with clinico-pathological characteristics in colorectal cancer, Clin. Chem. Lab. Med. 39 (2001) 121–128.
[40] M. Macaluso, C. Cinti, G. Russo, A. Russo, A. Giordano, pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-alpha in breast cancer, Oncogene 22 (2003) 3511–3517.