MODULATION OF GENE EXPRESSION IN OSTEOSARCOMA BY DEMETHYLATION

By

Khaldoun Al-Romaih

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ABSTRACT

Osteosarcoma (OS) is an aggressive tumor where a significant subset of patients responds poorly to the current treatments. Methylation-silencing of gene expression is a feature of many tumors and is reversible by demethylation drugs, such as decitabine. Demethylation reactivates methylation-silenced genes and leads to induction of differentiation, growth arrest, and apoptosis. Since genes associated with tumor progression and aggressive disease may be repressed by hypermethylation, it follows that decitabine treatment may influence tumor growth, and can be used to identify gene targets for novel epigenetic therapies in OS.

In the first part of this thesis, microarray expression profiling was used to identify decitabine-dependent changes in gene expression in U2OS cells. In parallel U2OS xenografts were used to study the effect of decitabine on tumor growth and differentiation. Microarray expression profiling of U2OS cultured cells showed that decitabine treatment caused a significant induction of more than 50 genes. Six of these genes were shown to be involved in apoptosis, and were induced in U2OS xenografts when mice were treated with decitabine. Importantly, drug-treatment of U2OS xenografts significantly reduced tumor size, decreased mitotic activity, increased bone

matrix production, and increased the number of apoptotic cells. Promoter regions of these genes were demethylated in response to decitabine treatment and they were re-expressed.

In the second part of my thesis I present an analysis of therapeutic modulation of Gadd45a using decitabine. Nuclei from the decitabine-treated U2OS xenografts exhibited increased amounts of Gadd45a proteins. *GADD45A* was also found demethylated in response to decitabine treatment in the OS cell line MG63. This demethylation was associated with elevated expression of *GADD45A* and induction of apoptosis. To show the specificity of this gene for decitabine-induced apoptosis in OS, *GADD45A* mRNAs were disrupted using siRNA and the ability of the drug to induce apoptosis was found to be reduced.

Collectively, these data provide novel insights regarding the use of epigenetic modifiers in OS treatment. Understanding the role of demethylation of *GADD45A* in re-expression of this pathway and restoration of apoptotic control have important implications for understanding OS oncogenesis and for development of new targeted therapeutic approaches involving demethylation drugs.

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Glossary

(5mC)	5- Methyl- Cytosine
(Decitabine)	5-Aza-2'-Deoxycytidine
(AML)	Acute Myeloid Leukemia
(alpha-MEM)	Alpha-Minimum Essential Medium
(AS)	Angelman Syndrome
(BWS)	Beckwith-Weidemann Syndrome
(BMP)	Bone Morphogenic Protein
(CIN)	Chromosomal Instability
(CDP)	cis-Platinum
(CGH)	Comparative Genomic Hybridization
(CT)	Computed Axial Tomography
(DMR)	Differentially Methylated Region
(DNMTs)	DNA (Cytosine-5) Methyltransferases
(DOXO)	Doxorubicin
(EFS)	Event-Free Survival
(FISH)	Fluorescent in situ Hybridization
(GADD45A)	Growth Arrest and DNA Damage Inducible- Alpha
(IFO)	Ifosfamide
(IHC)	Immunohistochemistry
(IH)	Isolated Hemihyperplasia
(MTP-PE)	Liposomal Muramyl Tripeptide Phosphatidyl Ethanolamine
(LOH)	Loss of Heterozygosity
(MRI)	Magnetic Resonance Imaging
(MTX)	Methotrexate
(MBDs)	Methyl binding domain proteins
(OS)	Osteosarcoma
(PCR)	Polymerase Chain Reaction
(PWS)	Prader-Willi Syndrome
(PI)	Propidium Iodide
(Pyro Q-CpG)	Quantitative Methylation-CpG Pyrosequencing
(RT)	Room Temprature
(SINE and LINE)	Short and Long Interspersed Nuclear Elements
(siRNA)	Short Interference RNA
(SKY)	Spectral Karyotyping
(TUNEL)	Terminal Uridine Deoxynucleotidyl Transferase
(TSS)	Transcriptional Start Sites

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CHAPTER ONE

1 INTRODUCTION

Modified in parts from:

Genomic mechanisms and measurement of structural and numerical instability in cancer cells. Jane Bayani, Shamini Selvarajah, Georges Maire, Bisera Vukovic, **Khaldoun Al-Romaih**, Maria Zielenska, Jeremy A. Squire. *Seminars in Cancer Biology 17 (2007) 5–18.*

1.1 Overview of OS

In 1901, Dr Leonard Freeman wrote: "*In the latter part of July, 1899, I examined an emaciated anemic Swedish woman, thirty-eight years of age, referred to me by Dr. Maxwell, of Elizabeth, Colorado. An enormous sarcoma occupied the upper half of the right thigh, and extended well into the pelvis ... springing from the periosteum of the femur bone upon its anterior surface ...*" [1]. For treatment he proposed that: "*our main weapon of defense is the knife. We wish that it were more reliable and less objectionable, but we are forced to accept it as it is*".

Sarcoma of the bone, is called osteogenic sarcoma or osteosarcoma (OS). One of the earliest evidence of OS presence in man was reported in the proximal extremity of a Celtic Warrior's left humerus (800-600BC), found in Muensingen (Switzerland) [2]. To-date there have been over 15,000 published articles on OS, of which many have discussed improved treatment regimens and, yet surgery still remains the most effective treatment option. In this introduction text I have reviewed the histopathologic and clinical features of OS, and briefly describe some of the novel therapeutic approaches to treatment of OS.

1.1.1 Definition of OS

OS is defined as a spindle cell malignant mesenchymal tumor of bone that produces malignant osteoid (bone extracellular matrix) **(Figure 1.1)**. It is the primary malignant tumor of osteoblasts (differentiated bone cells) and accounts for approximately 20% of primary bone cancers. It is diagnosed most frequently in patients in their second decade of life, coinciding with a period of increased bone growth [3-5]. Primary OS tumors are often located in the anatomical bone sites that are associated with maximum growth, the distal femur, the proximal tibia and the proximal humerus **(Figure 1.1)**.

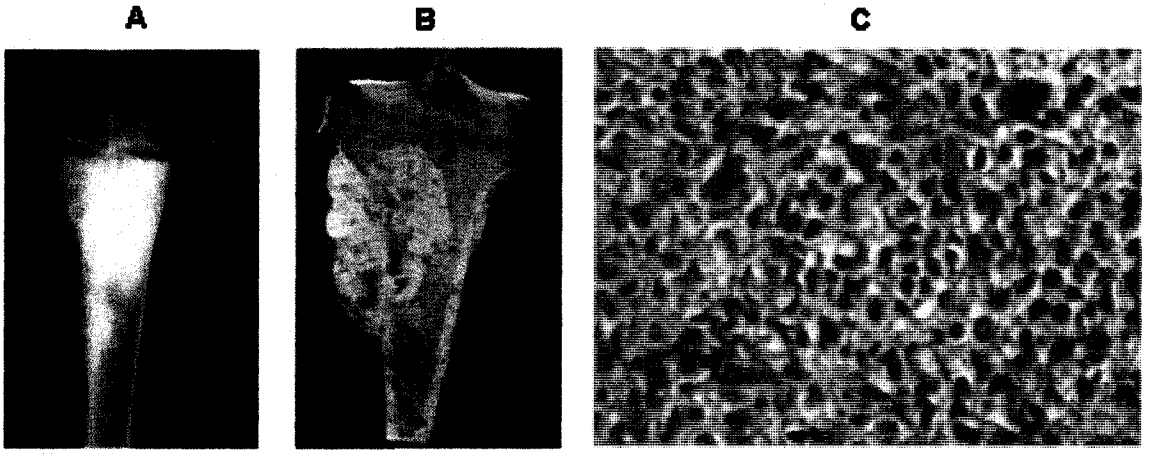

Figure 1.1: A) Radiograph of OS at the distal humerus. **B)** Gross specimen of high-grade intramedullary OS at the metaphyseal region of the proximal tibia. **C)** H&E stained section of high-grade OS. Pink areas of osteoid surround the pleomorphic osteoblasts. (Images adapted from [6]).

1.1.2 Epidemiology

The latest report from Health statistics Division, Health Canada and the National Cancer Institute of Canada indicated that the OS incidence rate is 3.8 per million and there were 159 new children with OS between the year 1999 and 2003 (total of 6,446 new children with any childhood tumor, age 0-19 years). Fifty four of the 159 OS patients have died (mortality rate = 1.3 per million per year) and the death-to-case ratio is 0.34, higher than that of the majority of childhood tumors including, leukemia (0.19), lymphoma (0.07), and retinoblastoma (0.03) [7]. Furthermore, a study at the Mayo Clinic of 8,542 cases of primary bone neoplasms, OS accounted for 19% of all bone tumors [8]. Another study reported a wide irregularity in the geographic pattern of incidence for OS. Low rates are observed in some Asian and Latin American populations with incidence rates rising steeply with age [9].

1.1.3 Etiology

OS may arise sporadically, or be associated with hereditary diseases such as Rothmund-Thomson [10] [11], Bloom [12], and Li-Fraumeni syndromes [13]. In addition OS in older patients may be associated with Paget's disease [14]. Other factors such as chemical agents, viruses containing the *src*-oncogene or the SV40 T-antigen, and radiation were shown to be potent inducers of OS [12]. Overexpression of oncogenes (such as c-*FOS* and *ERBB-2*) or growth factors (such as TGF-beta) leading to self sufficiency in growth signals and the loss of anti-tumor growth signals (loss of tumor suppressor genes such as *RB* and *P53*) are also amongst OS predisposition markers

(discussed in sections 1.2.2 through 1.2.5). Also, disruption of pathways leading to the evasion of apoptosis is emerging as another marker for OS predisposition. This includes (but is not restricted to) aberrations of survival factors (IGFs) or death factors (TNFs), although more investigation is needed to confirm the implication of these pathways in OS pathogenesis (discussed in sections 1.2.2 through 1.2.5).

1.1.4 Classification and Histopathological grading

OS is a malignant neoplasm of bone that demonstrates focal osteoid production by neoplastic cells. The predominant histological patterns are osteoblastic, fibroblastic, or chondroblastic. OS can be classified by histologic grade of malignancy [15] [16]. In this classification scheme, several histologic variants of OS have been defined including: high-grade OS, telangiectactic OS, small cell OS, giant cell OS, periosteal OS, parosteal OS, multifocal OS, well-differentiated OS, high-grade surface OS, and intracortical OS [17] **(Table 1.1)**. Of these variants high-grade OS mostly affects children and accounts for 90% of all OS cases. Another approach for grading OS is based on cellular irregularities and mitotic index. With this classification scheme, OS is one of three grades: grade I, II, and III where grade III is considered the highest that is almost always associated with OS metastasis. Grades I and II are associated with better prognosis and fewer (if any) metastatic disease [18].

Table 1.1: Histologic grades of OS as summarized from 'Tumors of the Bones and Joints' *Mills et al, 1993* [6].

Class	Occurrence	Age	Comments
Conventional high-grade OS	>90% of OS	2nd decade 80% (Childhood OS)	Mostly high-grade & aneuploid
Multifocal OS	Rare	Childhood & adult	Mostly High-grade
Telangectatic OS	Rare	Childhood & adult	
Small cell OS	Rare	1st, 2nd decade (70%)	90% diploid
Well-differentiated OS	1 to 2 % of OS	Adult	
Intracortical OS	Rare	10-30 years old	Mostly High-grade
Periosteal OS	<2% of OS	> 21 years old	Good prognosis
Parosteal OS	Up to 5% of OS	More in adults	Good prognosis
High-grade surface OS	Rare	Childhood & adult	Mostly High-grade

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1.1.5 Diagnosis

Although OS may rise from any bony site, it is usually a metaphyseal lesion. The distal femur accounts for approximately one third of all cases and is the single most common site, followed by the proximal tibia and the proximal humerus [8]. Most patients are in the second decade of life and present in clinic with pain, increased bone mass, and occasionally with decreased joint motion [19]. OS grows rapidly, where the median doubling time for tumor volume is 34 days [20] [21].

Radiographic assessment of the affected bones usually demonstrates bone irregularities of lytic and/or sclerotic nature [5]. Additional radiographic examination is usually required to evaluate the lesion in detail. Both computed axial tomography (CT) and magnetic resonance imaging (MRI) are superior to plain radiographs in determining the extent of the lesions [22] [5]. The typical radiograph finding is a large, infiltrating metaphyseal lesion that arises in medullary bone and erodes through the cortex to form a large soft tissue mass **(Figure 1.1)** [22] [5]. In addition a biopsy is important in distinguishing OS from other malignant neoplasms (Ewing's sarcoma, lymphoma, metastatic tumor) as well as from benign tumors (osteochondromas) and non-neoplastic conditions [23, 24].

1.1.6 Metastasis

Gross metastatic disease in the lung or bone is present at diagnosis in 10 to 20% of all patients with high-grade OS. Lung and (less often) bone are the most common sites of distant spread. Metastases to regional lymph nodes, to colon bowl or to the kidney is uncommon [25] [26, 27]. Metastastic lesions usually have similar histological pattern of the primary tumor [24], and patients who have had primary OS require regular radiological evaluation of the lungs and removal of any suspicious lesions for histological analysis [22] [28] [29].

1.1.7 Chemotherapeutic management of OS

Most commonly used agents for OS treatment include, high-dose methotrexate (MTX), doxorubicin (DOXO), cis-Platinum (CDP), and Ifosfamide (IFO) either alone or with etoposide [30]. Drugs are primarily given intravenously or rarely intra-arterially. Distant metastases remain the major cause of reoccurrence and mortality. Multiple trials employing either a single chemotherapy agent or multi-drug regimens showed wide disease-free-survival rate of 30 to 70% [31]. Several clinical trials have been conducted [32-36], and the overall outcome of those trials have confirmed that the combination of multiple chemotherapy agents and surgery results in long-term event-free survival (EFS) and survival rates of 60% and higher.

The high toxicity of currently used OS chemotherapy regimens is a concern. Early toxicity may be hematologic, or involve acute liver (mainly due to MTX) and/or renal (due to cis-Platinum and ifosfamide) sites. Longer survival has led to the increase in late chemotherapy toxicity. The main late toxicities are: second tumor, sterility, chronic renal failure, cardiac and neurological toxicities (mainly due to CDP) [37] [38]. For example in a series of 755 patients 13 patients (1.7%) developed Doxorubicin cardiomyopathy (dose range 300– 480 mg/m2) and all these patients experienced congestive heart failure and the risk of developing a second malignant neoplasm was calculated to be 8–10%, 20 years after initial diagnosis [39].

The current combination treatment of chemotherapy and surgery has significantly improved the OS mortality and morbidity rates. The 5-year survival rate has increased in the last 30 years from 10% to 70%. In patients with poor prognosis, such as those with

metastases at diagnosis, the 5-year survival rate has reached 20-30% due to chemotherapy and the surgical removal of metastases and primary tumor [39, 40]. However, drugs used for OS treatment are still the same as those employed over the last 20 years, and no standard, second line therapy exists for those who relapse [40-42]. At relapse surgery is still a main option [39].

Trials to identify the most effective treatment regimen are still ongoing. Most recently, the results of a four-arm study on ~700 osteosarcoma patients were reported [35]. In this study MTX, DOXO, CDP ± IFO and ±MTP-PE (liposomal Muramyl Tripeptide Phosphatidyl Ethanolamine, a biologic response modifier, and a monocyte activator) were added to chemotherapy: the arm that received MTP together with Ifosfamide had better results (78% 3-year EFS) compared to the standard arm CDP-MTX (68%) and the CDP, MTX, Doxo + Ifosfamide arm (61%). Since in this study the difference in EFS between the treatment arms was not highly significant ($P = 0.04$) additional clinical investigations was concluded by the investigators to be necessary to identify the most effective regimen [35].

There are several new therapeutic approaches being considered for future treatment of OS. These include immune-based therapy, antiangiogenesis agents, tumor-suppressor or suicide gene therapy [39, 43]. In addition, there has been increasing interest in anticancer drugs not commonly used in OS such as epigenetic modifiers which will be discussed in the second half of this chapter.

1.2 Chromosomal basis and molecular genetics of OS

Malignant transformation requires the accumulation of genetic and epigenetic alterations leading to loss of tumor suppressor genes, activation of oncogenes, abnormalities in genes that control DNA repair pathways, abnormalities in chromosome number and structure, and overall genomic instability. In OS, a number of studies have described a variety of alterations resulting in chromosomal abnormalities, tumor suppressor gene inactivation, and oncogene overexpression. Particularly, ploidy changes, chromosomal rearrangements, and genomic imbalance, have been reported frequently in OS and have been linked to its tumorigenesis. OS tumors also have been shown to harbor genetic lesions that inactivate both the p53 and/or Rb tumor suppressor pathways. These genetic alterations directly or indirectly suppress the normal function of p53, Rb, or both, which impairs control of cell cycle progression and shifts the balance in favor of cell proliferation. In addition, a variety of oncogenes including FOS, MYC, and ERBB2 are activated in some OS tumors. This section will review some important chromosomal and molecular lesions in OS and discuss some of the potential genes and pathways with possible roles in OS development.

1.2.1 Chromosomal alterations in OS

In contrast to sarcomas, such as Ewing's sarcoma, synovial sarcoma, and alveolar rhabdomyosarcoma with recurrent chromosomal translocations that are diagnostically useful, OS has karyotypes that are highly complex with multiple chromosome

rearrangements **(Table 1.2)**. Conventional and molecular cytogenetic approaches have been of value in observing chromosome structure and number aberrations in OS, and a number of surveys of structural and numerical aberrations have been performed on OS [44-50]. Chromosomal analyses and conventional G-banding studies have shown that OS tumors possess karyotypes with a high degree of aneuploidy observed as elevated chromosome numerical changes. Several reports have shown that OS is featured with chromosome number range from near haploid to near- tetraploid or even up to octaploid range [44, 46, 49-53]. Moreover, the high range of ploidy is more frequent in high-grade OSs than that of the low-grade OS [45].

Comparative Genomic Hybridization (CGH), array-CGH, and Spectral Karyotyping (SKY) analysis have been systematically applied to OS to address the inherent difficulties associated with classical cytogenetic analysis [53]. CGH studies have identified chromosomal gains at 1p, 1q, 4q, 5p, 7q, 8q, 14q, 19 and losses at chromosome 2q, 3p, 6q, 8p, and 10p. Cytogenetic studies including spectral karyotyping, M-Banding, and FISH studies have identified chromosome bands or regions 1p11-13, 1q11-12, 1q21-22, 11p14-15, 14p11-13, 15p11-13, 17p and 19q13 to be most frequently rearranged [50, 54-56]. As more tumors are studied using more advanced cytogenetics and microarray methods, recurrent anomalies affecting specific chromosomal regions and/or distinct patterns of chromosomal imbalance are becoming more apparent.

In summary, OS is characterized with high level of ploidy changes, numerical aberrations and very complex karyotypes with multiple rearrangements, which are thought to contribute to the aggressive behavior of this tumor. These characteristics imply

that the genome of OS is subject to an unusually high level of chromosomal instability (CIN) and that CIN may be intrinsic to OS oncogenesis. CIN refers to a series of chromosomal changes occurring at an accelerated rate in cell populations derived from the same ancestral precursor. [51] [57] [58]. CIN is characterized by grossly abnormal karyotypes, featuring both structural and numerical chromosome abnormalities [58]. The heterogeneity of numerical changes in OS is believed to directly reflect an underlying CIN, which may result from a persistent underlying defect leading to a constantly elevated rate of chromosome anomalies arising at each cell division [59, 60]. In addition to the role of chromosome segregation defects there is increasing evidence that links epigenetic aberrations to CIN [61] (discussed in section 1.5.3).

Table 1.2: Recurrent chromosomal translocations in sarcomas.

Tumor	Translocation	Fusion partners
Ewing sarcoma	t(11;22)(q24;q12)	EWS/FLI-1 [62]
Clear cell sarcoma	t(12;22)(q13;q12)	ATF1/EWS [63]
Desmoplastic small round cell tumor	t(11;22)(q13;q12)	EWS/WT1 [64]
Myoxoid chondrosarcoma	t(9;22)(q31;q12) [65]	EWS/TEC [66]
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	PAX3/FKHR [67]
Embryonal rhabdomyosarcoma	LOH/ loss of imprinting [68, 69]	

1.2.2 Alterations in the *P53* pathway in OS

Human *P53* is a commonly mutated tumor suppressor gene in human cancers. It was localized to chromosome 17p13 and its inactivation is central to the pathogenesis of OS. Inactivation of p53 in OS can be by mutations in the gene itself or by alterations of its regulatory genes. Alterations of p53 gene seen in OS tumor samples consist of point mutations (20-30%), gross gene rearrangements (10-20%), and allelic loss (75-80%) [12, 70]. *P53* mutations can be detected before or after the development of metastasis in OS tumors, an indication that *P53* mutations are an early event in OS [71]. Furthermore, the effect of *P53* alterations in mice and humans provide strong evidence of its involvement in OS pathogenesis. Studies showed that up to 70% of heterozygous *P53*-dificient mice develop OS by 16 months of age [72, 73], in addition to increased incidence of metastatic OS [74]. Germ-line mutations in *P53* predispose patients to a variety of tumors, particularly sarcomas including OS, a phenomena known as Li-Fraumeni syndrome [75, 76].

Loss of *P53* function can also result from upstream defects such as overexpression of its regulatory protein *MDM2*. *MDM2* is located on chromosome 12q13 and encodes a protein that modulates p53 negatively. Copy number analysis has shown that 5-10% of OS have amplification in the 12q13 region, encompassing not only *MDM2* but also often the closely linked *CDK4* gene [77, 78]. Alterations in *MDM2* are more evident in metastatic OS than it is in primary OS [79]. Another defect upstream of the p53 pathway in OS is deletions of the *INK4* locus [80]. The *INK4* locus on chromosome 9p21 encodes at least three genes: $p14^{ARF}$, $p16^{INK4A}$ and $p15^{INK4B}$ [80]. The $p14^{ARF}$

protein regulates p53 function by binding mdm2 protein and sequestering it in the nucleus. As a result, $p14^{ARF}$ prevents mdm2 from targeting p53 for degradation [81]. The *INK4A* locus is deleted in approximately 10% of OS, and most deletions will inactivate $p14^{ARF}$ expression [82] representing another mechanism for inactivation of p53 protein function.

1.2.3 Alterations in the RB pathway in OS

The retinoblastoma (Rb) protein is a major regulator of G1 to S cell cycle transition [83]. *RB* gene is located on the long arm of chromosome 13, a region with loss of heterozygosity (LOH) in approximately 60% of OS tumors [84, 85]. Gross structural rearrangements of the *RB* gene are present in up to 30% of OS tumors, and point mutations appear to be far less frequent, occurring in less than 10% [85, 86]. Heterozygosity for germ line mutations in *RB* predisposes patients to the hereditary form of retinoblastoma, and these patients have a significant increase in the frequency of primary and radiation related OS [87]. Other studies on sporadic OS reported the presence of *RB* gene alterations in about 70% of the cases and correlated alterations in this gene to late stages of OS development [88]. These alterations included structural rearrangements, complete deletions or less frequently point mutations. Nevertheless, OS tumors with no *RB* alterations might have defects in other proteins in the *RB* pathway. These proteins include $p16^{INK4A}$, and *CDK4*.

As mentioned above the *INK4* locus on chromosome 9p21 encodes for $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$. While $p14^{ARF}$ is involved in the p53 pathway, $p16^{INK4A}$ is a

tumor suppressor gene that inhibits cdk4, which in a complex with cyclin D1, facilitates the transition from G1 to S phase in cell cycle by phosphorylating Rb. Deletion of *INK4* locus was reported in 10% of OS cases [82]. Loss of $p16^{INK4A}$ function results predominantly from *INK4A* deletions rather than point mutations [82, 89]. Moreover, chromosome region 12q13 is amplified in a subset of OS [82]. This region is a genomic location for *CDK4* (in addition to *MDM2*) genes. Higher levels of Cdk4 may lead to Rb phosphorylation impairing its function in cell cycle control [90]. *CDK4* gene is amplified in 9% of OS tumors [82] making this event significant in OS pathogenesis as it can impact the Rb pathway.

1.2.4 Oncogene amplification in OS

In addition to alterations affecting tumor suppressor genes, other genes with oncogenic properties were found amplified in a subset of OS tumors including *MYC* and *FOS* oncogenes. *MYC* oncogene family plays a significant role in the pathogenesis of many human neoplastic diseases [91, 92]. c-*MYC* gene is located on 8q24 and encodes for a protein that is involved in a number of cell processes including DNA replication, modulation of cell growth, regulation of apoptosis and transcriptional regulation of specific genes [93, 94]. c-*MYC* gene is amplified in a small subset of OS [95, 96] and its product was found overexpressed more frequently in relapsed and metastatic OS [97].

The c-*FOS* oncogene is also amplified in OS [97]. It was isolated as the cellular homologue of the v-*FOS* gene found in the OS inducing FBR- and FBJ-murine sarcoma viruses (MSV) [98]. c-*Fos* oncogene is a transcription factor on chromosome 6q21 and its

activation induces transformation in cultured cells [99]. Moreover, when the viral homologue v-*FOS* was injected into mice, OS formation was enhanced [100]. Expression of c-*FOS* has been shown to be highly elevated in 60% of OS samples [101]. Elevated expression of c-*FOS* was correlated with high-grade more frequently than with low-grade OS [101]. Overexpression of c-*FOS* have been observed more often in patients who developed metastases than those who remained metastases free [97].

1.2.5 Other genes and pathways with potential role in OS pathogenesis

a) *GADD45A*

OS tumors typically have complex karyotypes with multiple structural and numerical chromosomal aberrations. There are several other factors whose aberrations have potential implication in OS pathogenesis. In particular, aberrations in factors that shift the balance toward increased tumor cell survival and decreased apoptosis. One downstream target for p53 protein that has been associated with genomic fidelity is the *GADD45* family member *GADD45A* [102, 103]. Gadd45a is a small protein involved in the G2/M checkpoint arrest [104], nucleotide excision repair [105] and apoptosis [106]. The involvement of this gene in OS pathogenesis and its therapeutic potential is one of the main subjects of this thesis. Detailed discussion of *GADD45A* involvement in caner in general and in OS in particular is given in Chapter 3.

b) Signaling pathways

There has been growing recognition of the importance of microenvironmental signals in cancers [107-110]. There are several examples of secreted factors with roles in the cell signaling pathways that are implicated in bone development. These factors have been implicated in normal bone development by being part of the signals exchanged in the bone microenvironment [111]. Examples of secreted signaling factors implicated in bone development include the Wnts and related proteins, notch, TGF-beta, and IGF binding proteins.

