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Methylation of the ER-alpha Promoter Is Influenced by its Ligand Estrogen in Osteosarcoma Cells SAOS-2 *In Vitro*

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Abstract. *Background/Aim: The aggressive fast-growing osteosarcoma is the most common primary malignant bone tumor. The relevance of estrogen as a key player in bone metabolism and bone tumor is well-known. At the molecular level, estrogen activates the estrogen receptor α (ERα) as a natural ligand of this receptor. ERα acts as a transcription factor by binding to the "estrogen response element" (ERE) and regulates the expression of a various number of genes. Epigenetic processes, e.g. the methylation of the "cytosine-phosphatidyl-guanine (CpG) islands" can change the transcription of target genes and subsequently the protein expression. As DNA methylation is generally associated with gene transcription repression, up until now little is known about the ERα methylation in osteosarcoma cells. The aim of the present pilot study was to evaluate the methylation status of ERα in osteosarcoma cells SAOS-2 and MG 63 after stimulation with estrogen. Materials and Methods: SAOS-2 and MG 63 cells were cultured in DMEM. After treatment with 10 nmol estrogen (E2) for 24 h, the expression of ERα was detected by immunocytochemistry (ICC). As controls we used untreated cells. Staining was evaluated semi-quantitatively by the immunoreactive score of Remmele and Stegner (IRS). To determine mRNA gene expression, extracted RNA was transcribed into c-DNA and a quantitative real-time-PCR (qRT-PCR) was carried out. The semi quantitative evaluation of the ERα mRNA was based on the $2^{-\Delta\Delta ct}$ method using untreated cells as reference control. One microgram of each extracted genomic DNA sample was converted with bisulfite and a real-time methylation-specific PCR (rt-MSP) was performed. Results: The estrogen-stimulated SAOS-2 cells showed a significant increase of ERα expression. A 7-fold up-regulation of ERα mRNA confirmed the results of immunocytochemistry. Methylation of the ERα promoter was not detected in treated cells. In contrast, we identified methylation of the ERα promoters in untreated cells. The staining of MG 63 cells showed a weak gain of ERα expression in the stimulated cells, as well as a weak increase of the ER-α mRNA (2-fold). Methylation of the ERα promoters was not detectable in either treated or untreated cells. Conclusion: The methylation status of ERα in osteosarcoma cells is affected by estrogen. These findings indicate that epigenetic changes of genomic DNA regulate ERα synthesis. Taken together, our results suggest that SAOS-2 cells can be an interesting model for further investigating ERα synthesis. In addition, the evaluation of ERα methylation in osteosarcoma specimens is in progress.*

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The most common form of primary bone cancer, osteosarcoma (OS), is a tumor of mesenchymal origin and comprises approximately 20% of all bone tumors (1). Depending on the cell type, osteosarcoma tumors are grouped into osteoblastic, chondroblastic and fibroblastic subtypes (2, 3). OS has revealed special characteristics concerning the age distribution; the peak incidence occurs in children under 15 years and a second smaller peak after the age of 50 years (4). Development and origin are not really clear. Disruptions of osteogenic differentiations were originally described; however, influence of the environment, cytokines, growth factors and transcription factors have been

also monitored. (3, 5-8). In addition, numerous studies document the importance of hormones, especially estrogen (E2) on bone development and remodelling and, hence, on osteosarcoma development (8-10). Recent data showed that epigenetic alterations have become important in understanding OS development (8, 11, 12).

Effects of E2 on bone metabolism, maintenance and bone mineral density, as well as osteoblast proliferation and differentiation have been well-known since decades (2, 13, 14). At the molecular level, estrogen activates the estrogen receptor α (ERα) as a natural ligand of this receptor. ERα acts as a fully functional transcription factor by binding to the estrogen response element (ERE) and regulates the expression of various numbers of genes, *e.g.* *RUNX2* (Runt-related transcription factor 2) as a master regulator of osteogenic differentiation and *WNT* as a key player in bone remodelling (9, 15-18).

Aberrant DNA methylation of gene promoter regions play a major role in different tumors, *e.g.* breast, ovarian, and cervix tumors (19-22). The methylation process is catalysed by DNA methyltransferases and results in methylated "cytosine-phosphatidyl-guanine (CpG) islands". The CpG islands lead to an inactive chromatin structure *via* histone modification (11, 23, 24). Methylation can change the transcription of genes; a high level of DNA methylation is generally associated with a lower transcription of target genes and, subsequently, lower protein expression (25). The aim of the present pilot study was to show the influence of estrogen on the methylation process of the *ERα* promoter region and to demonstrate the different methylation statuses in osteosarcoma cells SAOS-2 and MG 63 after stimulation with estrogen.

