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Methylation of histone H3 lysine 27 associated with apoptosis in osteosarcoma cells induced by staurosporine

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Summary. The relationship between histone methylation and apoptosis, programmed cell death, is beginning to be explored. The objective of this study was to investigate the effects of staurosporine, a PKC inhibitor on the methylation of histone H3 in osteosarcoma cells. Following stimulation by staurosporine in vitro of G292 cells, a human osteosarcoma cell line with fibroblast-like phenotype, methylation of histone H3 was evaluated by western blotting and immunocytochemistry. G292 cells revealed the expression of cleaved PARP after incubation with staurosporine for 3 hours. Monomethyl lysine (K) 27 was induced by staurosporine at a concentration of 1, but no monomethyl K4 or K9 in histone H3 was seen. Dimethyl and trimethyl histone H3 K27 were also identified. There was no expression of dimethyl or trimethyl histone H3 K4 and K9. Expression of monomethyl histone H3 K27 was dose-dependent. The morphologic changes of apoptosis induced by staurosporine were observed under microscopy. Immunocytochemistry of monomethyl histone H3 K27 showed a weak signal in controls, a strong signal in staurosporine-treated tumor cells and a denser signal in the apoptotic cells. Our studies demonstrated that monomethyl histone H3 lysine 27 is expressed in staurosporine-induced apoptotic osteosarcoma cells. The findings may provide novel bridge information between the epigenetic episodes and apoptotic process.

Key words: Methylation, Histone H3, Osteosarcoma, Staurosporine

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Introduction

The nucleosome, the fundamental unit of chromatin, is composed of approximately 147 base pairs of DNA wrapped around a histone (H) octamer core, consisting of two copies of H2A, H2B, H3, and H4 (Davey et al., 2002). Recently, epigenetic abnormalities, including histone modification, were identified as causative factors in cancers, genetic disorders, autoimmune diseases and even aging. The N-terminal "tail" of the histone protrudes externally from the core structure, thereby providing an opportunity for contact with adjacent nucleosomes and/or nonhistone proteins, such as HP1 or NuRD. Tail modification has been suggested as being important for regulation of chromatin structure and gene expression (Luger et al., 1997).

The histone tails can be modified by a number of mechanisms, including acetylation, methylation, phosphorylation, and ubiquitination of the lysine and/or arginine residues (Strahl and Allis, 2000). Unlike histone lysine acetylation, which may be correlated with transitional activation, histone lysine methylation is considered to be biochemically silent though reversible (Kubicek and Jenuwein, 2004; Shi et al., 2004). Histone lysine methylation occurs on lysines 4, 9, and 27 of H3 and lysine 20 of H4. Whether methylation represses or activates depends on which lysine residue is methylated. For instance, methylation of lysine 4 in histone H3 correlates with gene activation, whereas methylation of lysines 9 and 27 in histone H3 correlates with gene repression (Bannister et al., 2002; Fischle et al., 2003). During the early tumorigenic process, hypomethylation of DNA is associated with hypoacetylation of lysine 16 and trimethylation of lysine 20 of histone H4 (Fraga et al., 2005). Methylation of histone H3 lysine 27 is

associated with colon carcinogenesis (Kirmizis et al., 2004).

Osteosarcomas are osteoid-produced malignant bone tumors that usually occur in young adults. Osteosarcomas are pathologically divided into subtypes (including osteoblastic, chondroblastic and fibroblastic) on the basis of differentiation. The current treatment (chemotherapy, i.e., methotrexate, doxorubicin, cisplatin, etoposide, and ifosphamide followed by limb-salvage surgery) has significantly improved prognosis and quality of life during the past decades (Bruland and Phil, 1997). However, more than 30% of patients die of pulmonary metastases within 5 years (Shor et al., 2007). There is still no effective chemotherapeutic agent for patients who relapse following standard treatment. Recently, several studies have revealed that new chemotherapeutic agents that induce osteosarcoma cell apoptosis may be therapeutic (Orosco et al., 2007; Yin et al., 2007).