Wnt signaling pathway represents the potential role of microenvironmental signaling in determining the balance between proliferation and differentiation during bone development [112]. Wnt signaling regulates cell proliferation and is regulated tightly during bone development [113, 114]. It is active during the early stages and inhibited in the late stages of osteoblast differentiation [113]. Hence, Wnt signaling acts as a molecular switch influencing proliferation and differentiation balance in the osteoblast lineage. There is also good evidence that regulation of Wnt signaling pathway is coordinated along with that of notch, and TGF-beta pathways during osteoblast differentiation [115]. Coordination of the three signaling pathways during bone development is orchestrated by a class of genes know as bone morphogenic proteins (BMPs) [115]. Several family members of BMP family were found overexpressed frequently in OS [116] suggesting BMP implication in OS development. Other signaling molecules which are known as regulators of proliferation and differentiation in mammalian cells with potential role in bone development are the IGF binding proteins

(IGFBPs). The role of this signaling pathway in bone development remains largely undetermined and will be discussed in more detail in the second chapter of this thesis.

1.3 Preclinical *in vitro* and *in vivo* models of OS in drug testing

A full assessment of normal and tumor cell behavior requires integrating experimental data from novel drugs both *in vitro* and *in vivo* models. Such preclinical studies are usually performed prior to biological and clinical correlative studies as part of clinical trials. Numerous OS cell lines have been established and several of which have the biological characteristics of the disease [117]. Moreover, various therapeutic agents have been tested on those cells and the observed effects gave insights on OS cell sensitivity to chemotherapy [118]. Model systems for studying OS comprise: (1) human tumor cells grown in cultures (primary or immortalized); (2) experimental animals (xenografts); (3) animal OS tumors and derived cell lines; and (4) transgenic animal models with spontaneous or induced OS incidence.

One advantage for using animal hosts to propagate human cancer cells is to provide a microenvironment that resembles the physiological conditions in the original tissue. Such approaches have been proven to be particularly useful in studying the efficacy of therapeutic agents. This section focuses on discussing the use of human OS cells in drug testing *in vitro* and in xenografts.

1.3.1 Human OS cell lines

The most commonly used human OS cell lines are the human OS cell lines U2OS, MG63, SAOS2, HOS and 143B [119]. These cell lines have been extensively characterized in the literature. They were derived from young OS patients and have been confirmed to have some of the genetic lesions affecting pathways with biological relevance to OS tumors [119, 120]. Despite the similarity of OS cell lines in regards to their origin, they differ from one another in their response to treatment [120, 121]. This could be explained in one way by the differences in the genetic lesions observed in these cell lines. For example, the *P53* gene is known to be functional in U2OS but not in MG63, SAOS2 or HOS [122, 123].

In vitro studies of human OS cell lines have provided a better understanding of the molecular signaling and of the multitude of response to chemotherapy of OS derived cells, however they remain limited. One major limitation of the *in vitro* studies includes the inability of observing the effects resulting from the interaction between the tumor cells and the microenvironment that is provided by the host. This limitation is emphasized in experimental systems that investigate the efficacy of therapeutic drugs. Overcoming this limitation is possible through establishing these cell lines in animal models (xenograft), which would allow for the physiological and histopathological assessment in conditions comparable to those of the original disease.

1.3.2 OS xenografts

Therapeutic studies of OS xenografts in immunocompromised mice are needed to investigate the differential response of currently used drugs and to evaluate new drugs. Only a limited number of murine xenograft studies of OS have been undertaken [117, 118, 124]. Some researchers have generated xenografts from non-transformed primary OS cells [118]. They have shown that some of the *in vitro* observations are reproducible, and the tumor response to treatment across the xenografts closely parallels that of OS tumors.

1.4 Epigenetic Modifications

In the 1940s a new biological concept was described under the identification "epigenetic landscape". This concept was published in its *mature* form in 1957 describing a set of developmental choices that is faced by a cell in the embryo [125, 126]. This concept is one of Conrad Hal Waddington's greatest contribution to science and it is described in detail in his book "*The Strategy of the Genes*" [125, 126]. Waddington's "*epigenetic landscape*" is a metaphor for a dynamic system, one in which the axes represent concentrations of all the gene products in the cell. All the cells in the embryo would evolve according to the same laws, but because of the existence of inducing signals, cells in different regions would follow different pathways and end up at different attractors, which can be elegantly associated with different states of terminal differentiation [126]. Today, "*epigenetics*" is known as the mechanisms that result in heritable changes in gene expression without changes in gene sequence [127]. These heritable changes are maintained as covalent chemical signatures on the cytosine bases in the DNA and/or the histone proteins of the chromatin, resulting in regulation of chromatin organization and DNA accessibility, and ultimately regulation of gene expression [127].

Regulation of epigenetic signals ensures appropriate biological responses across the different stages of cell development [128]. Dysregulation of epigenetic signals, on the other hand, may lead to the occurrence of disease [129]. The most studied type of epigenetic signal in the literature is methylation of cytosine on DNA [130] **(Figure 1.2)**.

Histone protein modifications are another type of epigenetic signals that has gained increasing interest in the last two decades **(Figure 1.2)** [131, 132]. Both types of epigenetic signals are notably reversible, a feature that facilitated the targeting of these signals for human disease treatment [127]. This section of the introduction describes the key concepts of chromatin organization and DNA methylation.

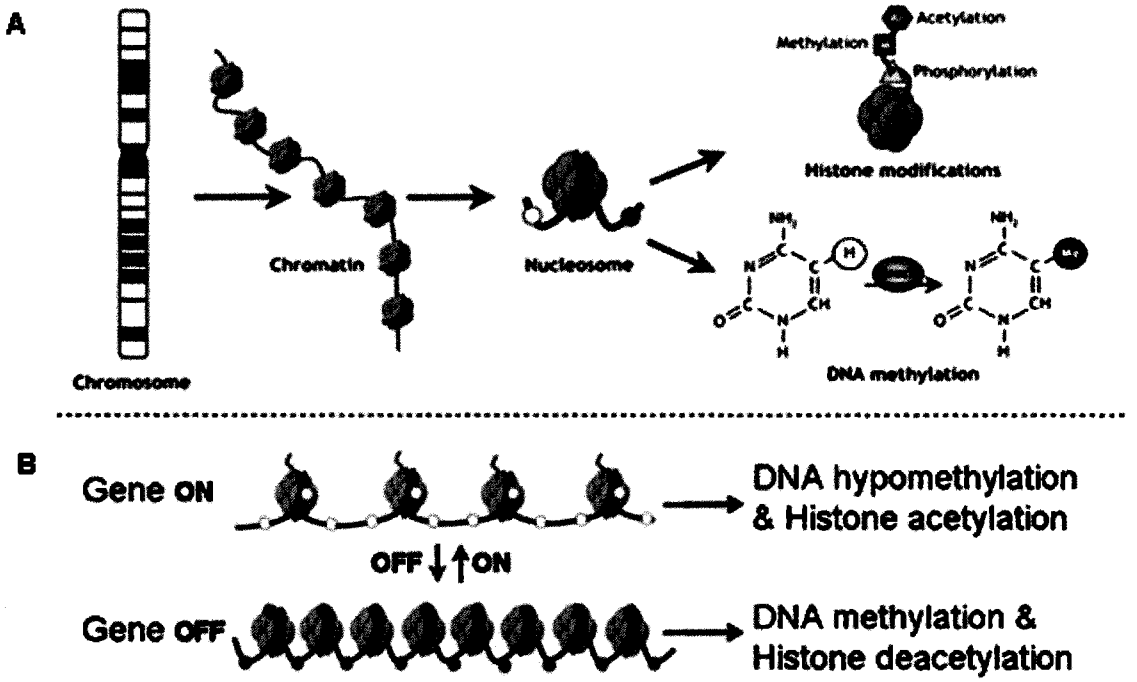


Figure 1.2: A) Epigenetic mechanisms: histone modifications and DNA methylation. Histone modifications can be one of several types including histone acetylation, histone phosphorylation and histone methylation. DNA methylation refers to the addition of a methyl group to the fifth carbon position in cytosine base (5mC). These processes orchestrate the opening or compaction of chromatin. **B)** Open and closed chromatin domains. Lower 5mC and higher acetylation are markers of open chromatin domains (active transcription domains). Higher 5mC and lower

acetylation are markers of closed chromatin domains (repressed transcription domains). (Adapted from [133])

1.4.1 Chromatin organization and histones

The largest unit of organization of the eukaryotic genome is the chromosome. Chromosomes are discrete nuclear bodies separated by an interchromatin compartment; a continuous space between adjacent chromosomes in the nucleus [114]. Chromosomes are essentially complex DNA structures that are packaged around core proteins called histones including H3, H4, H2A, H2B and H1. The packaging of DNA around histones is referred to as the chromatin. Chromatin and chromosomes are packaged in a number of distinct levels, each with its own packing ratio: the ratio of the length of the DNA to that of the structure into which it is compacted. The higher levels of DNA packing differ as the cell progress through cell cycle to mitosis. However, the lower levels of packing are generally the same with the exception of a few modifications of chromatin. Modifications of the lower levels of chromatin compaction are the subject of discussion herein [134].

The basic packaging units of chromatin are the nucleosomes, and each nucleosome is made of the core histones with the DNA thread wrapped around it. Each nucleosome is linked to adjacent nucleosomes by histone linkers forming the backbone for DNA packaging. The nucleosome contains 8 histone molecules, two of each histone, and is enough to wrap a DNA segment of ~200 base pairs; a structure known as the 'nucleosome fiber'. Nucleosomes are linked together by linker histones (H1 and its variants) to further stabilize the chromatin and link nucleosome fibers. Histones consist of unstructured highly basic N-terminal tail and a short basic C-terminal tail. Histone tails

contain amino acid residues (such as lysine, and arginine) that can be tagged with acetyl, methyl, or phosphate groups [134].

Histone proteins are the subject of several types of modifications. Histone modifications can influence the degree of DNA compaction. They are involved in the establishment of the chromatin state through facilitating opening or closing chromatin domains. Opened chromatin domains form the euchromatin, and closed chromatin domains form the heterochromatin [134]. Chromatin transcription activity depends on the state of the chromatin domain. Euchromatin is indicative of the accessible-transcriptionally active domains, whilst heterochromatin is indicative of silenced domains. This chromatin pattern is not random and is heritable leading to transmission of a number of biological phenotypes such as imprinting and X chromosome inactivation [135]. Although the function of the majority of histone modifications remains poorly understood, there has been considerable progress in uncovering the function of acetylation or methylation of lysine residues on histones [136]. For example, acetylation of histones on lysine residues has been shown, almost always, as a marker of active chromatin and a signature of actively transcribed genes [136]. In contrast, H3-Lysin 9 methylation has been consistently shown as a marker for repressed chromatin state leading to transcription silencing [137].

Chromatin degree of compaction is not only a result of histone modification, but also epigenetic modification of the DNA thread itself. The major epigenetic modification of DNA is methylation of the fifth carbon position (C5) of the cytosine base. In human, this mechanism has been strongly linked to normal development and to the development

of disease as discussed in the upcoming sections. Moreover, manipulating this mechanism has been shown to be fruitful in the treatment of human disease.

1.4.2 DNA methylation

Addition of a methyl group to C5 of the cytosine base is referred to as DNA methylation **(Figures 1.2 and 1.3)**. DNA methylation is the most extensively studied, thus the most understood epigenetic mechanism. In mammals, methylation of the C5 position of cytosines (5mC) within CG rich regions in the genome (CpG-islands: "p" denotes the intervening phosphate group) represents a major form of DNA modification [138, 139]. The distribution of CpG dinucleotides in mammalian genomes is not random. Globally, CG occurs at a low frequency of ~1 CpG/100 bp of the genome, lower than that of expected frequency at random, and these are predominantly methylated on both strands. The promoter and 5' sequences of about half of the known genes, however, have a higher CG frequency of occurrence than that of other regions of the genome (~1 CpG/10 bp; which is near the expected random frequency) [140]. These are called CpG-islands (~ 1 kb in size), which tend to be undermethylated in normal cells , with the exceptions of methylated CpG-islands that are associated with transcriptionally silent alleles of imprinted genes and the silent genes on the inactive X chromosome in females [140]. CpG-islands associated with imprinted and X-inactivated genes account for <10% of total genomic methylated CpGs. The bulk of methylated CpG in the genome (~70%) resides within CG-rich transposons that are not associated with 5' promoter regions of the genes (extragenic regions) [141, 142]. 5mC is mediated by proteins called DNA

(cytosine-5) methyltransferases (DNMTs) which are divided into two major classes: maintenance DNMTs and *de novo* DNMTs [143-146].

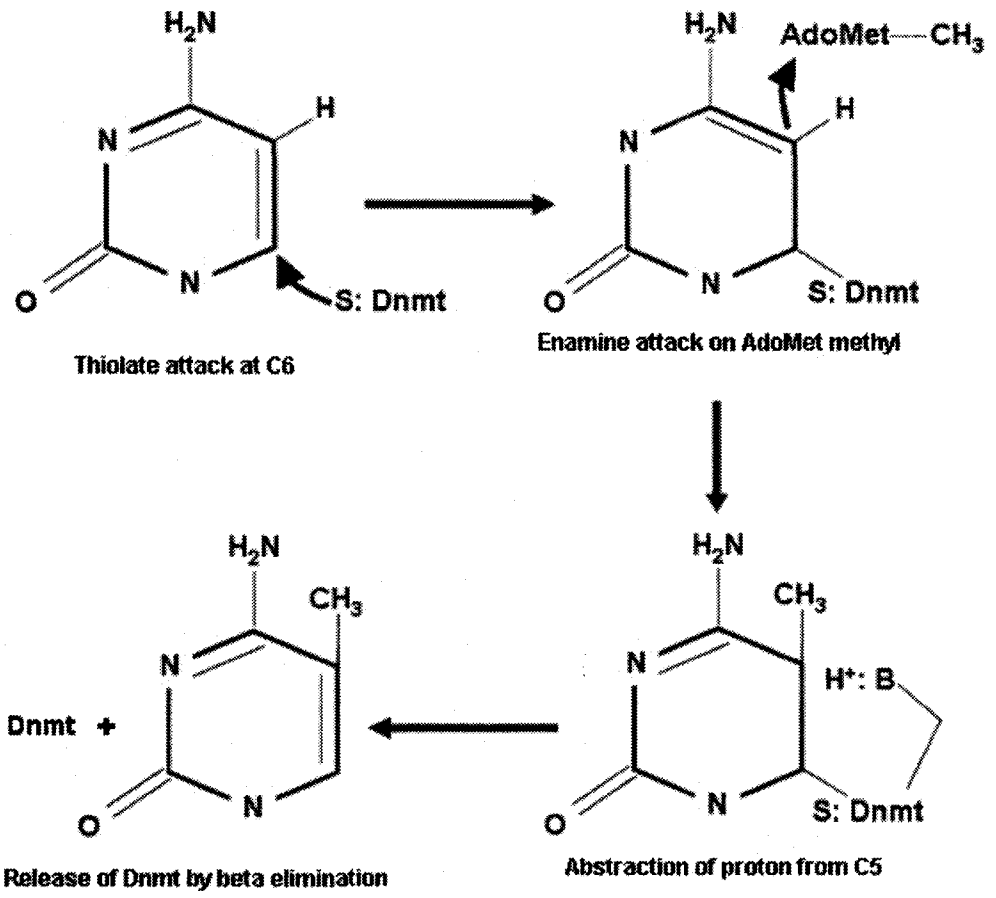


Figure 1.3: Proposed catalytic mechanism of Dnmts. The substrate in this mechanism is cytosine base in DNA and the methyl donor is S-adenosyl-L-methionine (AdoMet). Enzyme cysteine thiolate is added covalently to the C6 position; thereby pushing electrons to the C5 position to make enamine. Enamine then attacks the methyl (CH_3) group in AdoMet followed by methyl transfer. Abstraction of a proton from the C5 position was suggested to allow the 5-6 double bonds and the release of the enzyme from Cytosine. (Schematic adapted from [147])

a) DNMTs

The first eukaryotic DNA methyltransferase to be cloned is DNA (cytosine-5) methyltransferase-1 (Dnmt1) [148, 149]. It was identified based on the similarity of its biological activity to that of bacterial methyltransferases [149]. Several subsequent studies confirmed that the C-terminal region of Dnmt1 protein contains a series of motifs characteristic of the known DNMTs that are conserved from bacteria to humans [148, 150, 151]. These regions cooperate to form binding sites with the reaction substrates, S-adenosyl-L-methionine (AdoMet), DNA, and the DNMT catalytic domain. The DNMT catalytic domain catalyses the transfer of the methyl group from donor AdoMet to C5 of the CpG dinucleotide on the DNA. The proposed mechanism of this enzymatic reaction is depicted in **(Figure 1.3)** [147]. The enzymatic properties of Dnmt1 have been studied extensively using *in vitro* biochemical assays as well as *in vivo* genetic approaches. Although Dnmt1 can transfer a methyl group to symmetrically unmethylated CpG dinucleotides *in vitro*, it preferentially methylates hemimethylated target sequences [152-156], and little if any target sequence specificity has been seen outside the CpG dinucleotide [152]. These findings suggested that Dnmt1 functions in the maintenance of CpG methylation by methylating the daughter strand CpG in hemimethylated sequences.

Genetic studies in mice revealed that partial loss of Dnmt1 function results in embryonic lethality. However, *in vitro* Dnmt1 homozygous mutant ES cells are viable, and they exhibit no obvious growth or morphological abnormalities [157]. Genomic 5mC

content in these cells is reduced to levels significantly lower than those of ES cells expressing a partial loss-of-function of mutant dnmt1 [158]. Although these cells exhibit substantial demethylation, they retain ~30% of the normal level of genomic 5mC. In addition, they are able to *de novo*-methylate newly integrated retroviral DNA [158]. These studies confirmed the existence of additional DNA (cytosine-5) methyltransferases and, together with previous *in vitro* data, suggested that Dnmt1 functions to maintain rather than to establish patterns of CpG methylation.

The identification of Dnmt3a and Dnmt3b confirmed the existence of a family of mammalian DNMTs. These genes were identified in expressed sequence tag (ESTs) databases by their sequence similarity with the catalytic domain of Dnmt1. However, the Dnmt3 proteins have no homology to Dnmt1 outside this region [155]. The properties of the Dnmt3 family implicated these proteins as the long-assumed *de novo* DNMTs. Unlike Dnmt1, neither Dnmt3a nor Dnmt3b shows a preference for hemimethylated DNA target sites *in vitro* [155, 159]. Furthermore, the expression patterns of the Dnmt3 genes correlate with the timing of developmental *de novo* methylation. Although Dnmt1 is expressed ubiquitously in somatic cells, the Dnmt3 genes are expressed at a high level in undifferentiated embryonic stem cells but at low levels in differentiated somatic tissues [155]. The *de novo* methyltransferase functions of Dnmt3a and Dnmt3b have been confirmed by studies in genetically modified mice [160]. Embryonic stem cell lines with homozygous null mutations in Dnmt3a and Dnmt3b were produced, separately and in combination. Both single knock-out lines retained the ability to methylate foreign retroviral DNA, whereas the double knock-out cells completely lacked this activity,

demonstrating both the requirement and the redundancy of Dnmt3a and Dnmt3b for *de novo* methyltransferase activity [160].

Other DNMTs were discovered based on the similarities to the properties of bacterial methyltransferases and the structure of Dnmt1 or Dnmt3 including Dnmt2 and Dnmt3L. The function of Dnmt2 remains poorly understood since it has been shown to have no detectable methyltransferase activity [160, 161], whilst Dnmt3L is believed to assist *de novo* DNMTs in establishing the maternal imprinting patterns during development [162, 163]. The link between DNA methylation on CpG caused by DNMTs and transcription repression was missing until the discovery of a family of proteins that bind to methylated CpG dinucleotides and repress transcription activity [164]. The members of this family has been extensively studied in the last two decades, therefore I will briefly discuss the general characteristics of this family of proteins.

b) Methyl binding domain proteins (MBDs)

The correlation between DNA methylation and transcriptional inactivity is well established. However, a causative role for CpG methylation in repression of transcription had often been a subject for debate. The identification of a family of proteins that bind to DNA containing methylated CpG dinucleotides indicated the initial causative link between CpG methylation and repression of transcription. The members of this family are called methyl-CpG-binding proteins (MeCP or MBD) since they are all characterized with an N-terminal methyl binding domain MBD.

The presence of this family of proteins in human cell extracts was reported nearly two decades ago [164]. MeCP2 (MBD2) was the first to be purified and biochemically

characterized [165, 166]. This protein contains an N-terminal MBD and a C-terminal transcription repression domain (TRD) [167]. *In vivo*, MBD2 has been shown to associate with chromatin [166] and localizes to methyl-CpG-rich sequences [168]. *In vitro*, the full protein or the MBD alone selectively binds to DNA containing symmetrically methylated CpG dinucleotides with an affinity directly proportional to methyl-CpG density [165, 169]. TRD represses transcription with or independently of other MeCP2 sequences [170-173], through binding to the histone deacetylase corepressor complex Sin3a/HDAC [171, 172]. These findings suggested a chain of events by which DNA methylation promotes transcriptional silencing.

Today, an established model suggests that MeCP2 binds to chromosomal regions containing methylated CpG dinucleotides. A deacetylase corepressor complex is then recruited by the binding of Sin3a/HDAC to TRD. Histone deacetylation, in turn, results in condensation of chromatin leading to a local condensed chromatin structure and repression of transcription initiation [174]. Consistent with this model, *in vitro* transcription repression by MeCP2 is sensitive to histone deacetylase inhibitors [171, 172]. There is also an evidence for HDAC-independent mechanisms of transcription repression by MBD proteins, further extending the potential impact of DNA methylation on gene expression [173, 175-177]. In some cancer cell lines, hypermethylation silenced genes can be reactivated by simultaneous treatment with the HDAC inhibitor trichostatin A (TSA) and the demethylating agent 5-Aza-dC (decitabine) but not by TSA alone [178]. These findings suggest either that DNA methylation has additional repressive effects that

are independent of histone deacetylation, or that unknown TSA-insensitive HDACs are also involved.

Up-to date, *in silico* and cloning approaches identified at least five MBD family members including MBD1, MBD2, MBD3, MBD4, and MBD5 [179-181]. All MBDs have sequence similarities, however their capability of binding to methylated CpG differ. MBD3, for instance, has little if any methyl-CpG-binding activity *in vitro*, hence it is unlikely that mammalian MBD3 plays a role in methylation dependent transcription repression [180]. MBD4 has a sequence similarity to the bacterial DNA repair enzymes [180, 182], and although MBD4 is capable of binding to methyl-CpG sites, it has a higher affinity for 5mCpG-TpG mismatched sites [183]. The DNA repair domain in MBD4 provides DNA *N*-glycosylase activity at G-T mismatches, therefore, MBD4 is ideally suited to function in the repair of point mutations that result from spontaneous deamination of 5-methylcytosine to thymine [183, 184].

As discussed above, the establishment (*de novo*) and maintenance of appropriate DNA methylation patterns as well as the consequent remodeling of the chromatin structure is interplayed by a number of factors. The final product of this interplay is the regulation of gene transcription activity. Regulation of gene transcription activity by DNA methylation is essential and is coordinated in a timely manner for normal cell growth and development. Many normal cytological phenotypes are controlled by DNA methylation including X chromosome inactivation in female cells, silencing of imprinted alleles, and silencing of retro-transposable DNA elements **(Figure 1.4)**. In humans, disruption of normal methylation patterns may lead to initiation and to progression of

disease **(Figure 1.4)**. An overview on the importance of DNA methylation in the context of normal cell development is discussed below followed by another section dedicated to DNA methylation in the context of development and progression of human disease.

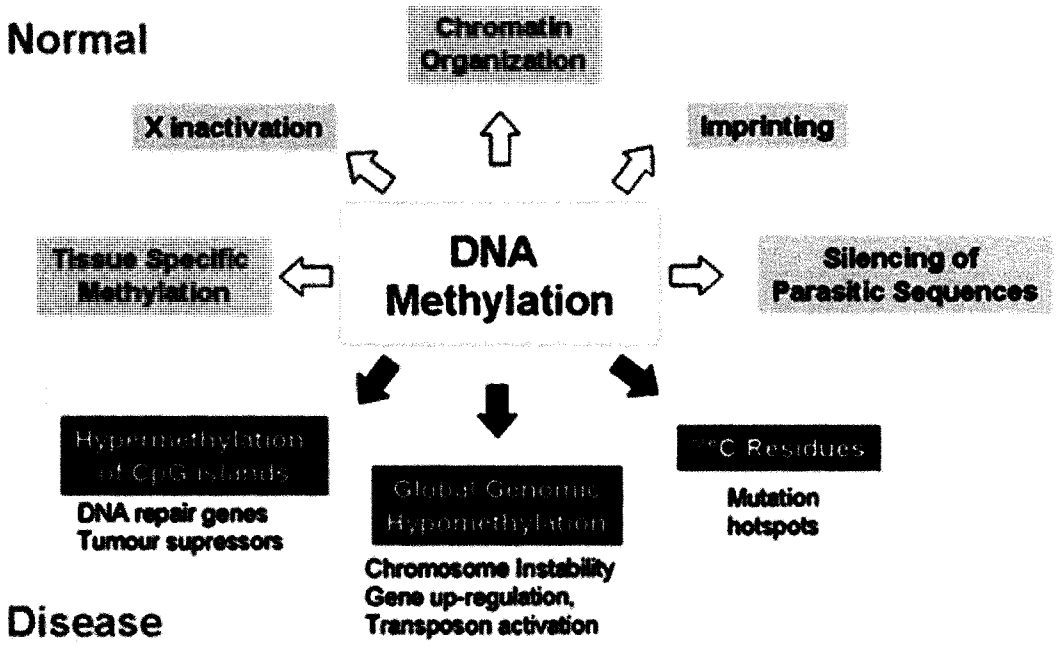


Figure 1.4: Normal and abnormal functions of DNA methylation. This schematic illustration demonstrate the variable functions of DNA methylation in normal cells and the role of DNA methylation in the development of disease as discussed in sections 1.4.3 and 1.5. (Illustration was adapted from [185]).

1.4.3 Normal function of DNA methylation

Mouse knockout for Dnmts have demonstrated that these enzymes are essential for normal embryonesis. Changes in DNA methylation during the early stages of embryogenesis provide evidence for the implication of DNA methylation in cell development. Methylation silencing of the transcriptionally inactive alleles of imprinted

genes, inactive X chromosome in females, and retro-transposable sequence elements in the genome are all indications of the important function of DNA methylation in mammalian cells.

a) DNA methylation in normal cell development

The Importance of DNA methylation in normal development is evident from the lethality effect seen in the Dnmt knockout mice [160]. In those experiments, Dnmt1, Dnmt3a, and Dnmt3b activity were believed to be essential for normal murine development. However, some domains of Dnmt proteins were found to interact with proteins involved in controlling DNA replication, the cell cycle, and gene expression [186, 187]. Hence, the non-viability of the Dnmt knockout mice alone does not prove that it is the loss of DNA methylation which leads to their phenotypes. Other observations in human disease, none-the-less, provided further evidence to the importance of regulation of DNA methylation in normal cell development. Point mutations in humans affecting only the catalytic domain on the C-terminal of Dnmt3b can give rise to a rare recessive syndrome characterized with immunodeficiency, centromeric region instability, and facial anomalies (ICF) [160]. The ICF mutation affects the domain of mammalian DNA methyltransferases that has been implicated only in controlling the catalytic activity of the enzyme. Moreover, ICF is characterized with loss of <10% of genomic 5mC [188]. This syndrome also involves immune problems affecting B-cell function and various other abnormal phenotypes such as, facial anomalies, growth retardation, and neurological dysfunction [146]. The finding that ICF mutations specifically alter the

catalytic domain of Dnmt3b indicates that normal levels of DNA methylation in humans are important for regulating expression of certain genes involved in differentiation during development.