Materials and Methods

Cell culture. Human osteosarcoma cell lines, SAOS-2 (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) and MG 63 (Sigma, St. Louis, MO, USA) were cultivated with Dulbecco's modified Eagle's medium (DMEM; Sigma) including 1 g/l glucose, 10% fetal calf serum (FCS Superior; Biochrom, Berlin, Germany), 20 mM HEPES (Biochrom), 2 mM L-glutamine (Sigma), 100 mg/l Primocin™ (InvivoGen, San Diego, CA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2. Cell media were changed twice a week. After reaching a confluence of 80%, cells were subcultivated by trypsinization with Trypsin/EDTA (Sigma) for 3 min at 37˚C and seeded in a concentration of 6×10^3 cells/cm^2.

DNA extraction and bisulfite conversion. Both cell lines, SAOS-2 and MG 63, were treated with E2 (Sigma) 10 nM for 24 h. Untreated cells served as reference control. Cells were washed with PBS 0.1M, trypsinized and, then, DNA was extracted using the NucleoSpin® Tissue Kit (Machery&Nagel, Düren, Germany) according to the manufacturer's instructions. Subsequently, concentration of DNA was measured with an UV spectrophotometer (Nano drop 2000; PeqLab, Erlangen, Germany). DNA methylation was assessed by bisulfite treatment using the EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany); cytosine residues in unmethylated DNA were converted to uracil, whereas methylated cytosine remained unaffected.

Table I. *Primer sequences for real-time methylation-specific PCR (rt-MSP).*

Primer	
Forward	5'-ggcgttcgttttgggattg-3'
Reverse	5'-gccgacacgcgaactctaa-3'
TagMan® probe	FAM 5'-cgataaaaccgaacgacccgacga-3' TAMRA

Real-time methylation-specific PCR (rt-MSP). Methylation-specific polymerase chain reaction (PCR) was carried out under the following conditions: enzyme activation 95˚C for 20 s; amplification at 95˚C for 3 s, annealing temperature 60˚C for 30 s; 40 cycles. The total volume of 25 μl contained 2.5 μl forward primer, 2.5 μl reverse primer, 2.5 μl TaqMan® probe, 12.5 μl TaqMan® Universal PCR Master Mix 2x (Applied Biosystems™ Life Technologies, Darmstadt, Germany), 3 μl H_2O (DEPC treated DI water, Sigma, Germany) and 2 μl bisulfite-treated DNA. Primer sequences are given in Table I. Untreated cells served as negative control. Positive amplification only for unmethylated primers was interpreted as unmethylation. Positive amplification only for methylated primers or for both methylated and unmethylated primers were considered as methylation. Water blanks were included in each assay. Three independent experiments were performed and all reactions were done in triplicate.

RNA extraction and quantitative real-time-PCR (qRT-PCR). Both cell lines were treated with E2 (Sigma) 10 nM for 24 h. Untreated cells served as reference control. After washing with PBS 0.1M, cells were trypsinized, counted and RNA was extracted using the RNeasy Kit (Qiagen) according to the supplier's instructions. Concentrations were measured with an UV-spectrophotometer (Nano drop 2000; PeqLab). Two μg RNA were transcribed into cDNA using the Quantiscript-Kit (Qiagen). Subsequently, qPCR was performed according to the manufacturer's instructions for fast reaction set up and the appropriate primer and Master Mix (Applied Biosystems™ Life Technologies, Carlsbad, CA, USA,), using the StepOnePlus™ Real-Time PCR System (Applied Biosystems™ Life Technologies). The results were normalized to the housekeeping gene glyceroaldehyde-3-phosphate dehydrogenase (*GAPDH*). The RNA of unstimulated cells served as calibrator. By employing the fast system, the reaction was carried out for 20s at 95˚C for enzyme activation and for 40 cycles of amplification with 3 s at 95˚C and 30 s at 60˚C. The results were analyzed by the $2^{-\Delta\Delta ct}$-method. Three independent experiments were performed and all reactions were done in triplicate.