Genetic mutations, particularly alterations in p53 and retinoblastoma (Rb) tumor suppressor genes, are fundamental to development of osteosarcoma (Bruland and Phil, 1997; Orosco et al., 2007; Shor et al., 2007), although epigenetic events are increasingly being recognized. Aberrant hypermethylation of several genes, including p14ARF, tumor suppressor genes (CDKN2A and RASSF1A), genes related to DNA repair/protection (MGMT), and genes related to apoptosis, angiogenesis and invasion (DAPK1 and TIMP3), have been demonstrated during osteosarcoma development (Hou et al., 2006; Oh et al., 2006). However, the exact role of epigenetic mechanisms in tumourogenesis and whether they influence apoptosis remains unclear and needs clarification.

In the present study, we investigated the epigenetic changes in osteosarcoma cells responding to staurosporine. Staurosporine induces cellular apoptosis by inhibiting multiple protein kinases, including protein kinase C (PKC) which regulates proliferation, differentiation, and cell survival (Spitaler and Cantrell, 2004). Staurosporine was found to induce methylation of lysine 27 in histone H3. This finding may have therapeutic implications for this frequently fatal disease.

Materials and methods

Reagents and antibodies

Staurosporine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal antibodies, including anti-PARP, anti-cleaved PARP, and fluorescein-conjugated anti-cleaved PARP (Asp214) antibody were from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-histone H3, anti-monomethyl histone H3 (Lys 4), anti-monomethyl histone H3 (Lys 9), and anti-mono-/di-/trimethyl histone H3 (Lys 27) were all from Upstate Biotechnology (Lake Placid, NY, USA).

Cell line and culture conditions

G-292 cells, a human osteogenic sarcoma cell line (ATCC CRL-1423) with a characteristic fibroblast-like phenotype was cultured in 90% McCoy's 5a medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine 37°C in a humidified incubator with an air atmosphere containing 5% CO_2 under ambient pressure. Cells were plated at a density of $3x10^5$/ml on 6 cm tissue culture dishes and subjected to different stimulations. Confluent cells were detached with 0.25% trypsin (Gibco) and 0.05% EDTA (Gibco).

Experimental protocol

Dishes of G292 cells rendered quiescent in serum-free media overnight were then incubated with staurosporine for 3 h. The control group was absence of staurosporine.

Protein extraction and Western blotting

Following staurosporine stimulation, cells were immediately washed with ice-cold PBS containing 100 μM Na_3VO_4 (Sgma) and lysed *in situ* with ice-cold lysis buffer at 4°C for 15 min. Lysis buffer contained 1% Igepa (Sigma), 100 μM Na_3VO_4, and one tablet containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Whole cell ysates were collected after centirfugation at 13,000 rp for 15 min.

NE-PER nuclear and cytoplasmic extraction reagents purchased from Pierce Biotechnology (Rockford, IL, USA) were used to separate nuclear and cytoplasmic extracts according to the manufacturer's protocols. Briefly, treated cells were washed twice with ice-cold PBS and detached with 3 ml of EDTA for 3 min in the incubator. Cells were scraped off the dish and centrifuged at 1,000 rpm for 10 min. After removing and discarding the supernatant, there was about 1 ml of packed cell volume, which was centrifuged at 3,000 rpm for 10 min to remove as much medium from the cell pellet as possible. To fully resuspend the cells, the cell pellet was vortexed at the highest setting for 15 sec after adding 200 μl of ice-cold cytoplasmic extraction reagent I (CER I)15,000 rpm for 10 min. The superatant (cytoplasmix extract) fraction was transferred to a clean pre-chilled tube and the pellet was resuspended in 100 μl of ice-cold Nuclear extraction Reagent (NER) and 20 μl of 10X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The tube was chilled on ice, vortexed 15 sec every 10 min for a total of 40 min, and centrifuged at 15,000 rpm for 10 min. The supernatant (nuclear extract) fraction was transferred to a clean pre-chilled tube and stored at -80°C until use.