Other evidence for the implication of DNA methylation in cell development comes from the observation of the dramatic changes seen in DNA methylation very early in embryogenesis. First there is active demethylation of the paternal genome soon after fertilization followed by a slow demethylation of the maternal genome [189]. Subsequent remethylation of DNA of the inner cell mass, but not the outer cell mass of the embryo, begins at the blastocyst stage. Also there is a suggestion that aberrant DNA methylation during embryo formation in mammals cloned by nuclear transfer may be partially responsible for the very high rate of prenatal lethality and growth abnormalities in surviving embryos [190].

b) Methylation of silenced alleles of imprinted genes

Most mammalian autosomal genes are expressed from two alleles, the maternally and the paternally inherited copies. A small fraction of the genes in the genome, however, are expressed in a parent-of-origin-specific manner and known as the imprinted genes [191]. The imprinted genes are regulated by epigenetic mechanisms, including, but not restricted to, DNA methylation of CpG dinucleotides. To date, ~ 50 imprinted genes have been identified in the human genome [192] and they tend to cluster together in certain imprinted chromosomal domains [191, 193]. Dysregulation of expression of imprinted genes is a critical cause of human disorders of growth and development [194]. Different combinations of epigenetic markers, including DNA methylation and histone

modifications, have been demonstrated to be critical for normal imprinted gene regulation. Allele specific differences in DNA methylation resulting in differential expression have been observed in the proximity of most imprinted genes [194].

A well established example of allele specific methylation is that of the *SNRPN* gene locus on chromosome 15q11 [195]. The allele specific methylation in this locus follows the paradigm known from gene expression studies where methylation is observed on the repressed allele [196]. Methylation patterns of imprinted genes, however, can be more complex. *IGF2R* harbor methylation marks on the maternally expressed allele and *IGF2* gene harbor methylation marks on the paternally expressed allele [197, 198]. In addition to allelic differences in methylation, the extent of differential methylation within a given locus differs greatly from one gene to another. For example, in somatic tissues the *H19* gene is methylated on the repressed paternal allele over an 8 kb region, whereas *IGF2R* has two independent regions of differential methylation that span 1.7 kb and 1.8 kb [197, 199]. Furthermore, differential methylation has been observed in every conceivable position in the imprinted loci, from core promoter regions, introns, and exons to regions that are distal to the transcribed sequence [194].

c) Inactivation of X chromosome

X chromosome inactivation in female cells is another important normal function of DNA methylation. Inactivation is achieved on one of the two copies of X chromosomes to ensure gene dosage compensation in females. The initiation of X inactivation is controlled by the X-inactivation center (Xic), which produces the noncoding *Xist* transcript responsible for triggering silencing in *cis*. *Xist* then recruits

many of the epigenetic features generally associated with heterochromatin, including histone modifications and DNA methylation [200, 201]. Histone modifications are believed to be an early event while DNA methylation appear to be relatively late events during X chromosome inactivation [201].

d) Methylation of transposable and retroviral elements

Majority of the 5mC are found in the repressed transposable and retroviral elements in the genome. These sequences include Alu repeats, short and long interspersed nuclear elements (SINE and LINE repeats) [202, 203]. Methylation of cytosine not only represses these elements, but also increase the rate of their mutational inactivation via cytosine to thymine conversion. The latter is thought of as an evolutionary process to contain these repetitive sequences and prevent their potential genomic harm [204].

1.5 DNA Methylation and human disease

DNA methylation is the best characterized epigenetic mechanism in mammals. After replication, Dnmt1 and other maintenance methylases restore the original pattern of CpG methylation in the daughter strands. This provides a simple mechanism for the perpetuation of epigenetic information in proliferating cells. Dysregulation of methylation alters gene expression leading to human disease of growth and development. Dysregulation of expression of imprinted genes is an example of human disorders that are associated with disruption of methylation patterns. In addition, the understanding of cancer as an epigenetic disease is evolving rapidly, and alterations of DNA methylation patterns are central to cancer epigenetics.

1.5.1 Methylation and human disorders of growth and development

Abnormal growth, irregular cell proliferation, defects in organ development, hyperplasia and occurrence of tumors are all amongst the manifestations of dysregulation of expression of imprinted genes. The strong evidence linking alterations of DNA methylation patterns to human disorders of growth and development comes from the extensively studied Beckwith-Weidemann syndrome (BWS,OMIM#13650). BWS is caused by dysregulation of expression of imprinted genes located on a cluster of the human chromosome 11p15 [205-207]. The regulation of imprinted genes on this cluster occurs on two distinct domains and controls the expression of genes in an area spanning 1 Mb. In one of these domains (domain 1), the imprinted genes *IGF2* and *H19* are found

and differential methylation of a region between the two genes (known as differentially methylated region 1, DMR1) orchestrate the expression patterns of the paternally expressed *IGF2*, and maternally expressed *H19* [191]. In BWS patients, gain of methylation within DMR1 for the maternal allele of the *H19* occurs at a frequency of ~10 % [191]. Sporadic loss of methylation of DMR2 in domain 2 of the 11p15 cluster occurs in 50% of BWS patients [206]. Other mechanisms are also implicated in BWS occurrences including microdeletions of the DMR or duplication [191].

Isolated hemihyperplasia (IH, OMIM#130650), and Russel-Silver syndrome (RSS, OMIM#180680) are other human disorders associated with dysregulation of imprinted genes. Altered methylation on the DMR2 of chromosome 11p15 was observed in these disorders [191]. Angelman syndrome (AS, OMIM# 105830), and Prader-Willi syndrome (PWS, OMIM#176270) are other disorders of human growth and development. They are two distinct neurogenetic disorders that are caused by the loss of function of imprinted genes on the proximal long arm of human chromosome 15 [208]. Imprinting defects in this region are caused by unique mechanisms leading to imprint mark disruption. In ~10% of cases the imprinting defects are caused by a microdeletion affecting the 5' end of the *SNURF-SNRPN* locus. In the majority of PWS and AS patients with an imprinting defect, the incorrect imprint arises without a DNA sequence change possibly including changes in DNA methylation [209].

1.5.2 Methylation and cancer

In cancers, methylation patterns are frequently altered, including hypermethylation of CpG islands in 5' regions of genes [210, 211], and hypomethylation of repetitive sequences of the genome [212, 213]. A candidate gene approach has been used to assess genes with roles in tumor development. This approach has identified methylation-mediated silencing of genes involved in most aspects of tumorigenesis, commonly affecting the cell cycle [214, 215], apoptosis [216], and DNA repair [217-219]. Aberrant CpG-island methylation may be focal affecting single genes [220, 221], or global affecting entire chromosomal domains in some human cancers (150 kb in breast cancer and 4 MB in colorectal cancer)[222, 223].

Many examples of CpG-island promoter hypermethylation of tumor suppressor genes, accompanied by gene silencing and presumably growth advantage for the affected cell, have been described in the literature [129]. Moreover, several lines of evidence indicated a direct causal role for DNA methylation in tumorigenesis. First, reduced DNA methylation suppresses the formation of intestinal polyps in $APC^{Min/+}$ mice [224]. Second, promoter region methylation of the *RB* gene and the von Hipple Lindau (VHL) gene have reported in familial cases of unilateral retinoblastoma and renal cancer, respectively [215, 225]. Third, studies of sporadic cases of colorectal carcinomas exhibiting microsatallite instability demonstrated a high frequency of promoter region hypermethylation of the mismatch repair gene *hMLH1* [226]. Importantly, there are now examples in which a tumor suppressor gene is wild-type, however silenced by hypermethylation, while the second copy is either mutated or deleted [227]. This

observation in particular strongly supports the hypothesis of DNA hypermethylation as a second hit on the road to tumorigenesis [228].

In addition to promoter hypermethylation, many human cancers exhibit a global decrease in 5mC, or genomic hypomethylation, relative to normal tissues [229-232]. Despite the knowledge of hypomethylation for more than two decades, the majority of genomic loci affected by cancer hypomethylation are unknown [61, 231-233]. Hypomethylation has been proposed to contribute to malignancy via at least three mechanisms including transcriptional activation of oncogenes, loss of imprinting and promoting genomic instability via unmasking of repetitive elements [61]. In mice, DNA hypomethylation is sufficient to induce tumor occurrence with consistent gain of chromosome 15, indicating that genome-wide hypomethylation plays a causal role in cancer [212, 234]. These observations suggest that DNA hypomethylation promotes cancer through effects on chromosomal stability (CIN).

1.5.3 Aberrations of DNA methylation and CIN

Epigenetic dysregulation is central to cancer development and progression. Hypomethylation may lead to oncogene activation and hypermethylation to silencing of tumor suppressor genes. Several studies have addressed the relation between global hypomethylation and CIN in human cells [235]. In most studies a correlation was observed between the degree of global hypomethylation and the frequency of chromosomal aberrations [212, 234]. For example, hypomethylation of satellite repeats located in the sub-centromeric regions of chromosomes 1 and 8 has been shown to be

associated with an elevated frequency of rearrangements of these chromosomes in the ICE syndrome [188, 236-238]. This observation led to the suggestion that hypomethylation could lead to chromatin de-condensation and this could facilitate chromosomal breaks and recombination.

Genetic mutations or deletions that lead to silencing of genes with tumor suppressor properties have been implicated in the rise of CIN in mouse and human cells [102, 103, 239]. Epigenetic silencing of such genes may presumably have similar effects on chromosomal structure and number in the affected cells. Indeed, the role of hypermethylation-silencing of specific genes in the occurrence of CIN has more recently emerged. Some researchers have reported that coordinate hypermethylation at specific genes with roles in cell cycle checkpoints precedes global hypomethylation in prostate carcinoma [240]. Others, suggested that genomic hypomethylation may be an early event in breast carcinogenesis [238]. Further characterization of the relations between DNA methylation, chromatin composition, and chromatin structure may allow for a better understanding of how DNA hyper- and hypo- methylation affects chromosome structure and integrity.

1.6 Therapeutic modulators of DNA methylation

DNA methylation refers to the addition of a methyl group on cytosine. DNA hypermethylation refers to the same process, however, occurring on the normally hypomehtylated promoter-associated CpG-islands of specific genes [241]. Several genes including many that are critical to cancer development have DNA hypermethylation in

their promoters. This process is associated with epigenetic silencing of expression and loss of gene function [242]. Early reports on DNA methylation changes in cancer described global loss of methylation, which was suggested to drive tumorigenesis through induction of oncogenes and CIN [230]. The idea of inhibiting DNA methylation therapeutically came from the subsequent studies showing that, in parallel to global hypomethylation, the promoters of genes with tumor suppressor activity are hypermethylated in cancer cells [242]. This led to revival of interest in drugs discovered decades ago to be potent inhibitors of DNA methyltransferases [243]. Some of these drugs have been approved for clinical use by FDA in the treatment of cancers of haemopoietic origin[243].

1.6.1 Targeting DNA methylation in cancerous cells

It is estimated that hundreds of genes are silenced by hypermethylation in every cancer and silencing of many of these genes play crucial role in the advantage of cancerous cells [244]. In addition, several lines of evidence indicated a direct causal role for DNA hypermethylation in tumorigenesis (discussed in section 1.5.2). However, the number of genes and pathways that are affected by DNA hypermethylation makes therapeutic targeting highly problematic. It is likely that demethylation treatment could affect many genes in the same cell. Identifying oncogenes that are hypermethylated in cancer cells [245] added even one more dimension of complexity to the applicability of demethylation drugs use in patients. None-the-less, the overall effect of demethylation treatment has been shown consistently to induce favorable outcome *in vitro*, in mouse

models, and in patients. Whether this is because that tumor cells are more affected by tumor suppressor gene re-activation, or that the lack of specificity is actually advantageous (multiple defects are corrected simultaneously), is still under debate [246]. Some experts in the field favor the first explanation since low doses of demethylation drugs are known to reactivate tumor suppressor genes [247], and prolonged treatment using low doses gave a better outcome than higher doses [248].

The proposed mechanism of action of DNA methylation inhibitors in cancer treatment has been discussed in many published reviews [228, 246]. The mechanism is based on two critical components 1) inhibition of Dnmts and 2) re-activation of genes with tumor suppressor activity. In this mechanism, inhibition of the expression of Dnmts, or blocking of their protein function (that is methylating DNA on cytosines), would lead to progressive reduction of DNA methylation after each round of DNA replication. The promoter of a gene with tumor suppressor activity is then switched from the methylated to the unmethylated status. The eventual appearance of unmethylated alleles reactivates gene expression in the treated cells. This effect on gene expression is believed to have several effects on cancer cell biology, including induction of differentiation, and apoptosis. DNA methylation inhibitors fall into several classes, of which the nucleoside analogs class has been considered the most suitable for clinical use.

1.6.2 Three classes of DNA methylation inhibitors

The most studied class of DNA methylation inhibitors is the nucleoside analogs including 5-Azacytidine and 5-aza-2'-deoxycytidine (decitabine). 5-Azacytidine and decitabine are cytosine analogues that trap all DNA methyltransferases and target them for degradation [127]. At low doses, these drugs are effective demethylation agents and they have shown clinical activity as anticancer agents [249, 250]. Other nucleoside inhibitors include zebularine, [251], and 5-fluoro-2'-deoxycytidine [252], which have shown promising anti cancer effects *in vitro*. One limitation of nucleoside analogues is the requirement for active DNA synthesis after nucleoside analog incorporation to DNA, which limits the activity of the drugs in slow proliferating cells. This has led to an interest in developing different inhibitors for the DNA methyltransferases.

Among the potential new inhibitors are the non-nucleoside inhibitors, such as procainamide and hydralazine [253]. The mechanism of action of these drugs is not well understood and their low level of hypomethylation induction is limiting their clinical potential [254]. Another class of new methylation inhibitors is the inhibitors that are experimentally designed for blocking Dnmt proteins based on protein structure studies [255]. One limitation to this approach is the fact that three separate *DNMT* genes encode for proteins with DNA methyltransferase activity. Cooperation between different DNA methyltransferases implies the need to inhibit several of them simultaneously for optimal clinical benefit.

Of the above drugs, 5-Azacytidine and decitabine are the only DNA methylation inhibitors that have been approved by FDA for the treatment of cancer [243, 250, 256].

The approval was based on the benefits that were observed in patients with a neoplasm of haemopoietic origin (myelodysplastic syndrome) hence the use of these drugs is restricted for the treatment of that neoplasm specifically. However, the data from myelodysplastic syndrome on the use of these drugs provide a proof of principle for epigenetic therapy in cancer and for the use of these drugs in other tumors.

Current data suggest that myeloid malignancies are the most sensitive neoplasms to treatment by demethylation drugs [243, 250, 256]. There may be pharmacologic reasons that favor malignancies of haemopoietic origin in this regard (such as drug uptake, and proportion of proliferating cells). However, the small number of studies in solid tumors limits our understanding of the potential applicability of this approach. Although, a few older studies suggested lack of activity for these drugs in solid tumors, most of these were done with high doses, and limited number of exposure days. Typically response evaluation was done after only one cycle of treatment, which along with the other factors could influence the observed outcome of treatment with these drugs [257]. The activity of demethylation drugs in solid tumors deserves further evaluation *in vitro*, *in vivo*, and potentially in humans, with appropriate doses and schedules. Indeed, there is already some evidence for activity of decitabine in malignant melanoma at low doses [258]. The second and third chapters of this thesis also show some evidence for the potential of decitabine use in the treatment of OS.

1.6.3 5-Aza-2'-deoxycytidine (decitabine)

Decitabine was recently approved for the treatment of haemopoietic neoplasms. This drug is a nucleoside analog of cytosine that has been shown to produce clinical improvement in more than 50% of the treated patients [259, 260]. Optimizing the drug included reducing the dose to favor demethylation over cytotoxicity [248]. Prolonged administration schedules, and increasing the dose intensity (within low dose limits) were also amongst the optimization parameters [248, 250, 256, 260]. To-date, the reported side effects have been primarily haemopoietic, with no chromosomal changes or secondary malignancies [261]. The therapy is normally effective with complete responses lasting up to years in some patients, however resistance develops in a significant proportion of the treated patients, and the mechanisms of resistance are still unknown [248, 250, 256, 260].

At the molecular level, decitabine treatment induces demethylation and re-activates methylation silenced genes [262, 263]. Reactivation of methylation silenced genes has been shown to be required for optimal responses [260]. The *in vitro* and *in vivo* data accumulated so far are consistent with an epigenetic effect of decitabine. The mechanisms by which decitabine clear neoplastic cells may be due to decitabine ability to induce variable pathways that are silenced in cancer cells, including differentiation and apoptosis. More recent evidence also suggest that decitabine clearance of neoplastic cells involves immune activation [258].

1.7 Epigenetics and OS oncogenesis

Epigenetic processes contribute to carcinogenesis through perturbation of differentiation [264]. Many experimental observations support the notion that DNA methylation patterns are essential for normal development, cellular differentiation, and tumorigenesis [228, 265]. Studies regarding the implications of DNA methylation in OS are limited, but have suggested a role in bone differentiation [266-268], transcription factor expression, and histone modifications [269, 270]. The acquired knowledge so far of transcriptional regulation of osteoblast differentiation indicates that several cancer related genes are key players in bone development, including the Wnts and related genes, notch, transforming growth factor- beta, insulin growth factor family and their receptors, NFkb ligands and their receptors, and polycomb genes [271]. Given the impact of these genes in bone development, there is scant information on the role of their epigenetic regulation in OS tumorigenesis.

DNA hypermethylation regulates other biological processes in cancer cells including DNA repair, cell cycle checkpoints, and apoptosis (discussed in section 1.5.2). DNA hypomethylation, on the other hand, is linked to activation oncogenes and the rise of CIN in cancers (also discussed in 1.5.2). OS is characterized with a high number of chromosomal rearrangements, induction of oncogenes and increased CIN. It is possible that altered DNA methylation patterns are implicated in OS tumorigenesis through hypermethylation silencing of genes that regulate DNA repair, cell cycle checkpoints, and apoptosis making OS cells advantageous in their growth ability despite the high level

of genomic rearrangements. It is also possible that hypomethylation is implicated in OS leading to the frequently observed oncogenes activation and the increased CIN.

1.8 Rationale and Hypothesis

1.8.1 Rationale

OS is an aggressive tumor which is characterized by genomic instability and complex chromosomal rearrangements. OS chemotherapeutic approaches have changed little in the last 20 years. Epigenetic modifiers such as decitabine have the ability to reactivate genes silenced by methylation, and have the potential of restoring functional pathways that are antagonistic to tumorigenesis, such as differentiation, growth arrest and apoptosis. No study to date has utilized decitabine to study OS cell response at the molecular and cellular levels. Targeting methylation by decitabine in OS will facilitate identification of new targets for development of new targeted therapeutic approaches involving demethylation in this disease.

1.8.2 Hypothesis

Genes associated with tumor growth and progression may be repressed by hypermethylation in OS. Demethylating these genes may modulate gene expression in OS and provide targets for novel epigenetic therapies.

1.9 Specific aims

AIM 1: Identification of decitabine up-regulated genes in OS cells

AIM 2: Methylation status of a subset of genes up-regulated by decitabine

AIM 3: Identification of *GADD45A* as a specific target for decitabine with a role in apoptosis in OS cells

CHAPTER TWO

2 Modulation by decitabine of gene expression and growth of U2OS cells *in vitro* and in xenografts

This work is based on the published article:

Modulation by decitabine of gene expression and growth of osteosarcoma U2OS cells *in vitro* and in xenografts: Identification of apoptotic genes as targets for demethylation. Khaldoun Al-Romaih, Gino R Somers, Jane Bayani, Simon Hughes, Mona Prasad, Jean-Claude Cutz, Hui Xue, Maria Zielenska, Yuzhuo Wang, Jeremy A Squire. ***Cancer Cell International***, 2007. K A performed the *in vitro* treatment assays, the AffyChip expression assays, the AffyChip data analysis, the real-time expression assays and analysis *in vitro* and *in vivo*, the DNA methylation data analysis, the meth5-C data analysis and conceived and wrote the manuscript draft; G R S performed the histopathological analysis and TUNEL analysis; S H performed the immunostaining using the Methyl-C-Ab; H X and Y W performed the mice experiment;

2.1 Introduction

Epigenetic abnormalities contribute in several ways to oncogenesis and may activate oncogenes or silence tumor suppressor genes. In addition, abnormalities in epigenetic processes can enhance chromosomal instability [272], [234]. Typically, a general pattern of demethylation of the genome is observed in tumor DNA, while increased methylation of a subset of promoter-associated CpG islands associated with the transcriptional start sites (TSS) of genes may also be observed. There is increasing interest in the therapeutic modulation of epigenetic processes, since epigenetic alterations are amenable to physiological alteration by drugs that change patterns of DNA methylation or histone acetylation, [127].

Current dugs for OS therapy have been the same as those used over the last 20 years, and there is no second line therapy for patients who respond poorly. Studies regarding the implications of epigenetic modification in OS have been limited, but have suggested a role in bone differentiation [266] [267] [268], transcription factor expression, and histone modifications [269] [270]. Demethylation drugs are useful in therapeutic modulation of epigenetic processes in cancer cells, resulting in favorable response in experimental models as well as patients with cancers of haemopoietic origin [260] [250]. No study to date has utilized decitabine to modify gene expression in an OS cell to identify gene-specific targets for demethylation that may have therapeutic importance. The studies presented in this chapter deals with modulation of gene expression and growth by demethylation drug decitabine in OS cell line U2OS *in vitro* and *in vivo*, addressing **Specific Aims 1 & 2** of this thesis.

2.1.1 U2OS cell line as an appropriate model for the study

The genetics and cytogenetics of this cell line has been studied extensively in the literature and it has been utilized extensively in drug testing *in vitro* [117, 273]. For the above reasons, U2OS presented an appropriate model to study the epigenetic events that are associated with OS tumorigenesis and the evaluation of demethylation treatment. This cell line is known to have wild-type *P53* [122] [274] and *RB* genes [275]. U2OS still displays an elevated degree of chromosomal instability [56] [51] that is typical of OS despite the integrity of the *P53* and *RB* genes.

2.1.2 U2OS xenografts

A full assessment of tumor cell response to treatment requires integrating experimental data from both *in vitro* and *in vivo* observations. One major advantage of preclinical animal models of xenografts of human tumor cell lines is that they provide both tissue vascularization and a tumor microenvironment that is closer to human tumors so that an evaluation of the therapeutic impact on tissue differentiation, cell growth and proliferation levels is possible [277], [278]. Such analyses are providing opportunities for a detailed assessment of new classes of anti-neoplastic drugs that target the epigenome, such as decitabine. To date one tumorigenic xenograft model of the non-transformed OS cell line U2OS has been established under the skin of immunocompromised mice [117, 279]. Meantime, U2OS has been shown to be generally non-tumorigenic using heterotopic grafts under the mouse skin [273]. Importantly, there have been no studies for anti-osteosarcoma drug testing using U2OS xenograft model. Establishing the U2OS xenograft in mice and testing the efficacy of the epigenetic modifier decitabine on this xenograft in this study is the first of its kind. Our collaborators from the Breast Cancer Agency in British Colombia (Dr. Yuzhuo Wang, and Hui Xue) established U2OS cells in the renal capsule of immunocompromised mice. The sub-renal capsule site has been proved to be an excellent grafting site for xenografts in general [280] [281] [282].

2.1.3 Decitabine

The most powerful DNA methyltransferase inhibitor in clinical use is decitabine. Decitabine is a cytosine analog that inhibits DNA methylation and reactivates silenced

genes. Decitabine has shown favorable clinical efficacy in the treatment of myelodysplastic syndromes, with evidence of gene target expression modulation by demethylation with less toxicity than conventional cancer chemotherapies [260] [250] (discussed in Chapter One). A single dose of decitabine at 1 μM concentration was used to treat U2OS cells *in vitro*. This dose was shown in previous reports to suppress the growth of tumor cell lines [283] [284-286] and re-activate methylation silenced genes [287]. The mice were treated with 2.5mg/kg body weight decitabine. Decitabine dosing in the mice was based on what had been used in previous studies [288] [289]. A minimal effective dosage was used to minimize cytotoxicity on the mice while anticipating effect on tumor growth as was reported previously [290]. In addition, this dose was shown to demethylate and re-activate tumor suppressor genes and decrease tumor growth in xenografts from another tumor model [291].

In the following experiments I analyze the effects of decitabine on U2OS cells at the cellular and molecular levels. This work represents the initial steps in the elucidation of the cellular pathways OS cells that are affected by genome-wide demethylation.

2.2 Results:

2.2.1 Effects of decitabine on U2OS cells *in vitro*

Decitabine is a cytotoxic agent in addition to being a demethylation agent. U2OS growth rate was measured in the absence, or presence of 1 μM decitabine. This relatively low-dose was chosen to minimize cytotoxicity. The doubling time for U2OS in the absence of 1 μM decitabine was close to 2.75 days (66 hours) while the decitabine treated

U2OS cells doubled at 3.5 days (84 hours). This was accompanied by a minor reduction in viable cell numbers as a result of decitabine treatment **(Figure 2.2.1 A)**.

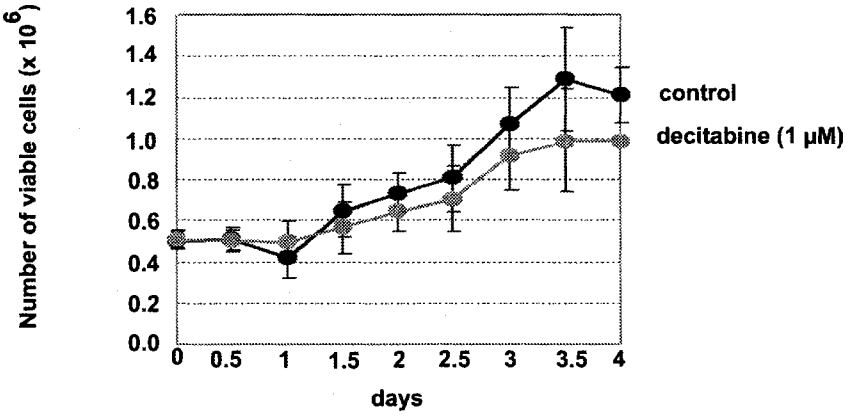


Figure 2.2.1 A: Viability and growth kinetics of U2OS *in vitro*. Growth curves of U2OS cells with growth medium alone (dark line), or growth medium with one dose of decitabine at a final concentration of 1 µM (light graph). The y-axis indicates cell number in millions and the x-axis indicates time points in days. The results are expressed as cell counts at each corresponding time point. Each data point is the Mean of cell counts from 2 experiments (5 passages apart) each consist of two independent cultures and the error bars indicate the standard deviation. The findings indicate a slight increase in the doubling time of U2OS cells and a decrease of 18% ($p=0.045$) in the viability of treated cells compared to the untreated control.