Immunocytochemistry. For immunocytochemistry, cells were seeded on chamber slides (Falcon™, BioCoat™; Becton Dickinson, Franklin Lakes, NJ, USA) in a concentration of 8×10^3 cells/cm^2. After adhesion overnight, cells were treated with 10 nM E2 (Sigma) for 24 h. Untreated cells served as reference control. Slides were washed with PBS 0.1 M, fixed in ethanol 100%/acetone (1:1) for

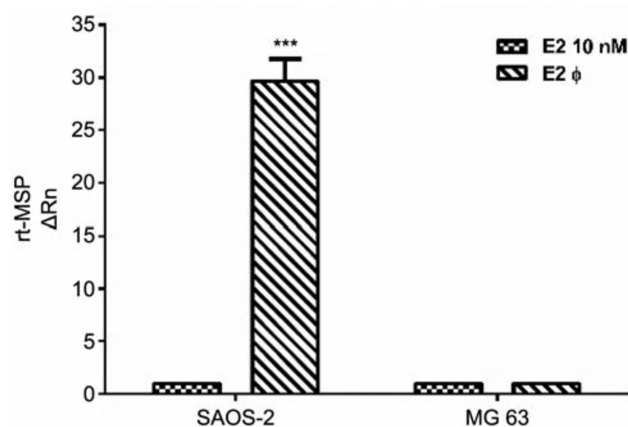

Figure 1. *Methylation of the ERα promoter in SAOS-2 and MG 63 cells. The stimulated SAOS-2 and MG 63 cells were cultured with E2 (10 nM) for 24h h. Real-time methylation-specific polymerase chain reaction (rt-MSP) was carried out using bisulfite-treated DNA. Unstimulated cells served as control. The stimulated SAOS-2 and MG 63 cells showed no methylation of the ERα promoter. The unstimulated SAOS-2 cells, in contrast to the unstimulated MG 63 cells, displayed a methylated ERα promoter.*

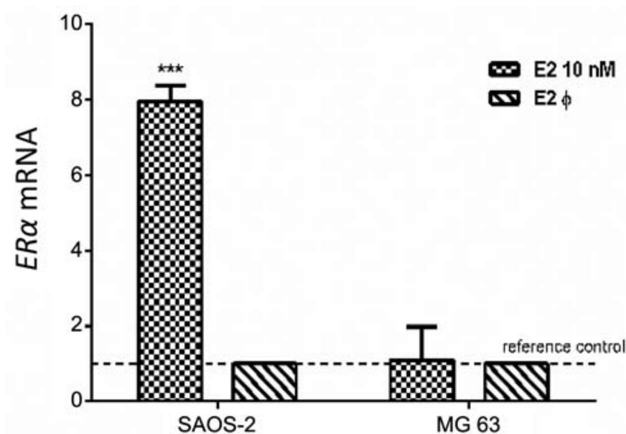

Figure 2. *ERα mRNA expression in SAOS-2 and MG 63 cells. The stimulated SAOS-2 and MG 63 cells were cultured with E2 (10 nM) for 24h h. Relative quantification of ERα mRNA was determined using untreated cells. The stimulated SAOS-2 cells showed a 7.8-fold increase of ERα compared to the unstimulated cells. E2-stimulated MG 63 cells showed a 2-fold increase compared to the unstimulated MG 63 cells.*

10 min at room temperature (RT) and air dried. Prior to staining, the slides were rehydrated in PBS 0.1M for 10 min at RT. To reduce non-specific background staining, slides were treated with Protein block (Dako, Glostrup, Denmark) for 20 min at RT. Subsequently, they were incubated with an anti-ERα mouse monoclonal antibody (clone 1D5; Dako) for 60 min at RT, followed by an incubation with a biotinylated secondary anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT. The slides were washed in PBS and incubated with avidin-biotin peroxidase complex (Vectastain-Elite; Vector Laboratories) for 30 min at RT. The antigen-antibody complex was visualized with the chromogen 3-amino-9-ethylcarbazole (AEC; Dako) and counterstained with Mayer´s hematoxylin. Finally, the slides were washed in tap water and coverslipped using Kaiser's glycerin gelatine (Merck, Darmstadt, Germany). The evaluation was based on the semi-quantitative immunoreactive score by Remmele and Stegner (IRS), which was calculated by multiplication of optical staining intensity (graded as 0, none; 1, weak; 2, moderate; and 3, strong staining) and the percentage of positively stained cells (0, no staining; 1, ≤10% of the cells; 2, 11-50% of the cells; 3, 51-80% of the cells; and 4, ≥81% of the cells) (26).