The protein concentratin was determined by the Lowry method. The protein (20 μg) samples were loaded onto a 12% SDS-polyacrylamide gel and the gel bands

were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P, Sigma). Membranes were blocked overnight at 4°C with 2% BSA in TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). After washing three times with TBST, blots were incubated for 1 h at room temperature with primary antibodies diluted in TBST. After washing 6 times with TBST, the blots were then incubated with horseradish peroxidase (HRP) labeled anti-rabbit secondary antibody (Dakocytomation, Glostrup, Denmark) for 1 h at room temperature. Membranes were rewashed extensively and the binding was detected using the Enhanced Chemiluminescence Western blotting detection system (Amersham Biosciences UK Limited, Amersham, UK), according to the manufacturer's instructions. Mouse monoclonal antibody tubulin Ab-4 served as internal control (NeoMarkers, Fremont, CA, USA).

Immunocytochemistry

G292 cells were fixed with a pre-chilled mixture of acetone and methanol (1:1) and then subjected to immunostaining. Immunostaining for monomethyl histone H3 lysine 27 was performed using a standard avidin-biotin-peroxidase complex detection kit (DakoCytomation). Fixed G292 cells were rinsed 3x with PBS, treated with 3% hydrogen peroxide and non-immune goat serum, respectively, to block endogenous peroxidase activity and non-specific binding, and then incubated sequentially with primary antibody (anti-monomethyl histone H3 lysine 27, 1:2000) for 60 min, biotinylated secondary antibody for 30 min, and peroxidase-conjugated streptavidin for 30 min. The chromogen 3-amino-9-ethylcarbazole (AEC) was used to detect peroxidase activity and localization of the reaction product was viewed by light microscopy. Control sections were also stained in parallel following the same procedure, but with the primary antibody omitted, or by adding the equivalent dilution of normal rabbit IgG (Santa Cruz Biotechnology, Inc., CA, USA) instead of the primary antibody to test for non-specific binding. The negative controls showed no positive signals (data not shown).

Immunofluorescence

G292 cells were fixed with a pre-chilled mixture of acetone and methanol (1:1). The cells were permeabilised using 2 ml 0.2% (v/v) Triton X-100 (Sigma) in TBS for 20 min, washed with 5 ml TBS three times with rotation, each for 5 min before incubation with 2 ml 1% (w/v) bovine serum albumin (BSA) in TBS for1 hour. Following removal of the BSA cells were incubated overnight at 4°C with human specific fluorescein-conjugated anti-cleaved PARP (Asp214) antibody diluted 1:100 in 2 ml TBS. In the negative controls the antibody was omitted. The cells were visualized using fluorescence microscopy and after taking pictures using an oil immersion lens, the dishes were washed with 5 ml TBST with rotation (at least ten times, each for 5 min) to remove the immersion oil. Cells were then immunostained for monomethyl histone H3 lysine 27 performed in the same dishes by using a standard avidin-biotin-peroxidase complex detection kit (see above). The cells were then viewed by light microscopy. Double staining was performed on three repeated experiments.

Results

Effect of staurosporine on apoptosis and the expression of monomethyl histone H3 lysine 27

Following 3h incubation with staurosporine at a concentration of 1 μM for 3 h poly (ADP-ribose) polymerase (PARP) degradation in whole cell lysates of G292 cells was seen (Fig. 1), indicating staurosporine induced apoptosis. Staurosporine caused marked histone H3 monomethylation at K27 but not at K4 or K9 as shown by western blotting of nuclear extracts (Fig. 2A). K27 methylation appeared to be predominantly through monomethylation, as dimethylation and trimethylation at K27 was comparatively less (Fig. 2B). Staurosporine-induced histone methylation at K27 was dose-dependent over the concentration range 0.1-10 M (Fig. 2C).