To determine whether a slower growth rate was accompanied by higher level of cell death following decitabine treatment, death assessment was studied by flow-cytometry after PI staining. The control (no-treatment) cells showed a 5% death rate

whereas the decitabine treatment significantly increased the death rate of 10% more than the control at day 3 ($p<0.05$) **(Figure 2.2.1 B)**.

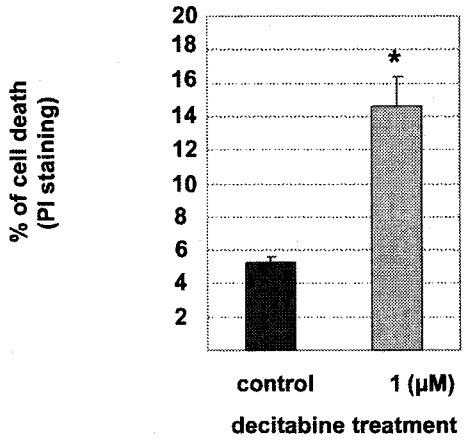


Figure 2.2.1 B: Cytotoxicity of U2OS after exposure to decitabine *in vitro*. Cell death in U2OS cells caused by decitabine treatment at 1 μM concentration (light column) compared to no-treatment (dark column). The results are expressed as percentage of cell death (fraction of cells with positive PI stain). The y-axis indicates the percentage of cells with PI staining (dead cells). Each column is the Mean of 3 experiments with error bars indicating standard deviation. The asterisk indicates significant increase in cell death ($p<0.05$) as a result of decitabine treatment.

To determine that the decitabine treatment conditions were effective at induction of demethylation we analyzed the SNRPN gene by methylation specific restriction endonucleases. This heavily methylated gene has previously been shown to undergo reduction in methylation following treatment with decitabine [292]. After 72 hours treatment of U2OS with 1 μM decitabine, ~60 % more DNA could be digested with a methylation-sensitive restriction endonuclease **(Figure 2.2.1 C)**.

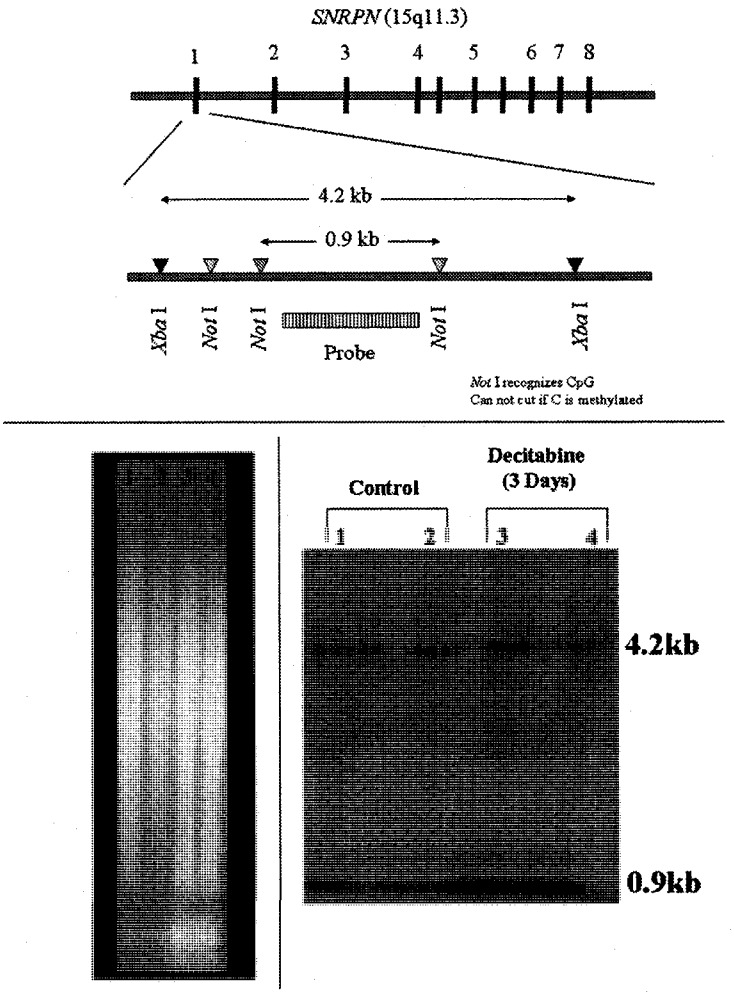
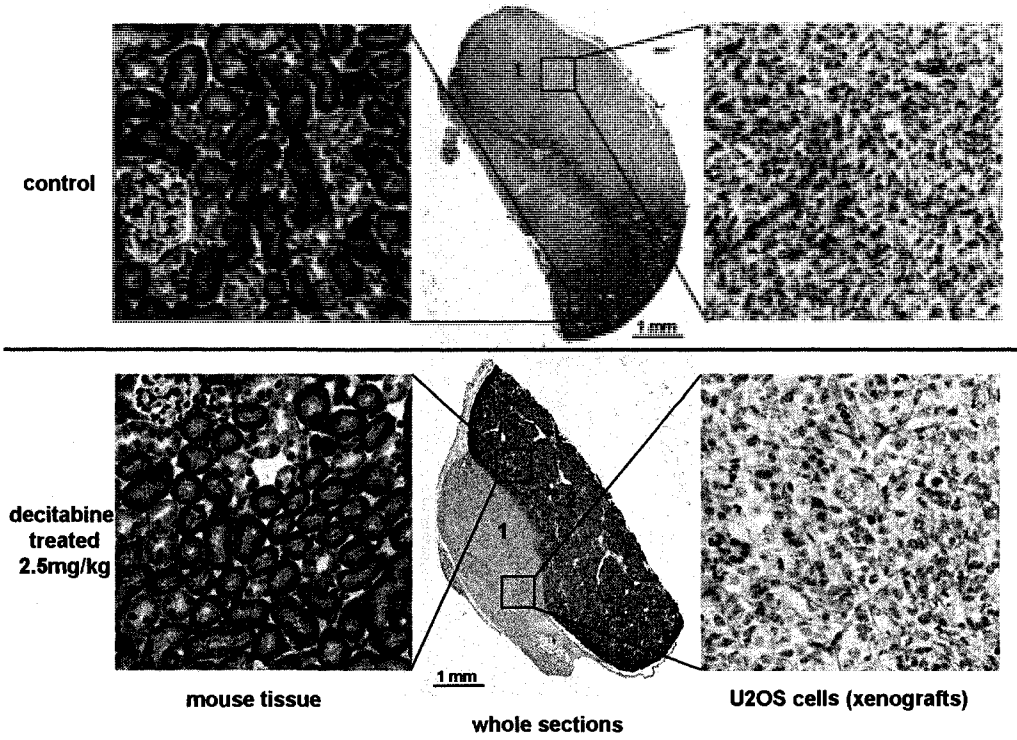


Figure 2.2.1 C: The effectiveness of demethylation following 72 hours treatment with 1 μM decitabine at the *SNRPN* gene locus. The imprinted *SNRPN* gene is located on human chromosome band 15q11.3 and alterations in DNA methylation at this locus are associated with individuals with the Prader-Willi and Angelman syndromes [209] [208]. It was utilized in this study to confirm that decitabine treatment reduced DNA methylation in U2OS cells. Upper panel shows a schematic figure of the *SNRPN* gene indicating the probe location relative to *Not*I and *Xba*I cutting sites. *Not*I is a methyl-sensitive restriction endonuclease that will only cut its recognition sequence when unmethylated. Lower panel shows

samples 1 and 2 (controls) and samples 3 and 4 (treated with decitabine for 3-days). Left panel shows the autoradiogram of the restriction digest of DNA samples 1-4 on a 0.8% agarose gel. Right panel is Southern blot showing an increase of 63% of the 0.9 kb *Not*I product as a result of decitabine treatment (63 % loss of CpG methylation at the locus in U2OS).

2.2.2 Effects of decitabine on U2OS *in vivo*

One group of three U2OS xenograft mice (two tumors per kidney per mouse) was treated with Decitabine (2.5 mg/kg) intraperitoneally on Days 29, 31 and 33. The other group of three mice was given saline as control at the same schedule. On Day 37, all 6 mice were sacrificed and xenografts were dissected from the mice, and the tumor volumes were measured and compared in both treatment arms. The effectiveness of demethylation *in vivo* was determined by analyzing the relative cellular distribution of 5-methylcytidine levels using a specific 5-methylcytidine antibody and 6 tissue sections from 6 xenografts (3 control and 3 decitabine treated). In these analyses, nuclei in host kidney stromal cells were used as internal positive controls (see dark staining nuclei in **Figure 2.2.2 A**). Negative controls obtained by omitting the primary antibody were also performed. Nuclear xenograft tumor staining was recorded by a semiquantitative and visual grading, considering both the intensity of staining and the proportion of positive tumor nuclei in the selected sections. Xenograft derived from the untreated control mice exhibited stronger nuclear staining than the staining present in nuclei from xenografts in decitabine treated mice ($p<0.05$). These results were consistent with a widespread loss of methylation in U2OS xenografts derived from the treatment group **(Figure 2.2.2 A)**.



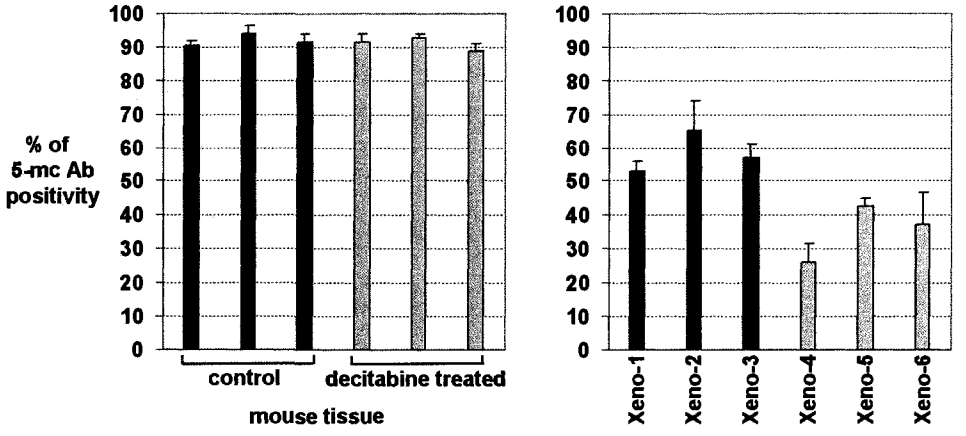


Figure 2.2.2 A: Analysis of differential 5-methylcytidine content of U2OS xenografts using histological sections. Analysis of the relative levels of cellular 5-methylcytsine within xenografts derived from representative control untreated mice (upper panel); or decitabine treated mice (middle panel) using immunohistochemical staining with 5-mc-Ab.

To the right a 20X enlargement of representative U2OS histology is shown. More 5-mc-Ab staining is evident in the nuclei from control sections (upper panel-right side) in comparison to nuclear staining in the sections derived from decitabine treated mice (middle panel-right side). These data are consistent with a reduction in 5-methylcytidine nuclear content in U2OS xenografts derived from mice treated with decitabine. Enlargements of darkly stained host murine kidney cells correspond to heavily methylated-differentiated renal tissue (denoted 2 in upper and middle panels). In the bottom panel the staining intensity from control (dark columns) and decitabine treated (light columns) is quantitated using Aperio scanning image analysis of sections. The graph to the right of the bottom panel confirms that the 5-mc-Ab staining in control untreated (dark columns) U2OS xenograft sections is more intense than the decitabine treated (light columns) U2OS xenografts. This decrease in staining intensity was significant ($p<0.05$). In contrast host kidney cells from both control and treated mice do not exhibit any significant difference in staining intensities.

Tumor volumes were determined for all 24 xenografts and shown in **Table 2.1**. The average volume size for the xenografted tumors in the control group was 49 mm^3 (±25SD). The xenograft tumors from the decitabine treated group had an average volume size of 27 mm^3 (±15.9SD) indicating a significant decrease ($p=0.0096$) of tumor volume as a result of the decitabine treatment **(Figure 2.2.2 B 'I')**. All U2OS xenografts were whitish in color and the texture of tissues from both treatment groups had a moderate-hard consistency, with no apparent kidney tissue invasion.

Table 2.1: Effect of decitabine on U2OS xenograft size (raw data)

Day 37		L	W	H	Volume (mm3)
	control	5.54	3.10	1.31	11.70
		5.07	3.50	1.81	16.70
		5.83	5.41	1.91	31.33
		5.69	4.42	2.74	35.83
		6.54	3.58	3.52	42.86
		6.21	5.04	2.96	48.17
		5.51	4.79	3.77	51.74
		6.92	4.85	3.01	52.53
		6.61	5.28	2.94	53.36
		7.42	4.70	3.25	58.94
		7.25	5.17	4.29	83.62
		8.00	5.72	4.31	102.56
				Mean	49.11
				Median	49.96
				SDV	25.52
	Decitabine	5.02	3.38	1.20	10.59
		5.24	3.44	1.46	13.69
		5.18	3.33	1.79	16.06
		4.31	3.27	2.66	19.49
		5.21	3.52	2.24	21.36
		5.39	3.60	2.19	22.10
		6.04	3.66	1.96	22.53
		5.76	3.71	2.37	26.34
		5.84	4.63	2.11	29.67
		6.41	3.78	2.37	29.86
		6.47	4.28	3.66	52.70
		7.49	4.68	3.54	64.53
				Mean	27.41
				Median	22.31
				SDV	15.90

Raw data measurements of 12 control and 12 decitabine treated xenograft tumors expressed as in tumor volumes estimated using this formula: volume (mm3) = (0.52) x (length 'L') x (width 'W') x (height 'H'). SD = Standard Deviation.

Comparison between the U2OS xenograft histology from the decitabine-treated mice and the control mice identified differences in bone matrix (osteoid) content. In these studies relative levels of osteoid were determined in 9 sections each from the control and decitabine treatment groups. As shown in **Figure 2.2.2 B 'II' and 'III'**, histological analysis of the stained xenograft sections identified an overall average osteoid area of 42% in the control group. The decitabine treated group, however, had a significant increase ($p<0.0001$) in osteoid formation reaching up to an average of 86%. With respect to tissue morphology, the tumors from the control mice showed solid sheets of poorly differentiated cells associated with a small amount of osteoid. In contrast, the xenograft tumors from the decitabine-treated mice showed a less dense cell population with increased areas of osteoid, seen as light pink and lacy matrix with the nuclei of osteoblasts sitting close to the matrix, a feature of differentiated osteoblasts in normal bone. The mitotic count was assessed in the same sections. The mitotic index showed an average of 11.9 mitotic figures in the control group and an average of 3.1 mitotic figures in the decitabine treated group ($p<0.0001$) **(Figure 2.2.2 B 'IV')**.

Since decitabine induced a higher level of cell death *in vitro* and there was a marked decrease in the size of U2OS derived tumors *in vivo*, the level of apoptotic cells in the xenograft tumors was analyzed using the TUNEL assay. Scoring was performed on digital images from 9 control sections and 9 decitabine treatment sections. The average apoptotic indices were 3.2% in the control group and 6.2% in the decitabine treatment group ($p=0.0329$) **(Figure 2.2.2 B 'V' and 'VI')**.

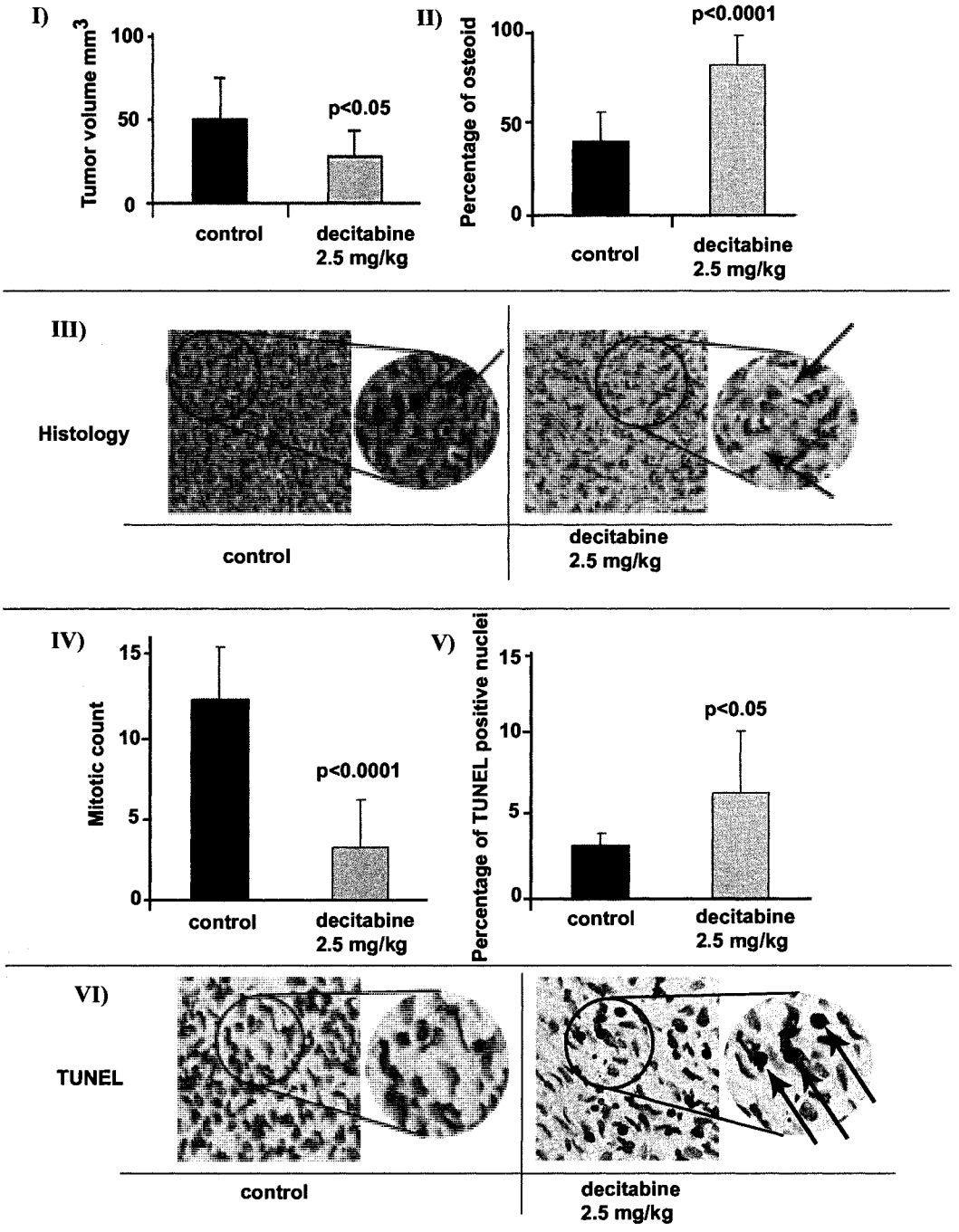


Figure 2.2.2 B: Induction of differentiation and apoptosis by decitabine treatment of U2OS *in vivo*. **I)**, Decitabine effects on tumor volumes *in vivo*. The results compare tumor volumes in control

xenografts to decitabine treated xenografts as described in methods. Each column is the Mean of tumor volumes measured in 12 xenograft tumors. There was a significant decrease ($p<0.05$) in tumor volumes as a result of decitabine treatment. **II)**, Osteoid assessment *in vivo*. Each column is the Mean of osteoid evaluation of 9 sections. There was significant increase ($p<0.0001$) in osteoid formation as a result of decitabine treatment. **III)**, Representation images of H&E sections. Control xenograft tumors (left) show solid sheets of poorly differentiated cells with minimal osteoid (image magnification x 100). Decitabine treated tumors (right) shows less dense cell population and increased areas of osteoid seen as light pink and lacy matrix with the nuclei of osteoblasts sitting closer to the produced matrix (image magnification x 100). The arrows in the enlargement (right) show the osteoid matrix surrounding osteoblasts (defined as eosinophilic osteoid-like material). **III)**, And **IV)**, Mitotic count was identified in the same sections used to asses for osteoid evaluation. Each column is the Mean mitotic count in 9 sections (a minimum of 1100 nuclei were scanned per section). Decitabine treatment resulted in a significantly lower mitotic count ($p<0.0001$). The arrows in the enlargement image in **III** (left) indicate mitotic nuclei. **V)**, Results for apoptosis analysis by TUNEL assay. Each column is the Mean count of TUNEL positive nuclei seen in 9 images representing 9 sections ($\geq$ 1000 nuclei were scanned per section). There was a significant increase ($p<0.05$) of apoptotic cells as a result of decitabine treatment. **VI)**, Representation images from TUNEL assay of control tumors (left) and decitabine treated tumors (right) (image magnification x 200). The arrows in the enlargement image show the TUNEL-positive nuclei (apoptotic nuclei). Error bars indicate standard deviation from the Mean values and p-values are based on comparison between control and decitabine treated tumors using student t-test.

2.2.3 Expression profiling of decitabine-induced changes in gene expression

Expression profiling of 14,500 transcripts with known gene function was performed using the Affymetrix U133A microarrays following treatment of U2OS cells with 1 μM decitabine. Eighty-eight (88) genes [available at Al-Romaih *et al* 2007: http://www.biomedcentral.com/content/supplementary/1475-2867-7-14-S2.xls] exhibited significant ($p<0.0025$) up-regulation after two independent treatments of U2OS with 1 μM decitabine. Within this large group of 88 genes there were 13 with a ≥2-fold change in both experiments. The expression of subset of 7 of these robustly reactivated genes was examined by real-time PCR and increased gene expression was consistent with the levels detected by microarray analysis **(Table 2.2)**.

Table 2.2: Genes up-regulated ($p<0.0025$ and ≥ 2 fold-change) after decitabine treatment identified using AffyChip.

Gene Symbol	Fold Change (Affy)	Fold Change (Real-Time)	CpG	known or proposed function
FAM65A	2.76	nd	yes	hypothetical protein LOC79567
GADD45A	**2.58**	**6**	**yes**	**Regulation of cyclin dependent protein kinase activity and DNA repair**
GAGE4	3.51	nd	yes	Unknown
HSPA9B	**2.32**	**5.3**	**yes**	**Implicated in the control of cell proliferation and cellular aging**
IGFBP6	2.22	3	yes	Insulin-like growth factor binding protein involved in negative regulation of cell proliferation
IMP-3	8.36	nd	yes	Binds to insulin-like growth factor II leader 3 mRNA and may repress its translation during late development
NFKBIA	**4.01**	**4.2**	**yes**	**Inhibits NF-k-B and involved in cell adhesion, apoptosis, differentiation and growth**
PAWR	**3.30**	**10**	**yes**	**Pro-apoptotic protein acts by down-regulating the anti-apoptotic protein BCL2 via its interaction with WT1**
PDCD5	**4.15**	**8**	**yes**	**Induction of programmed cell death**
PSG5	2.32	nd	no	Synthesized by syncitiotrophoblast of the placenta and has a role in maintenance of gestation
RAC2	5.09	nd	no	Involved in the regulation of the NADPH oxidase
TGM2	2.01	nd	yes	A peptide cross-linking factor
TNFAIP3	**3.44**	**4.5**	**yes**	**Negative regulation of I-k-B kinase/NF-k-B cascade**

Fold change (Affy) is the Mean from a duplicate AffyChip experiments.

The fold change using real-time PCR (TaqMan method) is the Mean

values from the same RNA from the same yields that were used for the AffyChip experiments and a third replica. The direction of expression was consistent in the Affy experiment and the real-time experiment for the tested genes. 11 out of 13 genes have CpG-island in there 5' region. Genes in bold characters are those identified in the apoptotic pathway as explained in "Methods" and "Results". The genes are listed in alphabetical order.

An *in silico* analysis of CpG islands associated with the 88 up-regulated genes was then performed. 63 genes (71%) were found to contain CpG-islands within their 5'-regulatory region. Of the 13 genes with a ≥2fold-change, 11 genes (84%) had a CpG-island within their 5' region **(Table 2.2)**. Pathway enrichment analysis was performed on the 88 up-regulated genes, and the three top enriched pathways included: "negative regulation of cellular processes" (p=0.007), "positive regulation of programmed cell death" (p=0.01) and, "organelle organization and biogenesis" (p=0.03). Interestingly, when the 13 genes with a ≥2-fold change were analyzed in the same manner, six genes were assigned to the "apoptosis pathway" with high significance ($p<0.0001$). These genes include *GADD45A*, *HSPA9B*, *PAWR*, *PDCD5*, *NFKBIA*, and *TNFAIP3*, which were selected for expression validation in the xenografts and in normal human osteoblasts (NHOst).

2.2.4 Real-Time expression analysis for apoptotic genes in U2OS xenografts and normal osteoblasts

Real-time PCR (TaqMan method) was used to validate expression of the genes involved in apoptosis shown in **Table 2.2** in the xenografts and NHOst. As illustrated in **Figure 2.2.4**, the change of expression was expressed as fold change relative to the control (no treatment), using beta-actin (*ACTB*) as a reference. Up-regulation, as a result of decitabine treatment was consistent in U2OS cells *in vitro* and *in vivo*. Gene expression of the apoptotic genes was up-regulated up to 8-fold for *GADD45A*, 8-fold for *HSPA9B*, 12-fold for *PAWR*, 8-fold for *PDCD5*, 9-fold for *NFKBIA*, and 5-fold for *TNFAIP3*. In addition, real-time expression for the six genes was examined in normal osteoblasts before and after treatment with decitabine. There was marginal increase of expression after treatment (<2-fold change) in normal osteoblast **(Figure 2.2.4)**.