Statistical analysis. This study was designed as a pilot study. Statistical analysis was performed by using GraphPad Prism 6.03 software (GraphPad Software, Inc., La Jolla, CA, USA). For statistical analysis 2-way ANONA and Sidak's multiple comparisons test were performed. Significance was assumed at $p<0.005$.

Results

rt-MSP. Unstimulated SAOS-2 cells showed a methylation of the *ERα* promoter, whereas the stimulated SAOS-2 cells were *ERα* promoter methylation-negative. MG 63 cells, stimulated and unstimulated, displayed an unmethylated *ERα* promoter (Figure 1).

qRT-PCR. The mRNA expression of *ERα* was increased 7.8-fold in SAOS-2 cells stimulated with 10 nM E2 compared to the unstimulated SAOS-2 cells. The E2-stimulated MG 63 cells showed a 2-fold increase compared to the unstimulated MG 63 cells (Figure 2).

Immunocytochemistry. SAOS-2 cells stimulated with E2 10 nM for 24 h displayed a stronger expression of the ERα receptor compared to the unstimulated cells (Figure 3A, B). Expression of ERα receptor showed no differences between stimulated and unstimulated MG 63 cells. Both treated and untreated cell types showed no significant expression difference. A summary of the staining results with the evaluation of staining is presented in Figure 4.

Discussion

Results of our pilot study show that the evaluation of methylation of the ERα promoter in osteosarcoma cells is possible. In addition, the results reveal that the methylation status of ERα in osteosarcoma cells is affected by estrogen. These findings indicate that epigenetic changes of genomic DNA regulate the ERα synthesis in the SAOS-2 cell culture system.

Promoter-Methylation is an epigenetic alteration that can be seen in multiple types of human neoplasias (27).

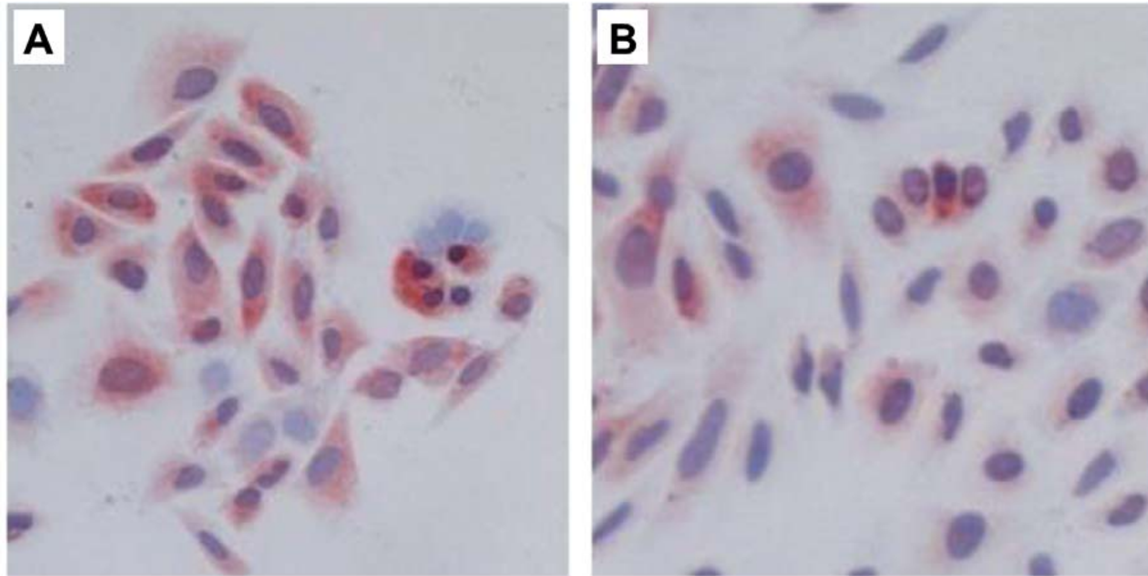

Figure 3. *ERα receptor is upregulated in SAOS-2 cells after stimulation with estradiol (10 nM) for 24 h. Stimulated cells show an intense expression of ERα in the cytoplasm as well as in the nucleus of the cells (A). Unstimulated SAOS-2 cells served as control and show a less intense staining of the ERα in only few cells (B).*

Hypermethylation of a certain gene usually inhibits gene expression. Within the last years methylation of a number of genes has been described in osteosarcoma, although correlations with clinico-pathological parameters are missing (11, 28-33). The fact that methylation rates increase from neoplasia to invasive cancer supports its role in carcinogenesis (8).