Morphological characterization of G292 cells treated with staurosporine

The above findings led us to investigate the relationship between histone H3 methylation and staurosporine-induced apoptosis of G292 cells by immunostaining for histone H3 monomethylation K27. No immunohistochemical signals were observed in the cytoplasm of control cells stained with secondary

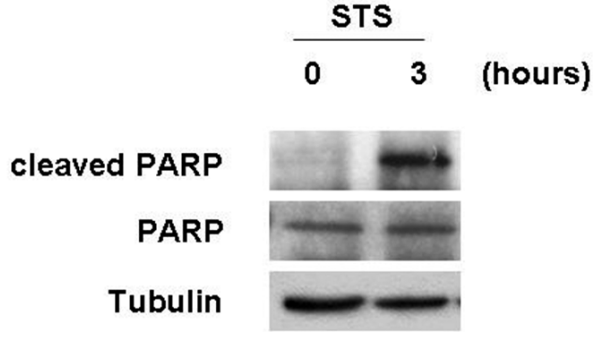

Fig. 1. Functional characterization of the effects of staurosporine (STS) on the osteosarcoma cell lune G292. Analysis of whole cell lysates showed that staurosporine (1 μM, 3h), a PKC inhibitor, induced PARP cleavage, a feature of apoptosis. Total PARP showed no significant change. Tubulin served as protien loading control.

antibody only (Fig. 3A), but there was a faint stippled nuclear immunoreactivity (Fig. 3B). Treated G292 cells (1μM staurosporine, 3 h incubation) ncubation) (in contrast to control [untreated] cells) exhibited morphological evidence of apoptosis, including cell detachment, loss of cell cytoplasmic processes, numerous floating dead cell nests, membrane shrinkage, and chromatin condensation. Strong immunoreactivity for histone H3 monomethylation K27 was observed in treated cells, and the signal was more intense in apoptotic cells (Fig. 3C).

We then examined the apoptotic cells induced by staurosporine in the same Petri dish. Immuofluorescent labeling first with fluorescein-conjugated anti-cleaved