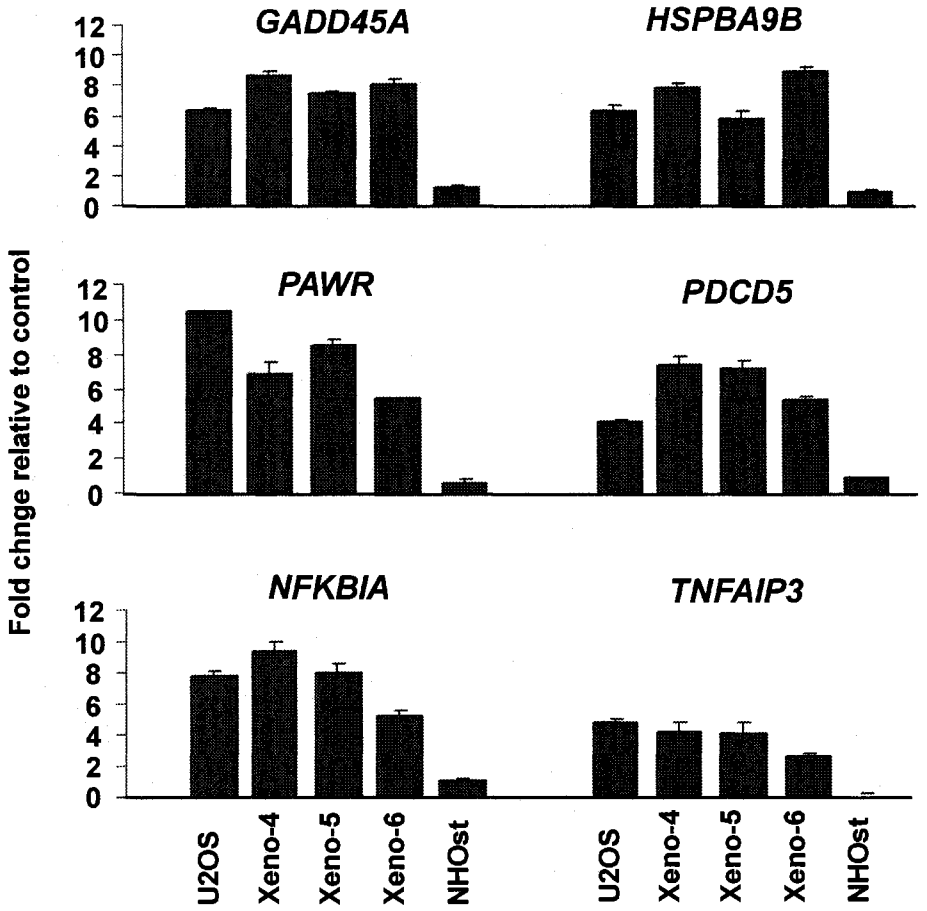


Figure 2.2.4: Real-time gene expression of 6 pro-apoptotic genes U2OS *in vitro* & *in vivo* and normal osteoblasts. Total RNA was extracted from U2OS cells *in vitro* and *in vivo* and reverse-transcribed as detailed in methods. TaqMan assays were used to determine relative expression using the cDNA from control (no treatment) as base lines and *ACTB* for a reference gene by applying the ΔΔCt method. Each column is the Mean of three replicas and error bars indicate standard deviation from the Mean. The data is expressed as fold change relative to control (no-treatment). Xeno-4, Xeno-5, and Xeno-6= decitabine treated xenografts. NHOst= normal human osteoblasts.

2.2.5 DNA methylation analysis of decitabine responsive genes using Pyro Q-CpG

Methylation Pyro Q-CpG sequencing analysis was performed on six genes with expression up-regulation after decitabine treatment including *GADD45A*, *HSPA9B*, *PAWR*, *PDCD5*, *NFKBIA*, and *TNFAIP3*. *GADD45A*, *HSPA9B*, *PAWR*, and *PDCD5*, but not *NFKBIA*, and *TNFAIP3*, showed reliable and reproducible results for the tested amplicons. *GADD45A*, *PAWR*, and *PDCD5* had a high methylation content (>70%) without decitabine treatment, while *HSPA9B* had an intermediate (50% - 70%) methylation content. Decitabine treatment lowered the methylation content in all CpG sites tested for the four genes *in vitro* and *in vivo* **(Figure 2.2.5)**. Induction of demethylation was most marked for *PAWR*, and *PDCD5*. *GADD45A* had intermediate loss of methylation and *HSPA9B* had the least change but overall methylation loss remained significant. Analysis of relative change in methylation was also performed on normal osteoblasts, which had an initial low methylation content comparable to the negative control (DNA from early embryos), and no change in this basal content was apparent following decitabine treatment.

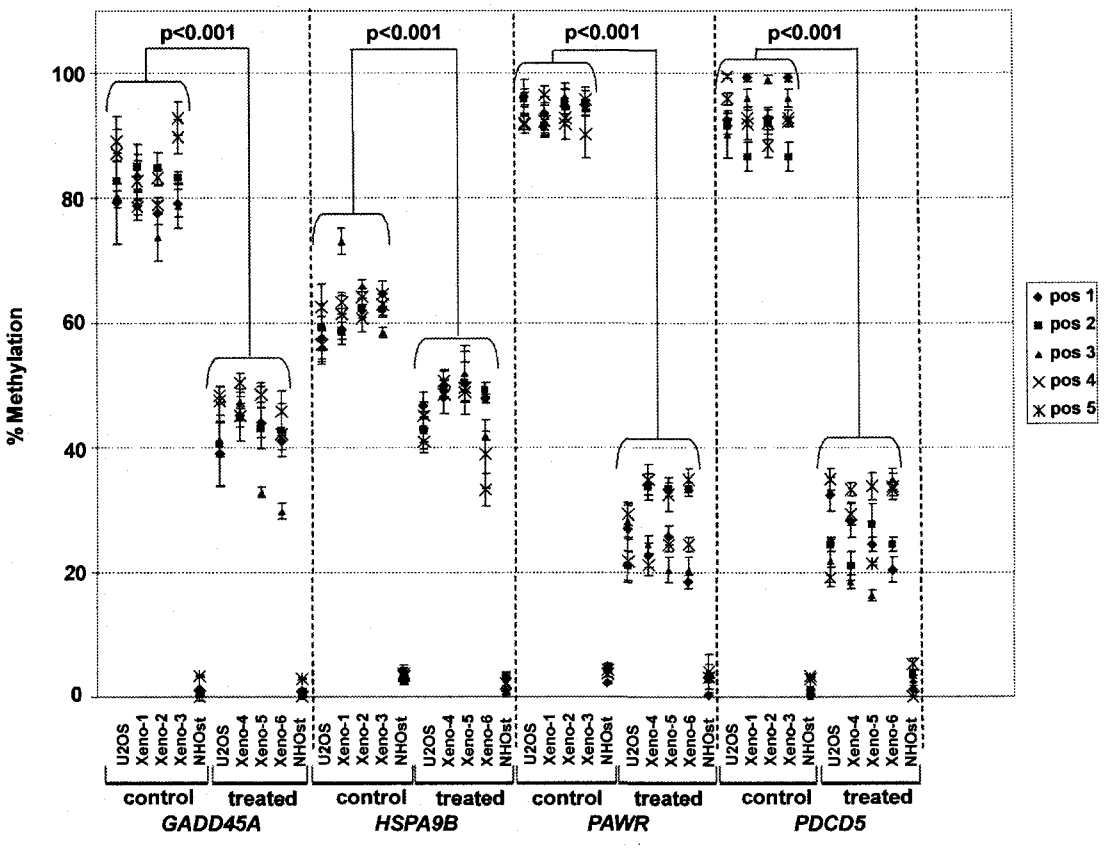


Figure 2.2.5: Comparison of percentage of methylation across 5 of the tested CpG positions *in vitro* and *in vivo*. Each data point is the average of methylation percentage for each CpG position in three experiments. Y-axis indicates the percentage of methylation and the samples are indicated on the x-axis. The location of CpG positions relative to the gene start site and to each other is shown in Figure 5. The results of 5 CpG positions are shown to represent the methylation percentage in the four genes across the samples. There was a significant decrease ($p<0.001$) in methylation quantity for each CpG position after decitabine treatment both *in vitro* and *in vivo* for the four genes in all sample but not in the NHOst (normal human osteoblasts). p-values were calculated by comparing the percentage of methylation for each individual CpG position in control

cells with the same CpG position in the treated cells using student t-test and they all resulted in $p<0.001$.

More detailed results on the methylation status for the same four genes for all tested CpG positions are provided in **Figure 2.2.6** [also see Al-Romaih et al, 2007: **http://www.biomedcentral.com/content/supplementary/1475-2867-7-14-S4.xls**]. For *GADD45A*, a CpG rich sequence at the promoter region containing a cluster of eight CpG positions was tested by Pyro Q-CpG. In U2OS cells, the methylation percentage on the eight CpG positions had an average of 83% in the no-treatment (control) cells. This was reduced to an average of 44% after treatment with 1μM decitabine ($p<0.001$). Similarly, the no-treatment xenograft tumors had averages of 84% in Xeno-1, 81% in Xeno-2, and 85% in Xeno-3, which was reduced after three doses of 2.5 mg/kg decitabine to 46%, 43% and 37% in Xeno-4, Xeno-5 and Xeno-6 respectively ($p<0.001$).

A CpG rich sequence at the promoter region of *HSPA9B* had a cluster of 13 CpG positions which were tested by Pyro Q-CpG. The overall methylation quantity seen in this sequence was lower than what was observed in *GADD45A*, none-the-less the difference between the no-treatment (control) samples and decitabine-treated samples was significant ($p<0.001$). U2OS cells (*in vitro*) had an average of 59% before treatment, and 42% after treatment ($p<0.001$). Before treatment, the xenograft tumors had averages of 61% in Xeno-1, Xeno-2, and Xeno-3 which was reduced after treatment to an average of 49% in Xeno-4 and Xeno-5 and 43% in Xeno-6 ($p<0.001$).

The analysis was done in the same manner for 13 and 12 CpG positions related to the CpG-island associated with *PAWR* and *PDCD5* respectively. These two genes had very high methylation percentage before decitabine treatment in U2OS, Xeno-1, 2, and 3 for the tested CpG positions. In the case of the 13 CpG positions tested in *PAWR* the average of methylation percentage before decitabine treatment was 93% in U2OS cells, 93% in Xeno-1 and Xeno-2, and 94% in Xeno-3. After decitabine treatment, this was reduced significantly ($p<0.001$) to an average of 26% in U2OS cells, 29% in Xeno-4 and 28% in Xeno-5 and 6. In the 12 CpG positions tested in *PDCD5*, U2OS cells had an average of 93%, Xeno-1 had an average of 94, Xeno-2 had an average of 93%, and Xeno-3 had an average of 92% before decitabine treatment. The methylation was reduced significantly after decitabine treatment ($p<0.001$) to 26% in U2OS cells, Xeno-4, and Xeno-5, and 27% in Xeno-6.

The normal low-passage osteoblast had a very low methylation percentage in all 4 genes. The range of methylation percentage had averages from 2.1% to 3.6% before treatment, and 1.4% to 2.7% after treatment with 1 μM decitabine across all the tested CpG positions in the 4 genes (**Figures 2.2.5, 2.2.6**). Importantly, the methylation status of the tested CpG sequences reflects the patterns of expression seen in all four genes after decitabine treatment in U2OS cells and xenografts. This is consistent with the possibility that decitabine treatment modulated the expression through reducing the amount of methylation on CpG-dinucleotides. To determine whether decitabine activated *GADD45A* methylation status and expression was also tested in two other OS cell lines, MG63, using identical decitabine treatment conditions. Similar to U2OS, induction of

GADD45A gene expression was associated with significant loss of methylation and increased transcript expression in MG63 cells.

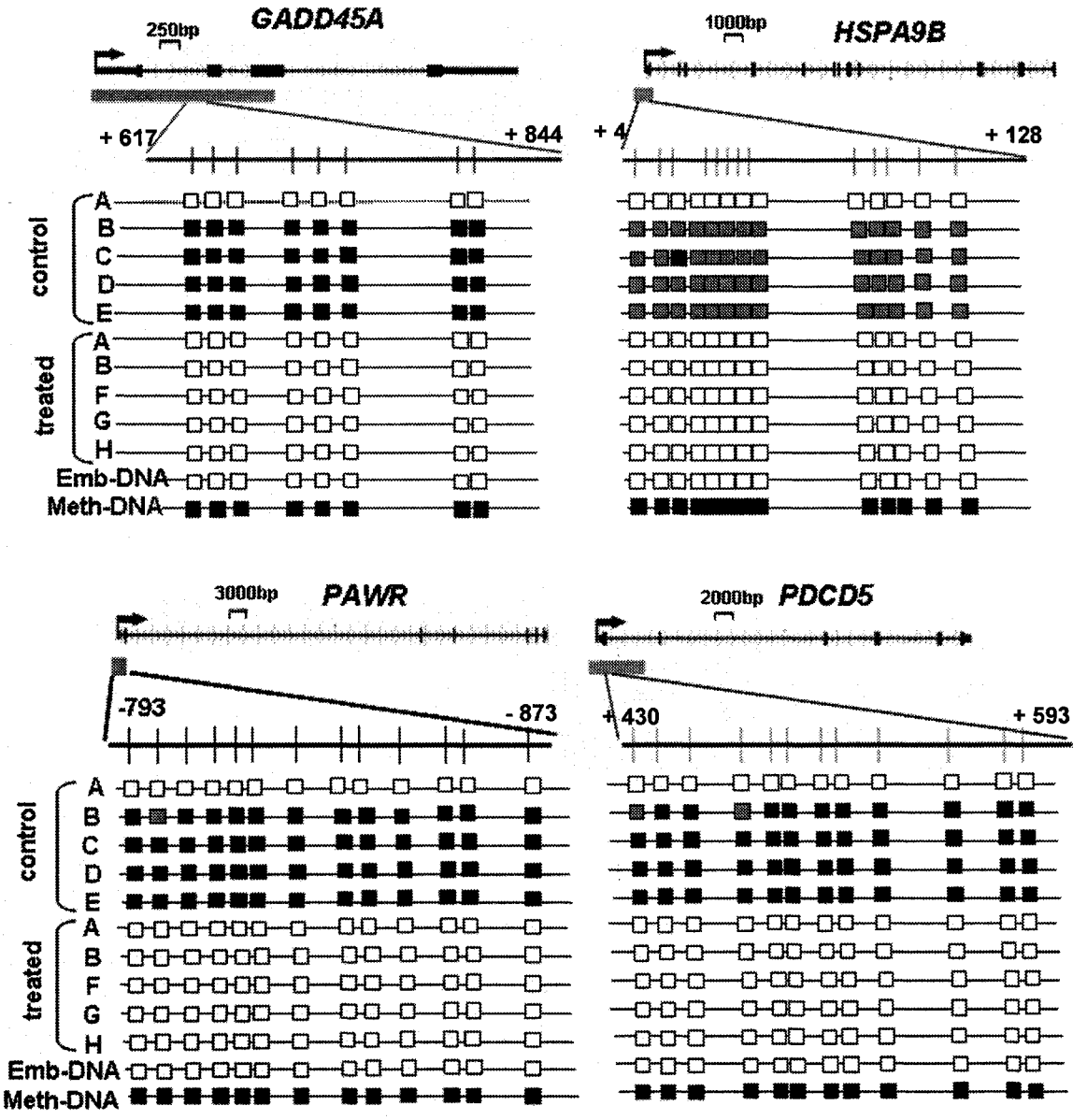


Figure 2.2.6 (previous page): Summary of Pyro-Q-CpG findings. Illustrated are the summary findings by Pyro-Q-CpG analysis of U2OS cells *in vitro* without treatment (control) and with 1 μM decitabine treatment (treated). The illustration also summarizes Pyro-Q-CpG findings in control U2OS xenograft tumors (Xeno-1, Xeno-2, and Xeno-3), and 2.5 mg/kg decitabine treated U2OS xenograft tumors (Xeno-4, Xeno-5, and Xeno-6). DNA from NHOst (normal low-passage human osteoblasts) was also analyzed for experiment control. DNAs from early embryonic DNA (Emb-DNA) and universally methylated DNA (Met-DNA) were used for negative and positive control respectively. CpG-islands are denoted by grey rectangles relative to the gene start site. The region further enlarged below corresponds to each tested CpG sequence. The tick marks denotes the individual CpG dinucleotides. The transcription start site is indicated by a directional arrow with the base pair numbers annotated for each tested sequence. The extent of methylation is represented by the scale bar (bottom right). *GADD45A*, *PAWR*, and *PDCD5*, had a high level of methylation before decitabine treatment while *HSPA9B* had an intermediate level of methylation before treatment. In all cases the methylation was decreased significantly ($p<0.001$) as a result of decitabine treatment.

2.3 Discussion:

This study draws attention to the possibility that therapeutic levels of decitabine could orchestrate the interplay between DNA damage genes, induce growth arrest, apoptosis and potentially modulate genomic fidelity. At present, neoadjuvant and adjuvant chemotherapy is favored in the treatment of OS and the agents most commonly used include doxorubicin, high-dose methotrexate, cis-platinum and ifosfamide either alone or with etoposide. The use of these agents in OS treatment has been well established and yielded 5-year disease-free-survival and overall-survival of greater than 60%, [293]. However, the lack of a near-complete response to chemotherapy in a sub-group of patients reflects inherent biologic resistance to these agents, hence poorer prognosis [41], especially since attempts at changing chemotherapy regimens for poor responders have generally not improved outcome [294] [295] [296].

Previous studies have implicated a role of epigenetics in OS biology; methylation of osteocalcin has been linked to bone differentiation [266] [267] [268], transcription factor expression, and histone modification [269] [270]. Abnormal promoter methylation of p16INK4a/p14ARF promoters was observed in OS–derived cell lines [275]. Aberrant methylation of specific genes was also correlated with poor survival in OS patients [297] [298]. The number of genes found methylated in OS is increasing [299] [300] which further supports the implication of DNA methylation in OS tumorigenesis. Up–to-date studies regarding the epigenetics of OS have either been based on a single gene or focused on a small number of genes, and limited with respect to elucidating the target

pathways suitable for epigenetic therapeutics in OS. Our study is the first to use demethylation treatment to modify global gene expression in an OS cell line in order to identify pathway-specific methylation targets that may have therapeutic importance.

Analysis of decitabine–induced cellular changes in U2OS xenografts suggested that apoptotic pathways may be the earliest pathways to be affected. The decitabine dose was based on previous studies [288] [289] [291] and has been shown to reduce the methylation of tumor suppressor genes and decrease tumor growth in xenografts. The xenografts in decitabine-treated mice decreased in volume size significantly ($p<0.05$) when compared to the xenografts in untreated control mice. Recent reports showed similar effects on xenograft size and growth parameters from melanoma tumors [301] and lung tumors [302] grown in mice treated with decitabine. In lung cancer, cell clones carrying conditional methylation of *FHIT* or *WWOX* transgenes showed significant suppression of xenograft tumor growth after induction of expression of the *FHIT* or *WWOX* transgene as a result of decitabine treatment, suggesting that treatments to restore endogenous Fhit and Wwox expression in lung cancers would result in decreased tumorigenicity [302]. Similar findings were also seen in melanoma xenografts where decitabine reduced tumor growth and that was correlated with re-activation of genes with tumor suppressor properties [301]. The observations in the lung cancer xenografts are particularly intriguing since it indicates decitabine effectiveness in the treatment of solid tumors.

The effect of decitabine on tumor cell mitotic index and apoptosis in OS has not been previously reported. In our series, decitabine treatment significantly decreased the

number of mitoses and increased the number of apoptotic cells. This is similar to decitabine effects in the lung cancer xenografts [302] where suppression of tumor growth was accompanied by low mitotic activity and high apoptosis. These observations suggest that decitabine reduce tumor growth by re-activating pathways that lead to cell arrest and/or apoptosis. In addition, treatment with decitabine significantly increased the amount of osteoid associated with the tumors. The effect of decitabine on extracellular matrix (osteoid) formation in OS has not been previously studied. Such results suggest that decitabine treatment reduces the proliferative capacity of U2OS cells, whilst concurrently driving the cells toward terminal differentiation and apoptosis.

Gene expression profiling by microarray analysis showed that 88 genes showed increased expression *in vitro* after decitabine treatment, which represented ~ 0.6% out of the 14500 genes on the array and this global modulation of genes is at a level comparable to other studies. The effect of decitabine on global gene expression has been previously reported in several studies [287] [303] [304]; comparable to our study, the number of up-regulated genes varied from 1.9% in ovarian cancer cells [303] to 1.1% in malignant glioma cells [304] and 0.6% in bladder cancer cells [287]. The differences in the number of decitabine up-regulated genes were possibly a result of cell line-to-cell line variations, microarray platforms, and experimental designs including differences in drug dosage and duration of treatment. Indeed, Karpf et al [305] screened the expression of approximately 38,000 human transcripts in several decitabine treated cell types and observed changes in genes expression for 0.2 to 1.4% of those transcripts, depending on the cell type treated [305] [306].

Decitabine-dependent reduced expression of genes was also observed in U2OS following *in vitro* treatment. Thirty-one genes out of the ~ 14,500 genes (~ 0.2%) were significantly down-regulated. In keeping with other reports [287] [303] [304], this percentage of down-regulated genes is markedly lower than the percentage of the up-regulated genes (~0.6% -this study). One possible explanation for this inequality in differential response to the drug is that an early indirect effect of decitabine is the demethylation of promoters regulating upstream transcriptional activation pathways; such an effect could lead to preferential re-expression of multiple genes. Another more general possibility is that repression of gene promoters by methylation is a more frequently used control mechanism *per se* than activation of genes by methylation [130] [307]. Up-regulation of gene expression after decitabine treatment was the main focus of this thesis since this approach provided a direct link between demethylation and gene re-activation. Further investigation of several OS cell lines will help to clarify the levels of global change of expression induced/reduced by decitabine.

The global expression profiling of U2OS represents the majority of cells that were viable at the time of harvest. These cells were undergoing fine molecular modulations of their epigenome eventually leading to changes in gene expression resulting in the observed cell death and apoptosis *in vitro* and *in vivo*. Decitabine treatment in U2OS induced a level of cell death that was comparable to observations in acute myeloid leukemia (AML) cells using similar treatment regimens *in vitro* [307]. In the AML study differentiation activation was considered to be an early effect of decitabine in AML. The findings of U2OS study, however, suggest that the cellular effects detected are a result of

up-regulation of apoptotic genes. These data are in general agreement with the increasing evidence that decitabine's antineoplastic effects may be through modulation of apoptotic pathways [308] [309].The genes identified in U2OS with a role in apoptosis were *GADD45A*, *HSPA9B*, *PAWR*, *PDCD5*, *NFKBIA*, and TNFAIP3. These proteins have potential roles in regulating a number of key apoptotic events including the p53 related apoptosis, bcl2 related apoptosis, and the nfkb related apoptosis. *GADD45A*, like p53, is considered to be involved in cell growth control, maintenance of genomic stability, DNA repair, cell cycle control, and apoptosis (discussed in Chapter Three).

In our study, 63 (71%) of the 88 decitabine up-regulated genes possessed CpG-island at their 5' region, a proportion that is higher than that observed in other reports; including AML [307], and human glioma cell lines [210], where decitabine re-activated 50% and 40%, respectively, of genes with potential CpG islands. This observation is particularly intriguing because it suggests that decitabine treatment of U2OS induced CpG-island associated genes more frequently than previously reported. Moreover, of the 13 U2OS genes with a ≥2-fold change, there were 11 genes (84%) with CpG-island in there promoter region. The further enrichment of the frequency of CpG-island-associated genes in the genes with a strong induction of expression (≥2-fold change) after decitabine treatment, further suggested that expression induction reflects either a more direct effect of decitabine through CpG-island demethylation, or indirect activation effects. In four of six apoptotic genes studied in detail, we showed a significant increase in expression following decitabine treatment was accompanied by a marked loss of promoter

methylation, which points out to the potential direct effect of decitabine on methylated CpG sequences.

The above data demonstrate that the re-activation of genes involves CpG-island demethylation. Dinucleotide clusters of CpGs in CG-rich regions of genomes or CpG-islands are present in the promoters and exonic regions of at least 40% of mammalian genes some reports, however, other reports indicate that up to 70% of mammalian genes have CpG islands in their promoter [310] [311]. Methylation of promoter associated CpG islands in the genome of cancer cells has shown non-random and tumor-type-specific patterns [210]. While some tumors exhibit hypermethylation of low number of specific CpG islands, other tumors possess hypermethylation of a higher number CpG island associated promoters [210]. It is important, though, to realize that not all genes with methylated CpG islands are re-activated by decitabine treatment possibly because some methylated CpG islands may have other chromatin structural alterations that does not respond to demethylation treatment [306].

Out of 88 significantly induced genes there were 25 genes with no apparent CpG island at their promoter region. Similarly, two genes out of the 13 genes with ≥2-fold change have no potential CpG islands close to the TSS and promoter region. Methylation-independent induction of gene expression has also observed in other studies [312] [307] indicating that genes without CpG islands may respond to this drug. Decitabine mechanism of action is not restricted to its demethylation capability and was reported to have effects on histone methylation and RB phosphorylation [313] [314].

Decitabine treatment re-activated several apoptotic genes in U2OS cells that were identified in this work. Other genes which are known as regulators of proliferation and differentiation in mammalian cells with potential role in bone development were also identified in this microarray screen. Decitabine re-activated the IGF binding proteins (IGFBPs) IGFBP6 and IMP-3 in U2OS cells (Table 1). IGFBPs modulate their receptors IGFs whose expression was shown recently as a possible significant risk factor for OS [315] [316] [317] [318] [319] [320]. Also, there is increasing evidence that induction of IGFBPs has anti growth properties in osteoblasts and in OS cells [321] [316] [322] [276]. However, more studies are yet to be performed to investigate the association between the IGF signaling pathway and bone development or OS pathogenesis.

Understanding the involvement of the pathways identified above in OS pathogenesis will provide more accurate implication of these factors in OS development and facilitate the opportunities to improve OS therapy. The finding of this study suggests that hypermethylation of several genes with anti-growth properties functions as a mechanism for increased proliferation capacity, reduction of apoptosis and loss of the differentiated phenotype in U2OS. Targeting hypermethylation in U2OS by decitabine indicated the potential of this drug for OS treatment. In the next chapter I show experimental evidence of the apoptotic role of one of the genes that was identified in the above screen. I also provided detailed discussion on the potential of targeting hypermethylation of this gene for OS treatment.

CHAPTER THREE

3 Decitabine demethylation induction of Gadd45a leads to apoptosis in OS cells

This work is based on the article:

Decitabine induced demethylation of 5' CpG-island in *GADD45A* leads to apoptosis in osteosarcoma cells (In press: *Neoplasia*) Khaldoun Al-Romaih, Bekim Sadikovic, Maisa Yoshimoto, Yuzhuo Wang, Maria Zielenska, and Jeremy A. Squire. K A performed all experiments in this section and wrote and conceived the manuscript. B S assisted in that writing of the manuscript. M Y assisted in the immunohistostaining experiments.

3.1 Introduction

As discussed in Chapter 2, one of the decitabine-reactivated genes of importance in OS oncogenesis was *GADD45A*. Gadd45a induces G2/M arrest after DNA damage and induces apoptosis in p53- dependent and independent manner [106] [323]. It is known as a conventional downstream effector of p53, however may play a role as an upstream regulator of p53 stabilization following DNA damage, thus has a defined positive feedback signal in the activation of the p53 pathway [324]. Significantly, Gadd45a activation was previously shown to be sufficient in the induction of apoptosis independent of p53 status in several cancer cell lines including COS, PC-3, DU145, and HeLa cell lines [325] [326] [219] [106].