Issa *et al*. (34) first described the methylation of CpG-islands in the promoter of *ERα* in colorectal cancer. Non-malignant tissues from thyroid, breast, lung, cervix and prostate were examined for the presence of promoter methylation and found to be negative (35). In contrast, *ERα* promoter methylation seems to play a role in the early steps of carcinogenesis in several tumor sites, including lymphoma, oesophageal cancer and colorectal cancer (34-36), being present in almost 100% of primary colorectal tumors.

Expression of ERα is intense in normal bone tissue (37); therefore, osteosarcoma cells *in vitro* are an interesting cell culture model for the investigation of *ERα* methylation and its changes. This cell culture model has already been established by our group (38).

The essential influence of E2 on bone mechanism and remodelling has been known since many years. E2 induces the commitment of precursor cells to the osteogenic lineage (39). Transcription factor *RUNX2*, a target gene of ERα, regulates the differentiation of osteoblasts as a master player of the osteogenic lineage (15, 40). Our finding that E2 inhibits the methylation of the genomic DNA of SAOS-2 cells is supported by a higher transcription level of *ERα* mRNA (7-fold). A moderate stronger expression of the ERα receptor was detected by immunocytochemistry. Considering the importance of E2 for bone, a highly methylated *ERα* promoter may lead to disruptions or defects of the osteoblastic lineage and subsequent OS development (8). In contrast to SAOS-2 cells, the genomic DNA of MG 63 cells displayed an

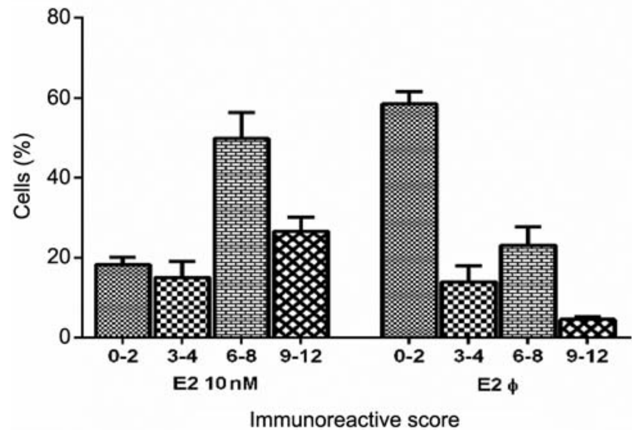

Figure 4. *Evaluation of immunocytochemistry of ERα receptor. The evaluation was performed by the immunoreactive score of Remmele and Stegner (IRS) using the following scale: 0-2, negative; 3-4, positive, weak expression; 6-8, positive, moderate expression; 9-12=positive, strong expression. Stimulated cells showed an IRS score of 6-8 in 50% of the cells whereas unstimulated cells showed an IRS of 0-2 in 60% of all cells.*

unmethylated *ERα* promoter. Additionally, the mRNA *ERα* levels were doubled and the expression of ERα receptor showed no differences between stimulated and unstimulated MG 63 cells. It has previously been reported that both osteosarcoma cell lines, SAOS-2 (female) and MG 63 (male) originating from different genders, show partially a different response to E2 treatment (41). We assume that the gender difference could play an important role in interpreting these results. The male-derived cell line MG-63 expresses both the estrogen (ER) and thyroid hormone (TR) receptors, whereas SAOS-2 cells express only the ER (42). This may indicate that MG-63 cells might not only be dependent on estrogen but also on thyroid hormones and the methylation status of the *ER* promoter may not be as important as for SAOS-2 cells.

In summary, the methylation status of *ERα* in OS cells, especially in SAOS-2 cells, is affected by estrogen. These findings indicate that epigenetic changes of genomic DNA regulate the ERα synthesis. In conclusion, our results suggest that SAOS-2 cells can be an interesting model for further investigating the ERα-dependent cell differentiation. In addition, the evaluation of *ERα* methylation in OS specimens is in progress.

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