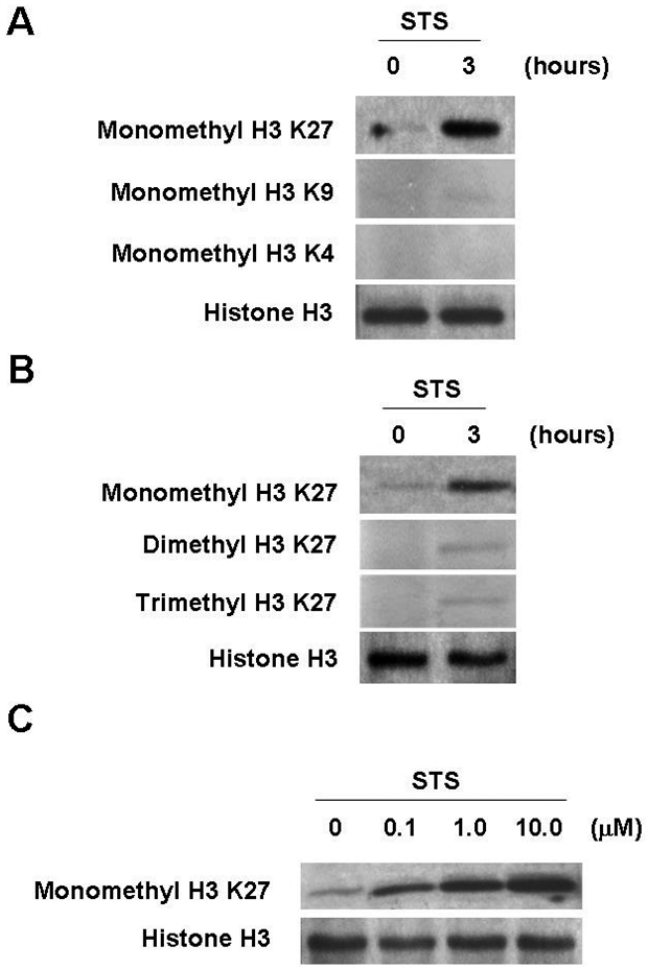

Fig. 2. Staurosporine induced histone H3 methylation. Nuclear extracts were subjected to Western blotting and immunoblotting. **A.** Histone H3 methylation was assessedx at lysine (K) 4, 9, and 27. Marked histone H3 monomethylation was demonstrated at K27, but none was demonstrated at K4 andK9. Total histone H3 was unchanged regardless of treatment. **B.** Staurosporine also induced dimethylation and thymethylation at K27 **(C)**. The increase in staurosporine-induced methylation was dose-dependent.

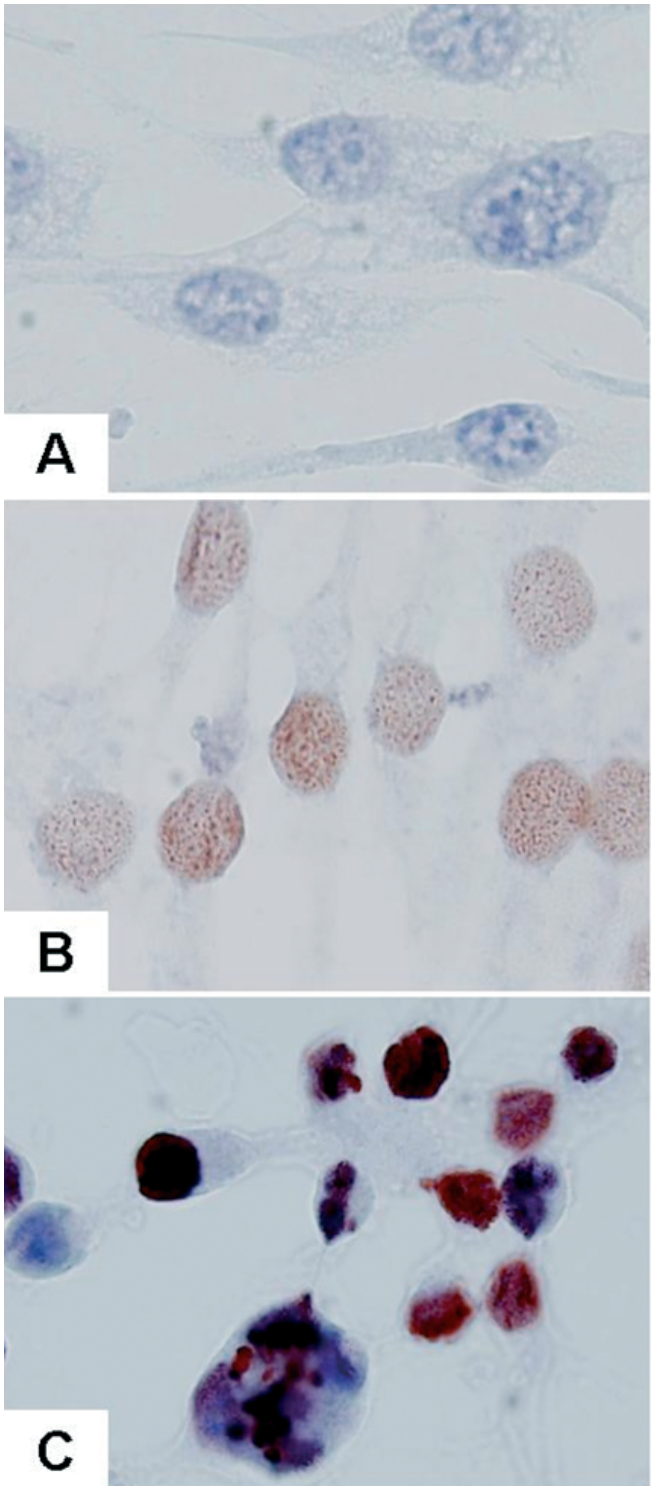

Fig. 3. Immunostaining of monomethylated histone H3 K27. **A.** Staining with secondary antibody alone revealed no signal. **B.** Staining with the histone H3 monomethylation K27 antibody revealed a very weak nucler signal in untreated cell. **C.** Stainign with histone H3 monomethylation K27 antibody revealed a sgtrong signal in the staurosporine-induced apoptotic osteosarcoma cells. Original magnification x 400