The work presented in this chapter discusses the involvement of *GADD45A* in apoptosis induction by decitabine in OS cells. This work comprises the objectives of **Specific Aim 3** of this thesis.

3.1.1 Relevance of *GADD45A* to OS tumorigenesis

Deletions of *P53* gene are common in OS. Interestingly, *GADD45A* promoter has a binding locus for *P53* in the third intronic region of the gene and its functionality is linked to activation of G1/S cell cycle arrest in response to ionizing radiation [327]. Also the promoter region of *GADD45A* has a repression binding site for *c-MYC* gene [328] which is known to be amplified in OS (section 1.2.4). Gadd45a is a central player in the maintenance of genomic stability and loss of the protein can lead to centrosome hyperamplification, chromosomal instability, and increased aneuploidy [102, 103, 329]. These effects are similar to those seen as a result of p53 loss, which include genomic instability, hyperamplification of centrosomes, gene amplifications, aneuploidy, and chromosomal aberrations [239]. Most of the Gadd45a- and p53- loss features are hallmarks to OS tumors [55] [56] [51] [53] (also discussed in the Introduction; section 1.2) suggesting that the role of this protein in OS is highly relevant and despite this striking relevance the work in this chapter is the first that investigated the role of *GADD45A* in the pathogenesis of OS.

3.1.2 Role of Gadd45a demethylation in decitabine induced apoptosis in OS

GADD45A belongs to the stress-responsive *GADD45* family which was reported to be methylated in multiple tumors [330], [219]. In OS, methylation within the 5' region of *GADD45A* is likely to be a mechanism of repression and inactivation of the protein's apoptotic function (chapter two). There is a region with dense repetitive CG sequence (CpG-island) near the transcription start site (TSS) of *GADD45A* that spans 1357 bp and

covers the first 3 exons of the gene [331] [332]. Methylation of a few clusters of CG dinucleotides in this CpG-island was reported in breast caner tissues [331]. One cluster of 8 CpG dinucleotides within the first intron of *GADD45A* was found to be methylated in the OS cell line U2OS *in vitro* and in xenografts [332]. In Chapter 2 of this thesis I demonstrated that demethylation induction of *GADD45A* was associated with induction of apoptosis in U2OS, but the precise role of *GADD45A* CpG island demethylation and the specificity of Gadd45a expression in the induction of apoptosis were not defined.

The experiments in this chapter were designed to examine the role of decitabine-dependent *GADD45A* CpG island demethylation on the expression of the gene, and on the subsequent induction of apoptosis in OS cell lines. The experiments were performed on U2OS cell line (discussed in section 2.1.1) and MG63 cell line. MG63 cell line is an OS cell line that was established spontaneously *in vitro* [333], and in contrast to U2OS, this cell line has a mutant p53. The data reported in this chapter provide evidence on *GADD45A* demethylation activation and induction of apoptosis by decitabine in p53-mutant background. The data also show that *GADD45A* demethylation activation by decitabine is central to the observed induction of apoptosis in U2OS and MG63 cell lines.

3.2 Results:

3.2.1 Methylation of GADD45A CpG-island in MG63

Reduction of *GADD45A* expression in U2OS *in vitro* and in xenografts was shown in chapter two to be associated with 5'-CpG-island hypermethylation of a cluster of eight CpG dinucleotides, within the first intronic region of the gene 620 bp

downstream of the transcription start site (TSS) and this CpG island is mapped in **Figure 3.2.1A**. Similar to U2OS, quantitative bisulfite pyrosequencing (Pyro-Q-CpG) showed that ~ 80% of the sequenced alleles from the eight CpG dinucleotides in *GADD45A* of MG63 were methylated **(Figure 3.2.1B)**. Exposure of MG63 to 1μM decitabine significantly decreased methylation ($p<0.001$) to ~55% of sequenced alleles at the eight CpG dinucleotides **(Figure 3.2.1B)**.

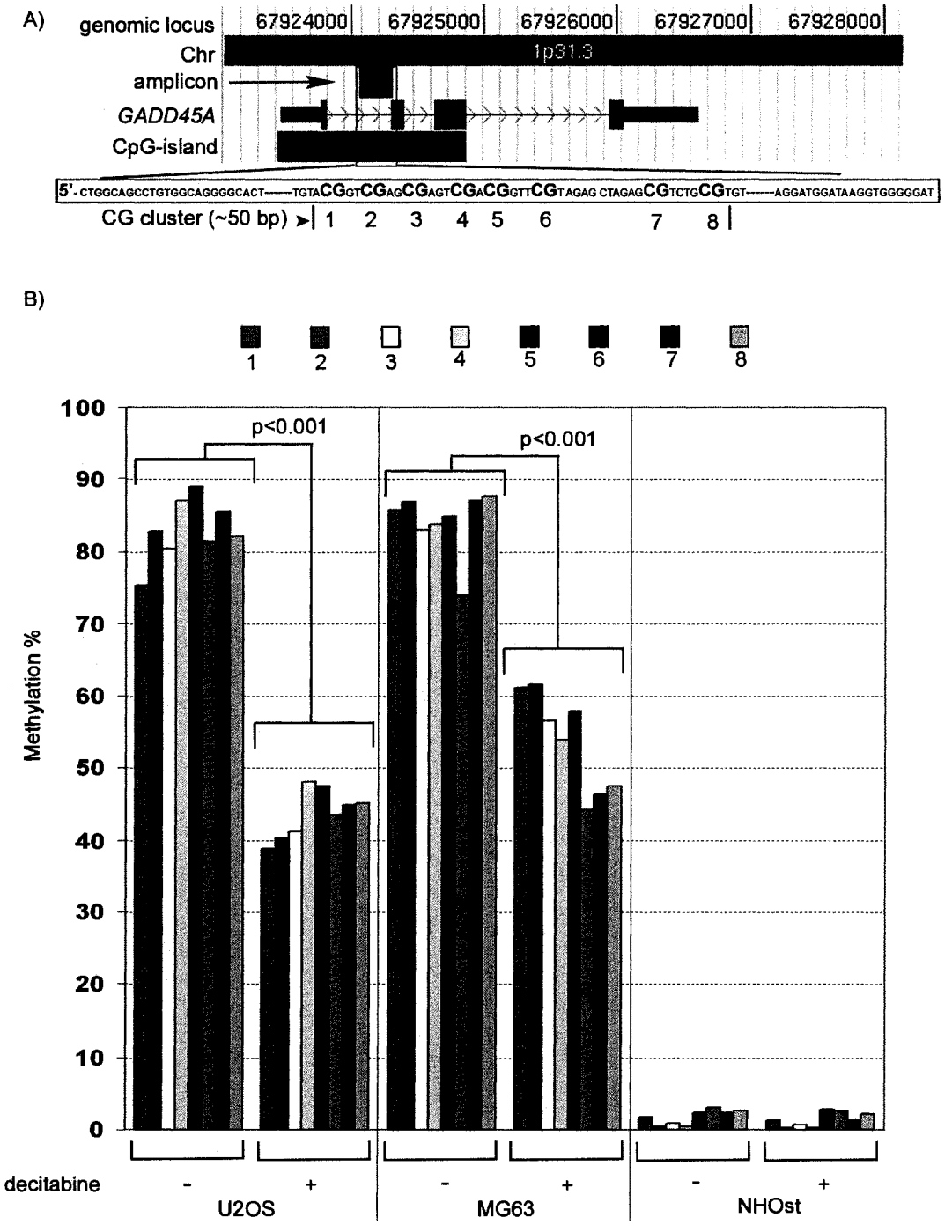


Figure 3.2.1: *GADD45A* CpG methylation in osteosarcoma cells. A) Schematic of GADD45A genomic locus. 5' CpG-island spanning the first 3 exonic regions of *GADD45A* is shown. An amplicon of ~ 250 bp was

amplified and a cluster of 8 CG dinucleotides in a ~ 50 bp region within the amplicon was analyzed by Pyro-Q-CpG. The schematic was based on the latest build of the Human Genome Browser data base: http://genome.ucsc.edu/cgi-bin/hgGateway . B) Percentage of methylated alleles on the 8 CpG positions in U2OS, MG63, and osteoblasts as detected by Pyro-Q-CpG. Each data column is the mean percentage of methylated alleles for each CpG position in three experiments. Y-axis indicates the percentage of methylation and the samples are indicated on the x-axis. Methylation percentage in U2OS, MG63 and normal osteoblasts (NHOst) with (+) and without (-) 1 μM decitabine is shown for 8 CpG positions in *GADD45A* promoter.

3.2.2 Methylation down-regulation of *GADD45A* expression

Methylation repression of *GADD45A* in MG63 was implicated since mRNA expression was found to be reduced, at levels slightly lower than those of normal low passage osteoblasts **(Figure 3.2.2A)**. Importantly, loss of DNA methylation was associated with a 3.5-fold increase in *GADD45A* mRNA expression relative to normal osteoblasts and 6-fold increase relative to untreated MG63 cells as detected by Q-RT-PCR **(Figure 3.2.2A)**. Interestingly very low methylation (range 0.1-2.9% methylation of sequenced alleles) was observed at all eight CpG dinucleotides in both treated-, and untreated osteoblasts **(Figure 3.2.1B)**. Moreover, *GADD45A* mRNA expression in osteoblasts was not induced after decitabine treatment **(Figure 3.2.2A)**.

To confirm that induction of mRNA expression led to a concomitant increase in Gadd45a protein levels, western blot analyses were performed on cell extracts from both decitabine treated, and untreated U2OS and MG63 cells **(Figures 3.2.2 B and C)**.In both

cell lines the levels of Gadd45a protein induction after decitabine treatment were similar to that observed in the mRNA levels.

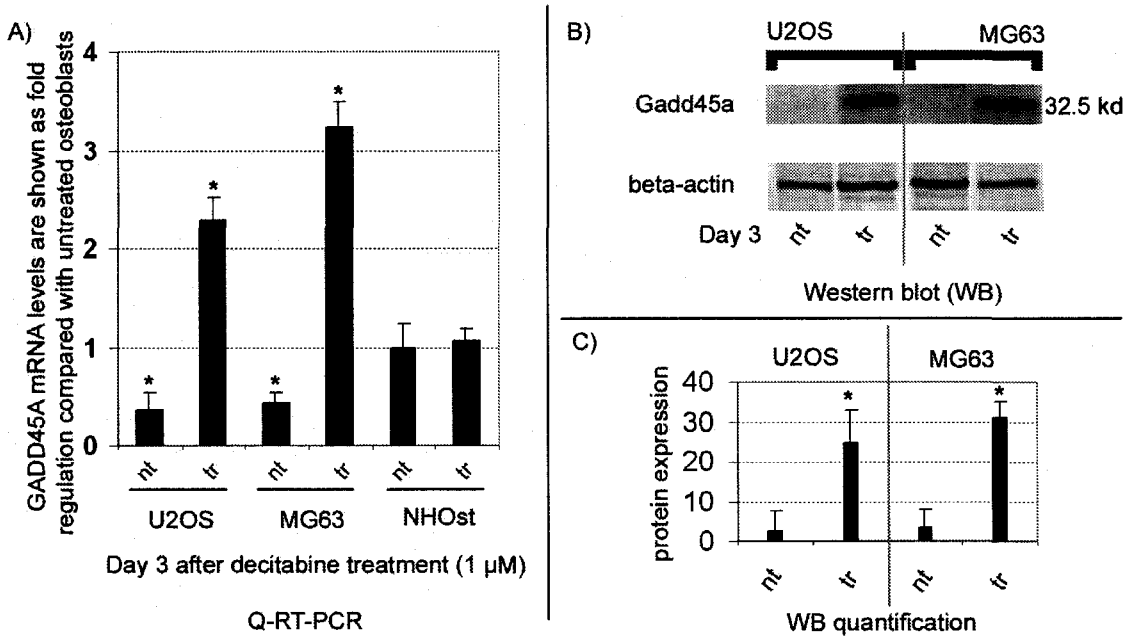


Figure 3.2.2: Down-regulation of GADD45A expression. *A) Induction of GADD45A mRNA expression in U2OS and MG63 after decitabine treatment.* Total RNA was extracted on Day 3 after decitbaine treatment was initiated. cDNA was then made as detailed in methods. TaqMan assays were used to determine relative expression using the cDNA from untreated osteoblast as baseline and beta-actin for a reference gene by applying the ΔΔCt method. Each column is the Mean of three replicates, and error bars indicate standard deviation from the Mean. In these analyses *GADD45A* mRNA levels are shown as –fold regulation compared with untreated normal osteoblasts. NHOst= normal human osteoblasts. nt = no treatment. tr = treated by decitabine. *B) and C) Decitabine-related Gadd45a protein induction in U2OS and MG63 cells.* Protein preparations from cells with or without treatment were done 3

days after initiating the treatment. For each sample a total of 50 μg protein was examined by western blotting and beta-actin was used as a loading control. The shown blot in (B) is representative from three experiments and the mean ± SD from quantification of 3 replicas after normalization to beta-actin is shown on the graph in (C).

3.2.3 Decitabine induces significant levels of Gadd45a protein in OS cells

In order to investigate the level of Gadd45a protein induction in U2OS xenografts, 6 xenografts (3 from no treatment and 3 from decitabine treated) were analyzed by IHC using a human Gadd45a antibody. Sections from cell pellets prepared from the Gadd45a activated- UV treated HeLa cells were used as positive controls. The staining of xenograft nuclei was quantitated using the intensity of staining and the proportion of positive tumor nuclei. As shown in **Figures 3.2.3 A and B**, xenograft sections from the untreated control mice exhibited low nuclear staining for Gadd45a, while the staining was stronger and more frequent in nuclei from xenografts in decitabine treated mice ($p<0.05$). Induction of Gadd45a was also found to be associated with increased apoptosis as shown in a previous analysis on the same U2OS xenografts (chapter two). In addition, caspase-9 activation was assessed by immunohistochemistry in U2OS xenografts using an antibody specific for cleaved (activated) caspase-9. In this analysis there was no significant induction of cleaved caspase-9 ($p > 0.05$) as a result of decitabine treatment when compared to no treatment xenografts.

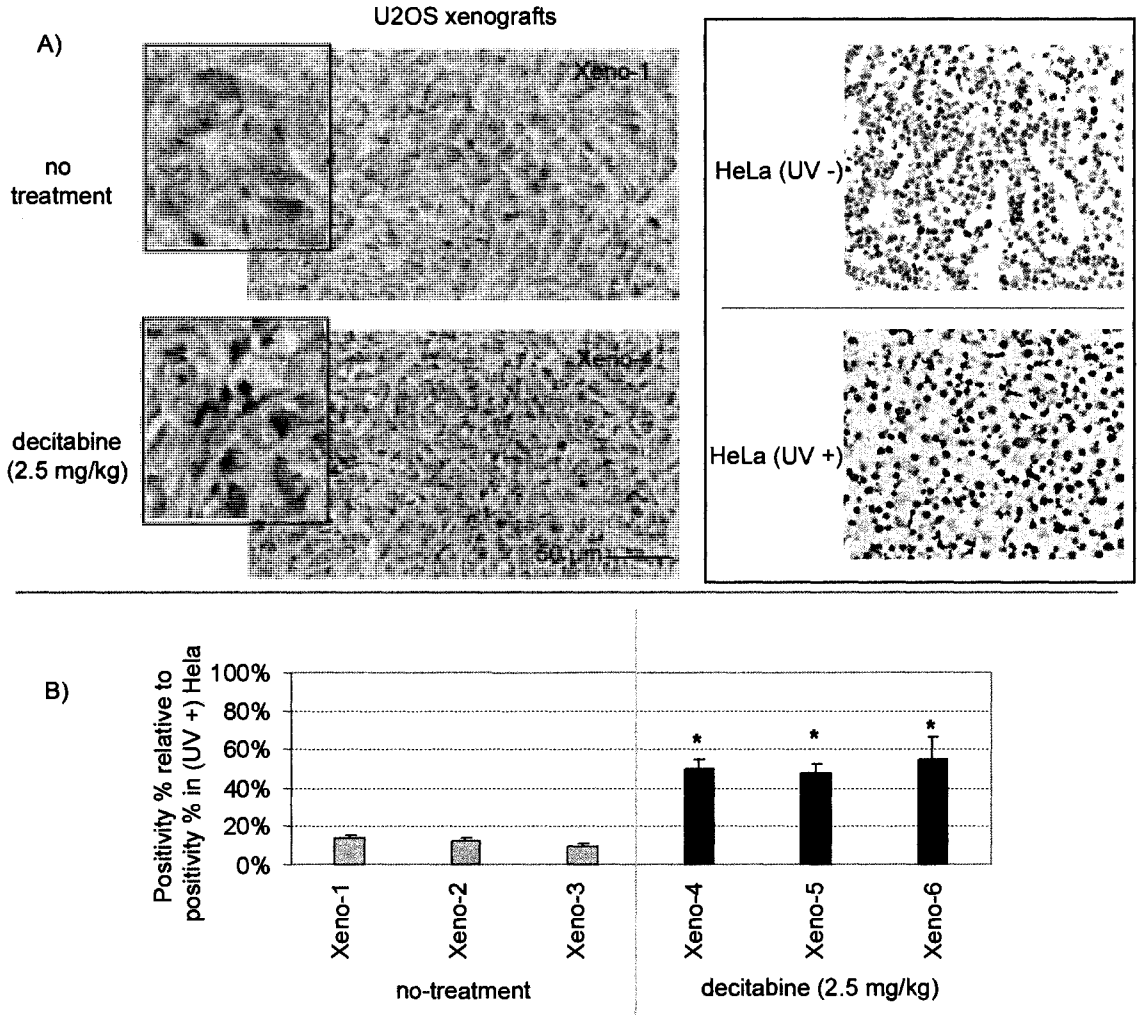


Figure 3.2.3: Induction of Gadd45a protein in OS xenografts by decitabine treatment. *A) Induction of Gadd45a protein levels in U2OS xenografts*. Analysis of the relative levels of nuclear Gadd45a protein within xenografts derived from representative control untreated mice (upper panel); or decitabine treated mice (lower panel) using immunohistochemical staining with human Gadd45a-Ab. A 20X enlargement of representative U2OS histology is shown inside boxed image. To the right (lower image) in (A) is the darkly stained HeLa cells correspond to induction of Gadd45a after UV exposure (UV+) which was used as positive control. *B) Quantification of Gadd45a-antibody staining.*

The staining intensity from control (light columns) and decitabine treated (dark columns) was quantitated using Aperio scanning image analysis of sections. Gadd45a-Ab intensity was calculated based on the percentage of positivity (total positivity/total negativity per snapshot) relative to positivity index in the positive control (UV irradiated HeLa cells) after normalization to negative controls (no Gadd45a Ab). Determination for the difference between the control and treated xenografts was done by applying the student t-test, and $p < 0.05$ was considered significant. The columns are the mean ± SD from 5 to10 (~ 0.3 mm^2) images after normalization to positive control.

3.2.4 Induction of apoptosis in *GADD45A*-transfected U2OS and MG63

Decitabine treatment at 1 μM induced apoptosis *in vitro* significantly ($p<0.05$) in both cell lines in comparison to no treatment cells **(Figure 3.2.4 A)**. Similarly, transient transfection of Gadd45a overexpression vector induced apoptosis significantly ($p<0.05$) in untreated U2OS and MG63 in comparison to transfection with an empty vector **(Figure 3.2.4 A)**. In both experiments, western blot analysis demonstrated activation of the Gadd45a protein **(Figures 3.2.4 B and C)**. In order to investigate the specificity of Gadd45a induction on decitabine-induced apoptosis, *GADD45A* mRNAs were disrupted using specific short interference RNA (siRNA) in U2OS and MG63 cells. The effect of 1 μM decitabine treatment on *GADD45A* mRNA and protein levels 3 days after treatment was analyzed alone or in combination with 60 nM Gadd45a-siRNAs.

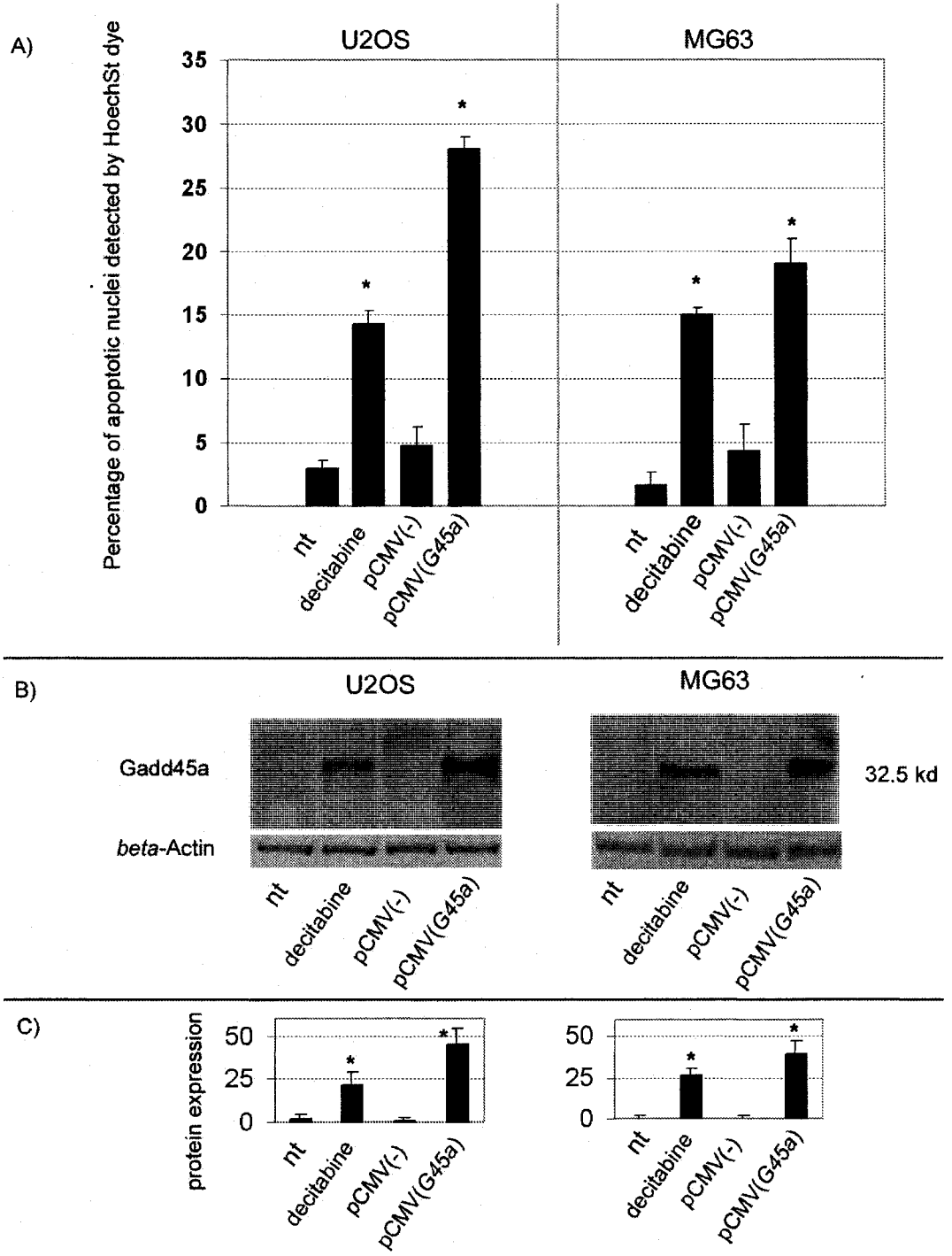


Figure 3.2.4: Apoptosis induction by pCMV-GADD45A in U2OS and MG63. *A) Percentage of apoptotic nuclei as detected by Hoechst stain in U2OS and MG63.* Columns are mean of three replicas and error bars are

standard deviation from the mean. *B) Induction of Gadd45a protein by decitabine treatment or by Gadd45a transient transfection.* Western blotting was used to detect protein levels after treatment with 1 μM decitabine or 2 μg of pCMV(*GADD45A*) transient transfection vectors (TrueClone™, OriGene, Rockville, MD, USA). 10 μl of Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) was used in the transient transfection experiments. *C)* The mean ±SD from quantification of 3 replicas after normalization to beta-actin.
nt= no treatment. pCMV(-)= empty vector. pCMV(*GADD45A*)= human *GADD45A* cDNA cloned in pCMV vector.

3.2.5 *GADD45A* specific reduction by siRNA

Transfection of Gadd45a-siRNA against a decitabine-treated background reduced *GADD45A* mRNA levels in both cell lines by >70% knockdown efficiency **(Figure 3.2.5 A)**. This knockdown efficiency was consistently observed in the protein level in U2OS and MG63 cells as analyzed by western blotting **(Figure 3.2.5 B and C)**. Decitabine treatment resulted in 5-fold induction of *GADD45A* mRNA in U2OS and 6-fold in MG63 cells. Similar levels of induction were seen in the presence of 60 nM of scrambled control RNA (ctRNA) in decitabine-treated cells.