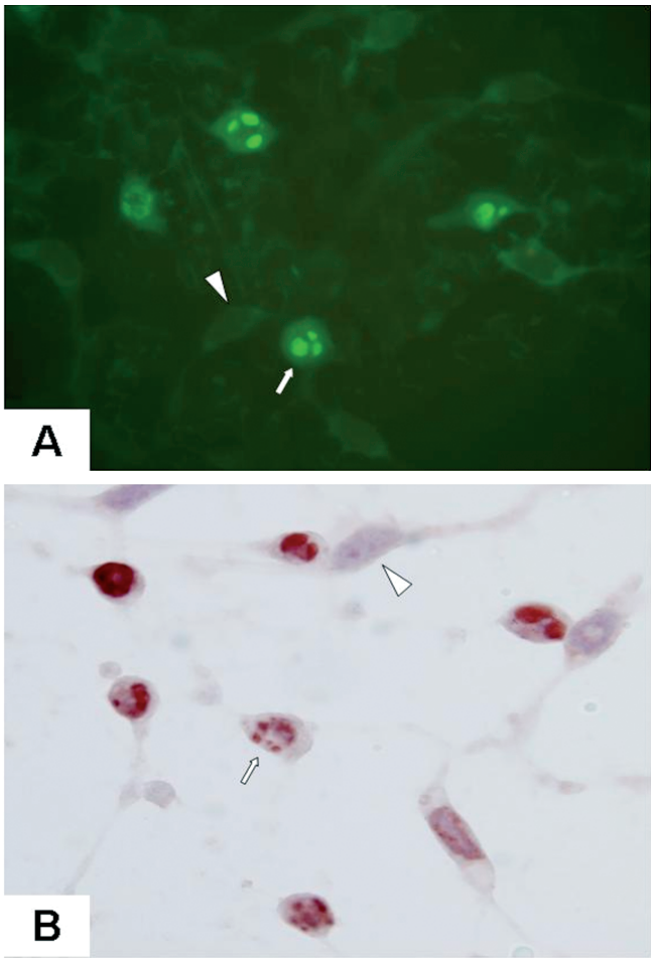

Fig. 4. Immunofluorescence of cleaved PARP **(A)** and immunocytochemistry of monomethylated histone H3 K27 (**B**) showed a similar positive signal in the apoptotic cells (arrows). There are no signals in the non-apoptotic cells (arrow heads). Original magnification x 1000

PARP (Asp214) antibody and then immunocytochemical staining with anti-monomethyl histone H3 lysine 27 were performed. Strong nuclear immunofluorescence for cleaved-PARP was visualized (Fig. 4A) and strong immunoreactivity for monomethyl histone H3 lysine 27 was also observed under light microscopy in the treated cells in the same dish (Fig. 4B). Apoptotic cells with nuclear fragmentation showed positive immunofluorescence of cleaved-PARP staining and positive signal of monomethyl histone H3 lysine 27 as well.

Discussion

Apoptosis is a programmed form of cell death involved in homeostasis (including development, differentiation, and morphogenesis) of eukaryotic multicellular organisms (Jacobson et al., 1997; Nagata, 1997). Our initial finding that staurosporine induced PARP cleavage in G292 cells, prompted us to investigate the apoptotic effect of epigenetic staurosporine-induced methylation of histone H3. We showed that apoptosis was associated with the epigenetic methylation of histone H3 in these cells.

The relationship between histone modification and apoptosis is beginning to be explored. In 2003, Konishi et al. demonstrated that histone H1.2 may play an important role in transmitting apoptotic signals from the nucleus to the mitochondria following DNA double strand breaks (Konishi et al., 2003). A global decrease in modification of histone acetylation has been explored during apoptosis (Allera et al., 1997). The release of hypoacetylated, trimethylated histone H4 and internucleosomally fragmented DNA from perinuclear heterochromatin during early apoptosis in leukemic cells provide links between epigenetic events and apoptosis (Biox-Chornet et al., 2006). Furthermore, apoptotic chromatin condensation and DNA fragmentation have also been shown to be associated with histone modifications, including phosphorylation of histone H2A, H2B and H3, dephosphorylation of histone H1, and deubiquitylation of histone H2A (Arends et al., 1990; Hengartner, 2001).

Histone lysine methylation can occur on lysines 4, 9 and 27 of H3 and lysine 20 of H4. Histone lysine methylation is considered to be biochemically silent (Kubicek and Jenuwein, 2004; Shi et al., 2004). Whether methylation represses or activates depends on which lysine residue is methylated. Methylation of histone H3 lysine 9 and lysine 27 and H4 lysine 20 are generally correlated with gene repression (Bernstein et al., 2007). For instance, cyclin E promoter activation could be repressed by H3 lysine 9 methylation in the G1 phase of the cell cycle (Nielsen et al., 2001). Another example comes from yeast, where active promoters are trimethylated at H3 lysine 4, but become repressed when dimethylated (Bannister et al., 2002). The observation that release of trimethylated histone H4 is associated with an epigenetic marker of early apoptosis was a novel finding that provided the first link between apoptotic nuclear events and epigenetic markers (Boix-Chornet et al., 2006). Subsequently, H3 lysine 4 trimethylation binding has been shown to be required for DNA repair and apoptotic activities of inhibitor of growth 1 (ING1) tumor suppressor (Peña et al., 2008).

H3 lysine 27 methylation has been shown to be a repression signal and associated with apoptosis. The presence of H3 lysine 27 trimethylation at transcriptional start sites is generally correlated with the repression of gene expression as a modification of transcriptional initiation by RNA polymerase II (Bernstein et al., 2007). H3 lysine 27 trimethylation contributes to the recruitment and/or stabilization of the polycomb repressive complex 1 (PRC1) and therefore mediates gene silencing (Schuetterngruber et al., 2007). Additionally, methylation of lysine 27 on histone H3 by PRC2 is associated with gene silencing in many

developmental processes (Kirmizis et al., 2004; Swigut and Wysocka, 2007). Chromatin immunoprecepitation analysis in mouse embryonic fibroblasts demonstrates that the level of H3 lysine 27 di/trimethylation is elevated in several X-linked genes (*Chic1, G6pd* and *Hprt)* in female mice. Both PRC1 and PRC2 can colocalize along with H3 lysine 27 trimethylation to around 1000 genes, many with developmental functions (Bracken et al., 2006). Transcription factors involved in regulation of pluripotency associated genes (*OCT4, NANOG, SOX2*) are repressed during differentiation by H3 lysine 4 and 27 trimethylation in human embryonic stem cells (Pan et al., 2007). In our study, one step of immunofluorescent study with fluorescein-conjugated anti-PARP antibody was performed first to characterize the apoptotic cells. The signal could not be recognized under light microscopy. We then examined the apoptotic cells with anti-monomethyl histone H3 lysine 27 antibody by immunocytochemistry to avoid antibody cross reaction. The total level of H3 lysine 27 monomethylation is increased, but dose not necessarily correspond to the situation at a specific gene promoter under staurosporine-induced apoptosis. The relationship between staurosporine-induced apoptosis and histone epigenesis has not been explored or addressed in the English literature.

In conclusion, our findings demonstrate for the first time that monomethylated histone H3 lysine 27 is expressed in staurosporine-treated osteosarcoma cells and is correlated with tumor cell apoptosis. Although only an accompanying effect during apoptosis, these events may provide further understanding of epigenesis and apoptosis, and might have therapeutic implications for this frequently fatal disease. The associated mechanisms, however, need to be further elucidated.

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