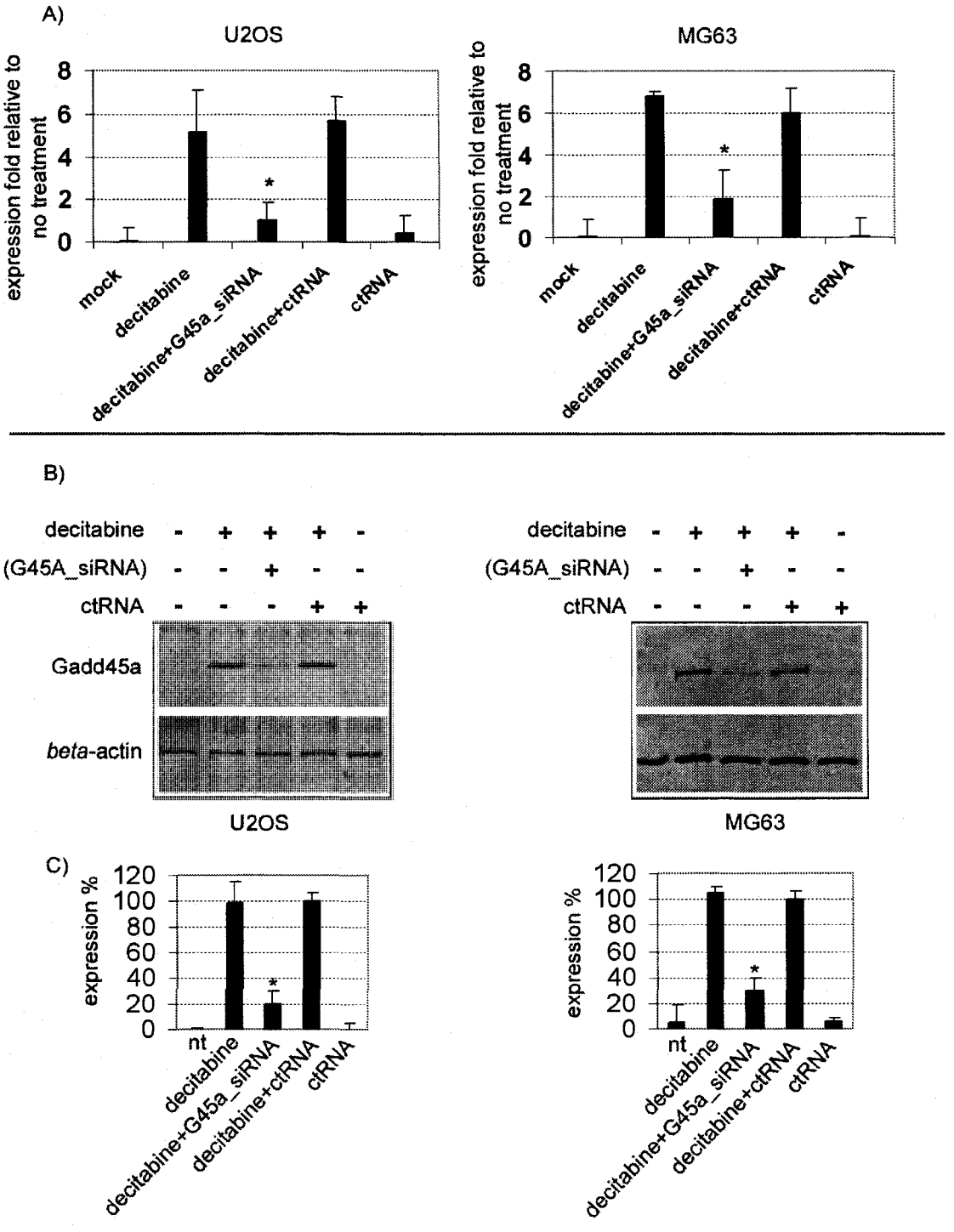


Figure 3.2.5: Reduction of GADD45A expression by *GADD45A*-siRNAs in decitabine treated U2OS and MG63 cells. *A) Effects of*

GADD45A-siRNA on mRNA levels in U2OS and MG63. Total RNA was extracted on Day 3 after the decitabine treatment was initiated. Preparation of cDNA and determination of mRNA relative expression is similar to that outlined in Figure 1 C and detailed in methods. Each column is the Mean of three biological replicas and error bars indicate standard deviation from the Mean. The data is expressed as fold change relative to no-treatment. Mock = transfection agent + medium (no siRNA or ctRNA). *B) Gadd45a protein levels at day 3 after decitabine treatment.* Total of 50 μg protein was examined by western blotting for activation of Gadd45a protein and beta-actin was used as a loading control. *C) Quantification of Gadd45a protein levels.* The level of Gadd45a in the decitabine treated and scrambled control RNA transfected (ctRNA) was set as 100%. The columns are the mean ± SD of three replicas after normalization to beta-actin.

3.2.6 Gadd45a specific induction of apoptosis

To investigate the effect of knocking down Gadd45a protein on apoptosis, nuclei from U2OS and MG63 were stained with Hoechst 33342 dye at day 3 after treatment in the decitabine alone and combination experiments **(Figure 3.2.6 A)**. Fluorescent microscopy demonstrated that the fraction of fragmented nuclei caused by 1 μM decitabine treatment were 14% in U2OS and 15% in MG63 while the no treatment cells or the ctRNA (60 nM) cells (in the presence transfection agent) had fragmented nuclei (apoptotic nuclei) percentage of less than 4% in both cell lines **(Figure 3.2.6 B)**. Decitabine also induced apoptosis to similar levels in U2OS and MG63 when used in combination with same amounts of ctRNA and transfection agent. Interestingly when

Gadd45a-siRNA (60 nM) was used in combination with decitabine, apoptotic nuclei were reduced from 14 % to 6% ($p<0.05$) in U2OS and from 15% to 8% in MG63 ($p<0.05$) **(Figure 3.2.6 A and B)** indicating that the ability of decitabine to induce apoptosis was reduced specifically by Gadd45a-siRNA. Same knockdown experiments were repeated on U2OS cells using two other siRNAs for human *GADD45A* for validation against off-target effect **(Figure 3.2.7 A and B)**.

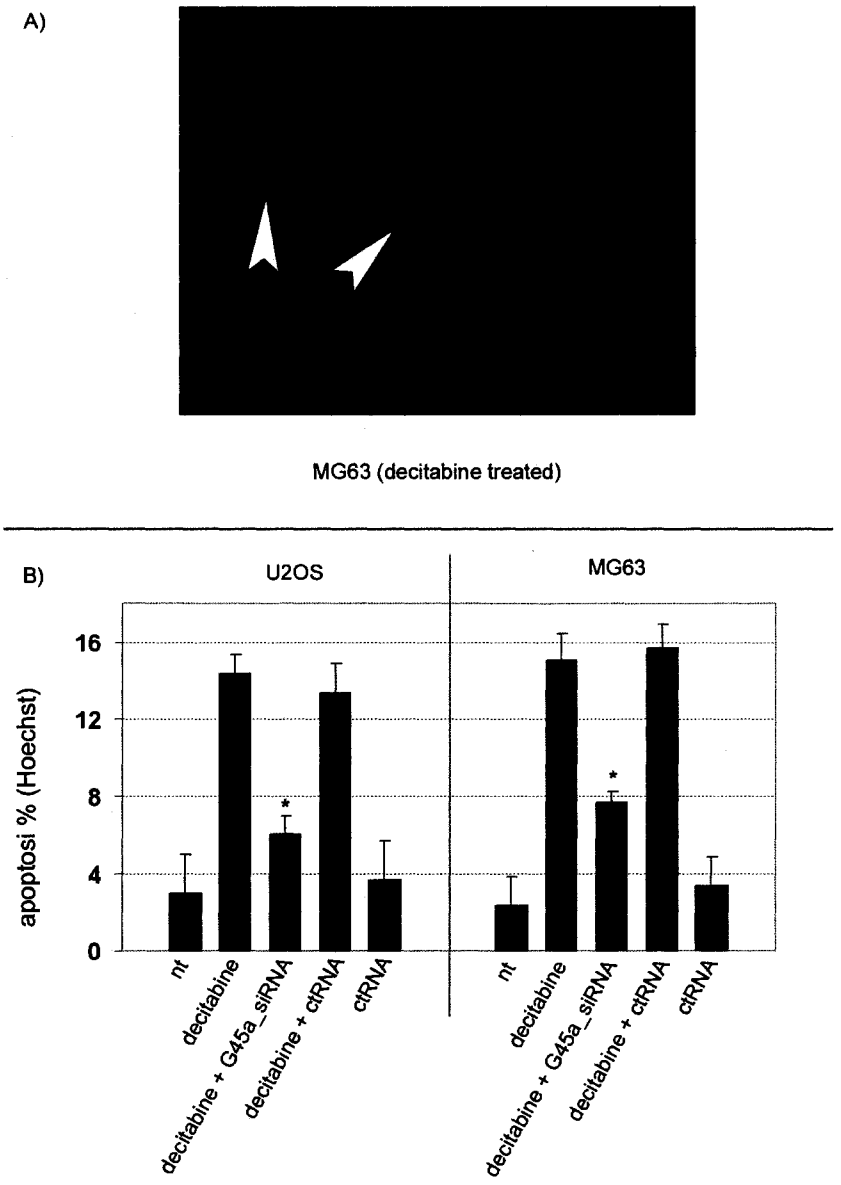


Figure 3.2.6: Gadd45a-siRNA treatment abolishes decitabine-induced apoptosis. *A) A representative image of apoptotic nuclei (arrows) from decitabine treated MG63 stained with Hoechst 33342 dye.* B) One hundred nuclei were counted per slide and 3 slides were prepared for each treatment condition. The columns are the mean ± SD of 3 biological replicas. When Gadd45a-siRNA (60 nM) was added to the medium in combination with decitabine, apoptotic nuclei were reduced to from 14 %

to 6% ($p<0.05$) in U2OS and from 15% to 8% in MG63 ($p<0.05$). G45a= Gadd45a. nt= no treatment. siRNA= short interference RNA. ctRNA= control non-targeting RNA

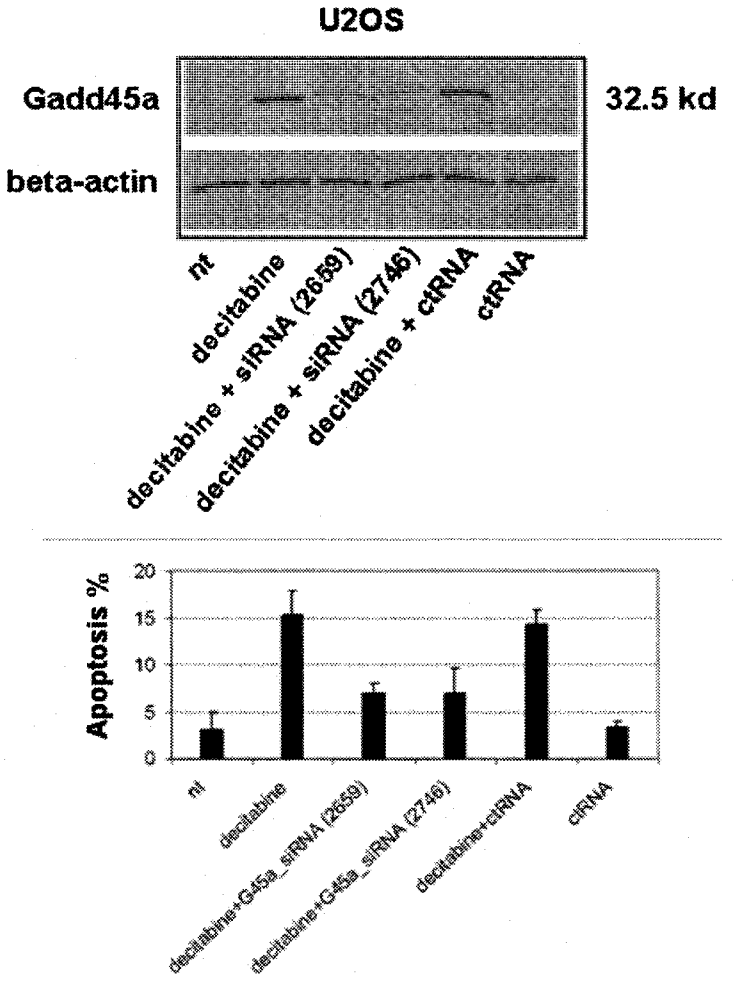


Figure 3.2.7. Reduction of Gadd45q protein expression by *GADD45A*-siRNAs (2659 and 2746) in decitabine treated U2OS cells abolishes decitabine induced apoptosis. Top) Gadd45a protein levels at day 3 after decitabine treatment. Total of 50 µg protein was examined by western

blotting for activation of Gadd45a protein and beta-actin was used as a loading control. Bottom) One hundred nuclei were counted per slide and 3 slides were prepared for each treatment condition. The columns are the mean ± SD of 3 biological replicas.

3.3 Discussion:

OS is a particularly aggressive cancer in which current treatment modalities result in a 5 year event free survival in 60-70% of patients [295, 334] [335]. Unfortunately there is a poor response to chemotherapy in a significant sub-group of OS patients and some patients have a high drug toxicity profiles [41]. In recent years, novel therapeutic approaches involving genome-wide epigenetic modification have been introduced [246]. Decitabine is one of the most popular in this class of new drugs and has been approved by the United States Food and Drug Administration [250]. The mechanisms of response to decitabine may vary in different patients, but are thought to include induction of senescence, differentiation and apoptosis [246]. The therapeutic potential of decitabine in OS tumors was first observed in the work presented in the previous chapter. Decitabine induced apoptosis and that was associated with induction of a number of apoptotic genes, one of which is *GADD45A*. To date, defects of the *GADD45* gene family have not been implicated in osteosarcoma (OS) oncogenesis, and the role of this pathway in regulating apoptosis in this tumor is unknown.

The GADD45 stress-response family has been shown to be inactivated by methylation in several types of tumors [330], [219]. The protein has been implicated in regulating genome stability [103], DNA damage response [324], DNA repair [329] [336],

apoptosis [106], and most recently, when overexpressed, DNA demethylation [337]. Despite the impact of *GADD45A* gene on all these processes, and its frequent methylation in some tumors, there is limited information regarding the role of *GADD45A* in the response to epigenetic modifications in general, and demethylation treatment in particular.

In this study we found that extensive methylation of the 5' CpG region of *GADD45A* was present in the MG63 and U2OS cell lines, and this epigenetic change was associated with reduced expression of *GADD45A* in OS. Furthermore, it was found that exposure of both OS cell lines to decitabine significantly decreased the methylated alleles to ~55% in this region of the gene. The relationship between loss of DNA methylation in this region, and elevated gene expression was demonstrated by a six-fold increase in both *GADD45A* mRNA and protein levels. The role of Gadd45a on cell cycle arrest is well established. The protein has been shown to play a role in G2-M checkpoint in response to DNA damage [105, 338, 339]. Gadd45a activates p53-dependent G2-M arrest providing a link between p53-dependent cell cycle checkpoint and DNA repair [338]. In this regard, it is noteworthy that p53 inactivation is one of the most common aberrations observed in human OS [340].

The role of Gadd45a in apoptosis is more complex than its role in cell cycle arrest. While *GADD45A* overexpression in both normal human cells and human cancer cells causes G2-M arrest [341] [338], *GADD45A* role in apoptosis induction in normal cells has been controversial. *GADD45A* induction of apoptosis has been observed more frequently in cancer cell lines than normal cells [106] [219]. For example, Gadd45a has

been shown to induce cell cycle arrest and fail to induce apoptosis in normal fibroblasts [38] Others, however, have shown Gadd45a dependent induction of apoptosis in normal epithelial cells [342].

In the present study we show that Gadd45a re-expression correlates with a significant induction of apoptosis in OS cells. The *in vivo* effects of decitabine treatment were studied by establishing xenografts and showing that tumor sections of xenografts from untreated control mice exhibited low nuclear staining for Gadd45a protein, while the nuclei from xenografts in decitabine treated mice exhibited much higher levels of Gadd45a protein. As with the *in vitro* studies, an increase in Gadd45a protein levels was associated with a significant increase in apoptosis.

To show the specificity for Gadd45a in decitabine-induced apoptosis, *GADD45A* mRNAs were disrupted using siRNA. This approach has been utilized previously in other cell types and inhibition of *GADD45A* led to disruption of functions including its ability to induce apoptosis [343] [344] [345]. *GADD45A* activation in OS was found to be central to the decitabine-induced apoptosis, and knockdown by siRNA demonstrated the specificity of this effect. This observation, however, does not eliminate the possibility of the involvement of the other apoptotic factors and pathways in the decitabine induced apoptosis in OS cells [332].

OS is known for having high levels of genomic instability with multiple chromosomal breakpoints, and increased incidence in genomic aberrations [56] [51] [53] [52]. Inhibition of the DNA damage response gene such as *GADD45A* by DNA hypermethylation could be one explanation on how OS cells escape apoptosis and

undergo survival, despite the numerous DNA breakpoints required to generate the complex karyotypes that characterize the OS genome. Interestingly Gadd45A has recently been shown to actively demethylate downstream target genes by promoting DNA repair [337] thus linking both processes. Finally, the findings of a potential role for *GADD45* repression by methylation in OS oncogenesis may encourage the development of novel therapeutic strategies that takes advantage of improved understanding of the genomics and epigenomics of this tumor.

CHAPTER FOUR

4 General conclusions and future directions

4.1 General conclusions

Currently, cancer chemotherapy is dominated by treatments that use cytotoxic agents which have had only a modest impact on mortality rates. This failure in part reflects the mechanism of action of current anticancer drugs which target proliferation, rather than specific abnormalities associated with tumor cells. Surgery remains the most important way of treating primary OS, and adjuvant chemotherapy plays an essential role in the control of metastasis. Unfortunately, one third of patients remain at high risk of eventual relapse. These patient groups, in particular, may benefit from investigations into agents, such as decitabine that may provide new opportunities for improved control and higher cure rates in OS.

This thesis is the first comprehensive analysis of the demethylation drug decitabine to modify global gene expression in OS cells. My approach led to the identification of methylation targets that may have therapeutic importance in OS. The analysis of U2OS cells after exposure to decitabine provided a link between genome-wide methylation and specific targets with therapeutic potential in these cells. Gene expression profiling by microarray analysis showed that 88 genes had significantly increase in expression after decitabine treatment, which represented ~ 0.6% out of the 14500 genes on the array. Although this global modulation of genes is at a level comparable to other studies, the findings of this study showed that six out of the 88 genes have been linked to apoptotic pathways. At the cellular level, decitabine treatment of

U2OS cells induced cell death and apoptosis. The findings of U2OS study suggest that the cellular effects detected are a result of up-regulation of the apoptotic genes. These cells were undergoing fine molecular modulations of their epigenome eventually leading to changes in gene expression resulting in the observed cell death and apoptosis *in vitro*.

Methylation-independent induction of gene expression by decitabine has been observed in a few studies indicating that genes without CpG islands may also respond to this drug. In my study, however, 63 (71%) of the 88 decitabine up-regulated genes possessed CpG-island at their 5' region, a proportion that is higher than that observed in other reports. This observation is particularly intriguing because it suggests that decitabine treatment of U2OS induced CpG-island associated genes more frequently than previously reported. In a subset of apoptotic genes I showed a significant increase in expression following decitabine treatment that was accompanied by significant loss of promoter methylation. This suggests the potential direct effect of decitabine on methylated CpG sequences demonstrating that the re-activation of these genes involves CpG-island demethylation by decitabine. 25 genes out of 88 (28 %) significantly induced genes had no apparent CpG island at their promoter region indicating that a smaller number of genes were induced independent of demethylation in the decitabine treated U2OS cells. Decitabine mechanism of action is not restricted to its demethylation capability and was reported to have effects on histone methylation and RB phosphorylation.

One of the decitabine-reactivated genes of importance in OS oncogenesis was *GADD45A*. Gadd45a induces G2/M arrest after DNA damage and induces apoptosis in

p53- dependent and independent manner. Because the effects of Gadd45a loss and p53 loss in mouse and human cells are strikingly similar, and resemble the genetic defects seen in OS tumors, and the findings that *GADD45A* re-activation associates with apoptosis induction in decitabine treated U2OS cells, the potential of *GADD45A* involvement in OS tumorigenesis was further investigated. In this study I found that extensive methylation of the 5' CpG region of *GADD45A* was present in the MG63 and U2OS cell lines, and this epigenetic change was associated with reduced expression of *GADD45A* in both cell lines. Furthermore, it was found that exposure of both OS cell lines to decitabine significantly decreased the methylated alleles to ~55% in the CpG-island of the gene.

The relationship between loss of DNA methylation in this region, and elevated gene expression was demonstrated by a six-fold increase in both *GADD45A* mRNA and protein levels. I also show that Gadd45a re-expression correlates with a significant induction of apoptosis in OS cells *in vitro* and in xenografts. To show the specificity for Gadd45a in decitabine-induced apoptosis, *GADD45A* mRNAs were disrupted using siRNA. This approach has been utilized previously in other cell types and inhibition of *GADD45A* led to disruption of its function including its ability to induce apoptosis. *GADD45A* activation in OS was found to be central to the decitabine-induced apoptosis, and knockdown by siRNA demonstrated the specificity of this effect.

Understanding the details of the various pathways identified by genome-wide demethylation in OS will identify additional opportunities for innovative OS therapies. Hypermethylation of pathways impacting on proliferation, apoptosis and OS

differentiation suggest that other therapeutic targets will emerge form this general approach. Targeting hypermethylation in U2OS by decitabine indicated the potential power of this drug for OS treatment. The findings from this study will encourage creative approaches to the development of novel and more effective epigenomic based therapeutic strategies, by taking advantage of our understanding of the genomics of this tumor. In addition, the concepts being addressed in this thesis have broader implications for more common classes of cancers also characterized by CIN.

4.2 Future Directions

The findings on methylation in cancer led to the re-evaluation of demethylation drugs *in vitro* and *in vivo*, resulting in FDA–approved drugs that are helping patients live longer with fewer side effects than conventional cytotoxic therapy. It seems likely that the field of epigenetic therapy will grow, with new drugs and new indications discovered through continued efforts in the laboratory and in the clinic. This thesis presents the initial genome-wide analysis of OS epigenetics. It shows the potential of decitabine use in the treatment of OS. It also reports specific targets for methylation silencing that could be informative to assess demethylation treatment or to serve as prognostic factors. The work presented in this thesis could be expanded further in order to translate the laboratory findings into clinical work where, ultimately, patients will benefit.

a) Demethylation therapy

The data in chapters 2 and 3 represent a proof-of-concept for epigenetic therapy using decitabine in OS cells. Decitabine produce cytotoxic effect that maybe an indication of possible remission or clinical improvements if tried on OS patients. A controlled clinical study using decitabine for a small number of OS patients (Phase II clinical trial) would facilitate evaluating the effectiveness of this drug on OS tumors. It will also allow one to determine the common side effects and risks of decitabine use, if exists, on OS patients. In addition, since decitabine shows the capability of inducing genes with roles in DNA damage response (*GADD45A*), a controlled study on a small number of OS patients that investigates the possible additive or synergistic antineoplastic effect of the current OS drugs (some of which are DNA damaging agents) and decitabine is of great importance. In these trials administration of the drugs in combination maybe designed to use lower doses of the current OS treatment drugs to reduce their high toxicity profile.

b) Combination of different epigenetic therapies

The literature indicates that decitabine and HDAC inhibitors, such as Trichostatin A, in combination are synergistic. This suggests that combination of these inhibitors may be an effective form of epigenetic therapy for OS. Initially, such possibility can be investigated in OS cell lines *in vitro* and in xenografts in the same manner as shown in this thesis. The objective would thus be to determine the synergistic antineoplastic activity of the two drugs on human OS cells and in xenografts. This study could also be taken to the next level by testing these drugs individually and in combination on patient

primary tumors after implanting them under the renal capsule of mice. The dosage and schedule of administration of the epigenetic drugs may then have application for future clinical trials on the treatment of OS with these agents.

c) Apoptosis as a therapeutic pathway in OS

Inhibition of the apoptotic gene *GADD45A* by DNA hypermethylation could be one of the mechanisms of how OS cells evade apoptosis and undergo survival, despite exhibiting high chromosomal re-arrangements. Other genes with roles in apoptosis and differentiation were also identified in this study. This indicates that these genes and possibly many others that are involved in these pathways are of possible importance for demethylation therapy in OS. Detailed analyses of these pathways after demethylation treatment is possible through the use of apoptotic pathway specific PCR Arrays. PCR Arrays are pathway specific arrays for real-time PCR analysis of a panel of pathway specific genes that utilizes SYBR-Green pre-optimized primer assays. mRNA from OS patient samples can be analyzed on this platform to identify the expression levels of multiple genes simultaneously. In addition, tumor samples can be evaluated after epigenetic treatment to determine tumor response to treatment at the molecular level involving multiple genes.

d) Role of GADD45A locus in OS oncogenesis

My studies also provide insights about acquisition of CIN in OS. It is possible that the reduced expression of the DNA damage response function of *GADD45A* by DNA hypermethylation could be an early event in tumorigenesis. Such "epigenetic mutations"

would allow OS precursors to escape apoptosis and undergo continued proliferation. This may account for the high degree of complexity of OS karyotypes. The genomic locus of *GADD45A* gene on chromosome 1p31 was not studied previously for genomic re-arrangements in OS. *GADD45A* locus in OS presents an appropriate genomic site to study the relationship between chromosomal re-arrangements and DNA methylation. One way to address this issue is by utilizing fluorescence *in situ* hybridization of several probes from *GADD45A* locus and its neighboring genomic sites on OS tissue microarrays.

Implicating *GADD45A* in the therapeutic response to decitabine in this study indicates the potential of using this gene as an indicator for response to epigenetic treatment. However, one necessary step to be undertaken is to analyze the mRNA and protein expression of GADD45A in a large number of OS tumors. Correlating the mRNA and protein expression to the methylation status in patient samples would provide additional proof-of- concept to the *in vitro* and *in vivo* data. Other genes in the apoptosis pathway that were reported in this study could also be analyzed for expression and methylation in OS patient samples. When such studies are performed, OS epigenetic biomarkers and epigenetic prognostic factors in future clinical trials maybe identified.

CHAPTER FIVE

5 Methodology

5.1 Cell line culture and treatment

The human OS cell lines U2OS (ATCC # HTB-96) and MG63 (ATCC # CRL-1427) and cervical adenocarcinoma (HeLa) cell lines (ATCC # CCL-2) were purchased from American Type Culture Collection ATCC (Rockville, MD) and maintained in alpha-Minimum Essential Medium (alpha-MEM) supplemented with 10% heat inactivated Fetal Bovine Serum and 2 mM L-Glutamine. Normal osteoblasts are primary osteoblasts from the hip bone of a normal male donor that were purchased from PromoCell (Heidelberg, Germany, Catalogue # C-12760) and maintained in medium provided by the manufacturer and used at culture passage 3. Treatment with decitabine was performed as described by Liang et al [287]; $5x10^5$ cells were plated in 56 cm^2 culture plates with 10 ml growth medium. 12 hours after plating they were treated with freshly prepared decitabine (Sigma Chemical Co., St Louis, MO) dissolved in growth medium to a final concentration of 1 μM without changing the medium. The use of a single dose decitabine to significantly reactivate methylation silenced genes was reported in bladder cancer cells [287]. Other treatment schedules analyzing demethylation in lung and head and neck tumors have involved including fresh decitabine when culture media is replaced [346]. Control (medium only) cultures were maintained and processed over the same period of time under the same condition as the decitabine treated cells. Three days after initiating the treatment, cells where harvested by trypsinization for DNA extractions, and for Propidium Iodide (PI) or Hoechst 33342 staining. For RNA and

protein extractions cells were washed 3 times by Phosphate Buffer Saline (PBS) and lysed in the culture vessel using TRizol (RNA) or RIPA (protein) agents.

To establish growth curves for U2OS cells with or without 1 μM decitabine, cells were plated at $5x10^5$ cells/56 cm^2 culture plates with 4 mm^2 grids. The cells were allowed to attach to the surface of the plates for 12 hours before the start of the treatment. Adherent cells were counted in 2 independent cultures in multiple 4 mm^2 grids every 12 hours after plating and the experiment was repeated after culturing the cells for 5 passages. When cell growth was near confluent, the cells were trypsinized, re-suspended in growth medium (10% serum) and a final viable cell count was determined using Vi-CELL™ XR (Beckman Coulter, Fullerton, CA) after Trypan Blue staining.

5.2 In vivo studies: U2OS xenograft and treatment

Six- to eight-week old male immune-deficient NOD-SCID and Rag-2M mice were bred and maintained by the Animal Resource Centre at the British Colombia Cancer agency, Vancouver, Canada. U2OS cells, in general, were considered as non-tumorigenic in mice while grafting the cells subcutaneously or orthotopically [273]. As such, in order to establish their xenografts, U2OS cells were grafted under the renal capsule, a site proven to be an excellent site for tumor engraftment [280] [281] [282]. Briefly U2OS cells were cultured in alpha-MEM and washed in growth medium containing 20% FBS. The viable cells were counted after trypan blue staining and $2x10^6$ cells were pelleted, re-suspended and grafted beneath the renal capsule of adult male SCID mice as previously

described by Wang Y et al [280]. After 5 months, a well-grown xenograft was selected for re-grafting to establish multiple stable U2OS xenografts under the kidney capsules of NOD-SCID mice (two per kidney per mouse = 4 grafts per mouse). The re-grafted U2OS xenograft had a 100% take rate, with a doubling time of ~10 days and exponential growth phase starting after ~ 2 of tumor growth doubling. After 5 tumor growth doublings the xenografts were surgically removed from the mice and cut into approximately 4 mm^3 portions then were re-grafted under the renal capsules of 6 male Rag-2M mice (two per kidney per mouse = 4 grafts per mouse). Four weeks after grafting (~2-3 doubling of tumor growth), the host mice were divided into two groups. One group (3 mice) was given decitabine (2.5 mg/kg body weight) dissolved in saline (0.9% w/v NaCl), intraperitoneally on days 29, 31 and 33. The other group (3 mice) was given saline alone as a treatment control over the same schedule. On day 37, mice from both groups were sacrificed. Tumor volumes were measured using a digital caliper, recorded and expressed in mm^3, using the formula: volume (mm^3) = (0.52) X length (mm) X width (mm) X height (mm). Data were presented as means ± Standard Deviation (SD) and student t-test was used to analyze the difference between the two treatment groups. The xenograft tissues were then snap frozen, or fixed in formalin and prepared in paraffin and sectioned according to standard procedures [280].

5.3 Immunohistochemistry and image analysis

5.3.1 Global methylation analysis using 5-methylcytidine antibody

Xenograft tissue sections were de-paraffinized using xylene and re-hydrated in a series of alcohols. The tissue sections were then incubated at room temperature (RT) in 3% H_2O_2 in PBS for 10 minutes to inactivate endogenous peroxidase. Following incubation the slides were washed 3 times in PBS for 3 minutes each. Antigen retrieval was obtained by heating in a microwave at maximum heat for 20 minutes in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) and cooling for 20 minutes at RT. Slides were again washed 3 times in PBS for 3 minutes each. The slides were blocked (30 minutes in a humid chamber at RT) with serum to reduce non-specific binding. Serum was removed from the slides and the slides were then incubated with the primary antibody 5-methylcytidine (5-mc-Ab) (Eurogentec, San Diego, CA) at 1:500 dilution at 4°C overnight.

Following incubation the slides were washed 3 times in PBS for 3 minutes each. The slides were then incubated with a secondary antibody [Polyclonal rabbit anti-mouse immunoglobulins/biotinylated rabbit F(ab')2; Dako] for 30 minutes at RT, followed by 3 washes in PBS for 3 minutes each. The slides were then incubated with StreptABCComplex/HRP (Dako, Glostrup, Denmark) for 30 minutes in a humid chamber at RT, followed by 3 washes in PBS for 3 minutes each. A 3,3'diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA) was used for detection and hematoxylin was used for counterstain. The slides were then dehydrated and mounted.

5.3.2 Analysis Gadd45a protein induction using Gaadd45a antibody

Xenograft tissue sections and sections from pelleted and paraffin embedded HeLa cells were de-paraffinized using xylene and re-hydrated in a series of alcohols. The tissue sections were then incubated at room temperature (RT) in 3% H202 in PBS for 10 minutes to inactivate endogenous peroxidase. Slides were then washed 3x in PBS for 3 minutes each (PBS wash). Antigen retrieval was performed at 95°C for 30 minutes in 10 mM Sodium Citrate buffer (pH 6.0), and cooling for 5 minutes in a running water bath, followed by the PBS wash. The slides were blocked using 3% skim milk for 30 minutes in a humid chamber at room temperature, which was followed by primary anti-human Gadd45a-Ab (Abnova, Taipei City, Taiwan) exposure (1:500 dilution) at 4°C overnight followed by PBS wash. After the PBS wash, slides were incubated with secondary antibody [Polyclonal rabbit anti-mouse immunoglobulins/biotinylated rabbit F(ab')2; Dako] for 30 minutes at RT, followed by the PBS wash. StreptABCComplex/HRP (Dako, Glostrup, Denmark) was applied for 30 minutes in a humid chamber at RT, followed by the PBS wash. A 3,3'diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA) was used for detection and hematoxylin was used for counterstain. To confirm that each section retained immunoreactivity, a positive finding for the Ki67 antibody stain indicated that adjacent xenograft sections were positive and informative.

5.3.3 Image analysis

Whole sections from xenografts were scanned by ScanScope CS (Aperio technologies, Vista, CA). The slides were digitized to 20x magnification (~ 0.5 microns/pixel). Images were then viewed with Aperio's image viewer software (ImageScope), which allows performing quantitative analysis of stain intensity on snapshots from the sections. Five to ten ~ 0.3 mm^2 snapshots (each containing 3,000 to 5,000 cells) were analyzed per section using the following parameters: compression quality = 30, and color saturation threshold = 0.04. Positivity thresholds were150 to 220 = high positive, 100 to 150 = low positive, and 0 to 100 = negative. Descriptive analysis such as mean and standard deviation for 5-mc-ab immunostaining intensity were calculated from the positivity indices of the scanned images (Positivity index = number of total positive nuclei /number of total positive + negative per image) after normalization to negative controls (no 5mc-Ab).

For Gadd45a-Ab intensity were calculated from the positivity indices of the scanned images relative to positivity indices in the positive control (UV irradiated HeLa cells) after normalization to negative controls (no Gadd45a Ab). Comparison between control and decitabine-treated sections was done using the student t-test and $p< 0.05$ was considered significant.

5.4 Histopathological analysis and TUNEL assay

Formalin-fixed paraffin-embedded xenografts tissues from the control (no treatment) and decitabine treated groups were stained with hematoxylin and eosin (H&E). Sections were assessed blindly and extracellular matrix was defined as eosinophilic osteoid-like material surrounding individual cells and small clusters of 3-5 cells, and the percentage of tumor with osteoid was then calculated. Mitotic counts were performed in areas with the highest mitotic rate, and ten high-powered fields (x 400) were assessed per section.

In situ hybridization for terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed on paraffin sections as recommended by the manufacturer (Ventana Medical Systems, Tucson, AZ). Scoring of the sections was performed using Simple PCI analytical software (Nikon, Tokyo, Japan). Sections were examined and the most intense areas of staining were photographed using a DXM1200 digital camera (Nikon) at a power of x200. The digital image was then scanned using the Simple PCI program and the numbers of positive and negative nuclei were obtained. Control and treatment images were all photographed at a uniform brightness, and all images were subjected to uniform binary image modification and size calibration prior to counting by Simple PCI. The positivity index was obtained by dividing the number of positive nuclei by the total number of nuclei (positive + negative). The number of nuclei counted was always over 1000, and ranged from 1100 to 2000. Positivity indices from the 2 treatment groups (control and decitabine treated) were compared by Student's t-test and a p-value of <0.05 was considered significant and shown in Figure 2.2.2 B V and VI.

5.5 Affymetrix expression analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Germany) after lysing the cells in the culture vessel. RNA was obtained from two replicate experiments of U2OS cells at day 3 after treatment with 1 μM decitabine or medium alone (control). In each experiment RNA yields were pooled from two independent cultures per treatment arm. For each case, 10 μg of RNA was labeled and hybridized to the Affymetrix HG-U133A GeneChips using the manufacturer's protocol (Affymetrix, Santa Clara, CA) by the Centre of Applied Genomics at the Hospital for Sick Children (Toronto, Canada). Data were extracted using the Microarray Suite (MAS) version 5.0 (Affymetrix) and linearly scaled to achieve an average intensity of 150 across each chip. The candidate gene list obtained from the MAS 5.0-extracted data was selected by eliminating genes that were not present in at least one experiment. The arrays were subjected to a pair wise comparison using MAS 5.0, with signal intensities from the no-treatment cells as the baseline. The statistical significance for the change of expression for each probe set between the decitabine treated and control was calculated by the MAS 5.0 software. The criteria for gene selection for real-time expression validation analysis was based on the statistically significant up-regulation ($p<0.0025$) and fold change of ≥ 2 for expression after decitabine treatment. The gene list was annotated based on the NetAffx data-base [347] and further verified using the Human Genome Browser data base [348]. All the raw data for expression arrays is available in [349] under the series record number (GSE7454).

5.6 In silico analysis of CpG-island association, gene annotation, and pathway enrichment

The criteria for a CpG-island was based on those outlined by Takai and Jones [350], where the GC $\geq$ 55%, Obs/Exp $\geq$ 0.65, and length > 300 bp which was reported to exclude most Alu-repetitive elements. We identified the genes that harbored CpG-island within a 2000 bp window upstream or downstream from the transcription start site based Human Genome Browser data base [348]. To be certain that there were no CpG island closer to the TSS and gene promoter regions, we submitted the sequences of interest (including a 2000 bp window upstream and downstream from TSS) to the CpG search engine available in reference [350] and verified that there was no CpG islands that are closer to TSS for the genes we tested. Up-regulated genes with CpG-island associations were further analyzed through the Microarray Literature-based Annotation tool MILANO [351] to look for evidence of epigenetic modifications in the literature. MILANO is a web-based tool that allows annotation of lists of genes derived from microarray results by user defined terms [351]. Using MILANO we searched for literature associations between our list of genes and the terms 'epigenetics', 'methylation' 'chromatin modification' 'cancer', and 'disease'. To identify the putative functional pathways for each gene list, we used the functional annotation enrichment tool. This tool utilizes the Gene Ontology database and uses GO Terms to identify enriched biological themes in the gene lists [352]. The Fisher Exact test was applied to determine the significance in the proportions of genes falling into a certain pathway in each gene list. We used this tool to

look for enriched pathways of up- or down- regulated genes with CpG-island associations from the gene lists from the cell lines.

5.7 Expression validation using reverse transcription and quantitative real-time PCR

Total RNA from xenografts and osteoblasts was extracted using the TRIzol reagent method. 1 ml of TRIzol (Invitrogen, Osaka, Japan) was used for every 50-100 mg of tumor tissue and homogenized in an RNase free environment. Chloroform was then added (200 µl for each 1 ml TRIzol) and the samples were centrifuged at high speed for 15 minutes at 4°C. The aqueous layer was then transferred into a new tube and RNA was precipitated with iso-propanol followed by one wash using 70% ethanol. The RNA precipitate was then dissolved in 10-15 µl of RNAse free water and analyzed for quantity and quality using a spectrophotometer. A two-step reverse transcription-PCR procedure was performed. Total RNA was reverse transcribed using the GeneAmp kit (Applied Biosystems; ABI, Foster City, CA). 20 ng of the resulting cDNA was then used in the real-time PCR step. Six genes were tested by real-time PCR including: growth arrest and DNA-Damage inducible, alpha (GADD45A), heat chock70KDA protein 9b (HSPA9B), parkc apoptosis wt1-regulatort (PAWR), programmed cell death 5 gene (PDCD5), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), tumor necrosis factor, alpha-induced protein 3 (TNFAIP3). We used the TaqMan primers (ABI) for all the genes that we tested (primer information is provided in

Table 5.1). All real-time PCR assays were performed in triplicate in a 96-well plate using the 7900 Sequence Detector System (ABI) according to the manufacturer's protocol. Data analysis was performed using the Sequence Detector System (SDS) software (ABI) and the results were expressed as fold-change in relative mRNA expression level, calculated using the ΔΔCt method with β-actin (ACTB) as the reference gene and the non-treated cells as baseline. The validation was carried out on RNA from three replicate experiments of U2OS cells, three decitabine-treated U2OS xenograft tumors (Xeno- 1, 2 and 3), three no-treatment (control) U2OS xenograft tumors (Xeno- 4, 5, and 6) and three replicate experiments of NHOst.

Table 5.1: TaqMan assays from Applied Biosystems (ABI, Foster City, CA) that were used for Real-Time PCR.

Gene	TaqMan assay#
GADD45A	Hs00169255_m1
HSPA9B	Hs00269818_m1
TNFAIP3	Hs00234713_m1
PAWR	Hs00169332_m1
NFKBIA	Hs00153283_m1
PDCD5	Hs00270435_m1
beta-Actin	4352935E

5.8 Quantitative-bisulfite pyrosequencing

Quantitative Bisulfite Pyrosequencing for CpG islands (Pyro Q-CpG) is a sequencing-based analysis of DNA methylation that quantifies multiple CpG sites per amplicon using Pyro Q-CpG software. 2 μg of DNA from the control and decitabine treatment were bisulfite-treated using the Zymo DNA Methylation Kit (Zymo Research,

Orange, CA). Bisulfite-treated DNA was amplified by PCR then sequenced according to the manufacturer's protocol (Biotage, Kungsgatan, Sweden). The target sequences inside the CpG-islands of the candidate genes and the primer sequences are shown in **Table 5.2**. The percentage of C content (methylated alleles) versus T content (unmethylated alleles) is calculated by the Pyro-Q-CpG software for each CpG position in each sample. Analysis was performed on DNA samples from 3 replicate experiments of U2OS and MG63 cells in vitro and six U2OS xenograft tumors; three decitabine treated (Xeno-1, Xeno-2 and Xeno-3), and three saline (control) treated (Xeno-4, Xeno-5 and Xeno-6). Universally methylated DNA was used as a methylation positive control. DNA isolated from early embryos (Biotage, Kungsgatan, Sweden) was used for methylation negative control. DNA from low-passage normal human osteoblasts (PromoCell, Germany) was used for experiment control.

Table 5.2: Methylation CpG Pyrosequencing primers.

		Amplicon primers		Sequencing primers 5'---3'
GENE	Sequence type	Forward 5'---3'	Reverse 5'---3'	
GADD45A	original	CTGGCAGCCTGTGGCAGGGGCACT	AGGATGGATAAGGTGGGGGAT	CTTGGGCGTGCAGGGGTCAT
	converted*	TTGGTAGTTTGTGGTAGGGGTATT	AGGATGGATAAGGTGGGGGAT	TTTGGGYGTGTAGGGGTTAT
HSPA9B	original	ACCTCCAACCACGTGGGGTGAGGG	ATGATGGTTGGAGAAAGCCTGCC	GGGGCGGGGGTTGGTCACT
	converted*	ATTTTTAATTAYGTGGGGTGAGGG	ATGATGGTTGGAGAAAGTTTGTT	GGGGYGGGGGTTGGTTATT
PDCD5	original	GGGCCTGGATCCAAGCACAATCTCAGC	GGGAAGCTTGGATGGATCACA	CTCAGCTTTTGGAGCCAGCA
	converted*	GGGTTTGGATTTAAGTATAATTTTAGT	GGGAAGTTTGGATGGATTATA	TTTAGTTTTTGGAGTTAGTA
PAWR	original	GGGGGCGGGGCCTCACTCTG	GGGGGYGGGGTTTTATTTTG	GGCCTCACTCTGCGATATAACTC
	converted*	GGGGGYGGGGTTTTATTTTG	TTYGGATAGTATGAGTTTTATTA	GGTTTTATTTTGYGATATAATT
NFKBIA	original	GGTGAGGGCTGCGGAGGAA	CGCGGCGCCCTATAAACG	AGCGTTCGGGGCGGTGCA
	converted*	GGTGAGGGTTGTGGAGGAA	TGTGGTGTTTTATAAATG	AGYGTTYGGGGYGGTGYA
TNFAIP3	original	AGAGCCGGCCCGAGGCCTAACCGG	AAGCTGCAGCTGACTGGTGAAGA	CCTTGACCAGGACTTGGGACTTT
	converted*	AGAGTTGGTTTGAGGTTTAATTGG	AAGTTGTAGTTGATTGGTGAAGA	YYTTGAYYAGGAYTTGGGAYTTT

* Bisulfate converted

5.9 Knockdown by Gadd45a-siRNA

Silencer® pre-designed siRNAs for human *GADD45A* (siRNA ID: 146174) and control non-targeting siRNA (ctRNA) were obtained from Ambion, Foster City, CA, USA. Transfection of OS cells with siRNA was performed using siPORTTM Amine transfection agent (Ambion, cat # 4502) as per manufacturers' recommendations, in 6-well plates. Cells (2x105) were suspended in 2 ml medium with transfection agent alone or with transfection agent plus siRNA (60 nM), or ctRNA (60 nM), prior to plating. Decitabine treatments were done 12 hours after plating. All experiments were performed in triplicate and knockdown efficiency was determined by western blotting. Cells from triplicate experiments were assessed for apoptotic nuclei using Hoechst 33342 stain. Same knockdown experiments were repeated on U2OS cells using two other Silencer® pre-designed siRNAs for human GADD45A (siRNA IDs:2659 and 2746 From Ambion) for validation against off-target effect. All target sequences for the siRNAs used in this study are within exon 4 of *GADD45A* gene.

5.10 Transient transfection of GADD45A

2 μg of pCMV(GADD45A) transient transfection vectors from TrueClone™ (OriGene, Rockville, MD, USA) with 10 μl of Lipofectamine 2000 from Invitrogen (Burlington, ON, Canada) was used in the transient transfection experiments. Cells (2x105) were suspended in 2 ml medium with transfection agent alone or with transfection agent plus pCMV(GADD45A), or the empty pCMV vector (TrueCloneTM) prior to plating. Cells were assayed by western blotting and Hoechst 33342 and all experiments were performed in triplicate.

5.11 Western blot analysis

Cells were lysed in TC plates on ice using Radioimmunoprecipitation (RIPA) Buffer. Lysates were transferred to 1.5 ml tubes, sonicated briefly (with tubes on ice), and centrifuged at 15,000 rpm for 15 minutes at 4°C. Total protein was quantitated using Bradford dye reagent (BIO-RAD, Hercules, CA, USA). 50 μg of total protein/well was separated on acrylamide gel, and transferred to PVD membranes (BIO-RAD) overnight at 25 volts at 4°C. Membranes were then blocked with 5% BSA for 8 hours at 4°C, followed by primary antibody incubation for 16 hours. Primary antibodies used were mouse monoclonal anti human Gadd45a-Ab (Abnova, Taipei City, Taiwan) at 1:12000 dilution and rabbit polyclonal anti human beta-actin-ab (Abcam, Cambridge, MA, USA) at 1:24000 dilution for loading reference. After primary antibody, the blots were washed using 5 ten-minute washes with TBS-Tween 20 and a final one wash in TBS without Tween 20. The blots were then incubated with perioxiase-labeled secondary antibody (ECL-Plus kit, Amersham, Buckinghamshire, UK) as per manufacturer instructions, and scanned on the Typhoon 9410 scanner (Amersham) at normal sensitivity, 600 laser power, and 200 micron resolution. Stripping of the blots was done using a solution that contains: 20 ml of10% SDS, 12.5 ml of 0.5 M Tris HCL (ph 6.8), 0.8 beta-mercaptoethanol, and 67.5 ml dd H2O for 45 minutes at 50°C. Stripping was followed by multiple washes in TBST. Quantitation was performed using Image-J software on blots from 3 replicates and significance was estimated using t-test at $p < 0.05$ using beta actin as a reference control.

5.12 Hoechst 33342 staining for apoptotic nuclei

The cells were harvested with trypsin, and re-suspended in 1 ml PBS. Staining was performed in 5 ml of 4% formalin solution with Hoechst 33342 dye (0.01 μg/ μl final concentration). Aliquots of the samples were bar coded prior to spreading the cells on glass slides. Hoechst stained cells were blindly analyzed using a fluorescence microscope. One hundred cells were scored per slide and the percentage of stained (apoptotic nuclei) out of total was recorded. Experiments were done in triplicate and statistics on 3 replicas were determined using student t-test at $p < 0.05$.

References:

1. **Freeman L: VIII. Periosteal Osteosarcoma of Upper Extremity of Femur; Ligation of Common Iliac Artery, with Amputation at Hip-Joint and Removal of Portion of Pelvis, etc.; Recovery, with Freedom from Recurrence at End of nearly Sixteen Months.** ***Annals of surgery*** **1901, 33(3):318-322.**
2. **Capasso LaDT, G: The Antiquity of Osteosarcoma.** ***International Journal of Osteoarchaelogy*** **1996, 6:512-514.**
3. **Miller SL, Hoffer FA: Malignant and benign bone tumors.** ***Radiologic clinics of North America*** **2001, 39(4):673-699.**
4. **Hayden JB, Hoang BH: Osteosarcoma: basic science and clinical implications.** ***The Orthopedic clinics of North America*** **2006, 37(1):1-7.**
5. **Picci P: Osteosarcoma (osteogenic sarcoma).** ***Orphanet journal of rare diseases*** **2007, 2:6.**
6. **Mills R: Tumors of the bones and joints. Washington DC: American Forces Institute of Pathology (AFIP); 1993.**
7. **Canada CCSNCIo: Cancer in Children and youth.** ***Canadian Cancer Statistics*****:67-69.**
8. **Dahlin DCUKK: Bone Tumors: General aspects and data on 8,542 cases. In., 4t edn: Springfield. III: Charles C. Thomas; 1986: 269-307.**
9. **Parkin DM, Stiller CA, Nectoux J: International variations in the incidence of childhood bone tumours.** ***International journal of cancer*** **1993, 53(3):371-376.**
10. **Anbari KK, Ierardi-Curto LA, Silber JS, Asada N, Spinner N, Zackai EH, Belasco J, Morrissette JD, Dormans JP: Two primary osteosarcomas in a patient with Rothmund-Thomson syndrome.** ***Clinical orthopaedics and related research*** **2000(378):213-223.**
11. **Hicks MJ, Roth JR, Kozinetz CA, Wang LL: Clinicopathologic features of osteosarcoma in patients with Rothmund-Thomson syndrome.** ***J Clin Oncol*** **2007, 25(4):370-375.**
12. **Fuchs B, Pritchard DJ: Etiology of osteosarcoma.** ***Clinical orthopaedics and related research*** **2002(397):40-52.**
13. **Khayat CM, Johnston DL: Rhabdomyosarcoma, osteosarcoma, and adrenocortical carcinoma in a child with a germline p53 mutation.** ***Pediatric blood & cancer*** **2004, 43(6):683-686.**
14. **Hansen MF, Seton M, Merchant A: Osteosarcoma in Paget's disease of bone.** ***J Bone Miner Res*** **2006, 21 Suppl 2:P58-63.**
15. **Bertoni F, Bacchini P: Classification of bone tumors.** ***European journal of radiology*** **1998, 27 Suppl 1:S74-76.**
16. **Furlow B: Radiography of bone tumors and lesions.** ***Radiologic technology*** **2001, 72(5):455-469; quiz 470-452.**
17. **Klein MJ, Siegal GP: Osteosarcoma: anatomic and histologic variants.** ***American journal of clinical pathology*** **2006, 125(4):555-581.**

18. Meister P, Konrad E, Lob G, Janka G, Keyl W, Sturz H: Osteosarcoma: histological evaluation and grading. *Arch Orthop Trauma Surg* 1979, 94(2):91-98.
19. Marcove RC, Mike V, Hajek JV, Levin AG, Hutter RV: Osteogenic sarcoma under the age of twenty-one. A review of one hundred and forty-five operative cases. *The Journal of bone and joint surgery* 1970, 52(3):411-423.
20. Mendelsohn ML, Shackney SE: The growth kinetics of solid tumors: summary report of meeting. *Cell and tissue kinetics* 1970, 3(4):405-414.
21. Shackney SE, McCormack GW, Cuchural GJ, Jr.: Growth rate patterns of solid tumors and their relation to responsiveness to therapy: an analytical review. *Annals of internal medicine* 1978, 89(1):107-121.
22. Ferguson WS, Goorin AM: Current treatment of osteosarcoma. *Cancer investigation* 2001, 19(3):292-315.
23. Gebhardt MC, Ready JE, Mankin HJ: Tumors about the knee in children. *Clinical orthopaedics and related research* 1990(255):86-110.
24. Raymond AK AAKS: Convential osteosarcoma. In World Health Organization Classification of Tumours. Pathology & Genetics, Tumours of Soft Tissue and Bone, Fletcher CDM, Unni KK & Mertens F editors. pp. 264-270: IARC Press, Lyon; 2002.
25. Ogose A, Morita T, Emura I, Nemoto K, Hirata Y: Osteosarcoma metastatic to the kidneys without lung involvement. *Japanese journal of clinical oncology* 1999, 29(8):395-398.
26. Panizo-Santos A, Sola I, Lozano M, de Alava E, Pardo J: Metastatic osteosarcoma presenting as a small-bowel polyp. A case report and review of the literature. *Archives of pathology & laboratory medicine* 2000, 124(11):1682-1684.
27. Misra A, Misra S, Chaturvedi A, Srivastava PK: Osteosarcoma with metastasis to orbit. *The British journal of ophthalmology* 2001, 85(11):1387-1388.
28. Uribe-Botero G, Russell WO, Sutow WW, Martin RG: Primary osteosarcoma of bone. Clinicopathologic investigation of 243 cases, with necropsy studies in 54. *American journal of clinical pathology* 1977, 67(5):427-435.
29. Goorin AM, Abelson HT, Frei E, 3rd: Osteosarcoma: fifteen years later. *The New England journal of medicine* 1985, 313(26):1637-1643.
30. Ferrari S, Palmerini E: Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma. *Current opinion in oncology* 2007, 19(4):341-346.
31. Bacci G, Lari S: Current treatment of high grade osteosarcoma of the extremity: review. *Journal of chemotherapy (Florence, Italy)* 2001, 13(3):235-243.
32. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, Pritchard J, Malpas JS, Baker AR, Kirkpatrick JA *et al*: The effect of adjuvant chemotherapy on relapse-free survival in patients with

osteosarcoma of the extremity. *The New England journal of medicine* 1986, 314(25):1600-1606.

33. Eilber F, Giuliano A, Eckardt J, Patterson K, Moseley S, Goodnight J: Adjuvant chemotherapy for osteosarcoma: a randomized prospective trial. *J Clin Oncol* 1987, 5(1):21-26.
34. Link MP, Goorin AM, Horowitz M, Meyer WH, Belasco J, Baker A, Ayala A, Shuster J: Adjuvant chemotherapy of high-grade osteosarcoma of the extremity. Updated results of the Multi-Institutional Osteosarcoma Study. *Clinical orthopaedics and related research* 1991(270):8-14.
35. Meyers PA, Schwartz CL, Krailo M, Kleinerman ES, Betcher D, Bernstein ML, Conrad E, Ferguson W, Gebhardt M, Goorin AM *et al*: Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. *J Clin Oncol* 2005, 23(9):2004-2011.
36. Ferrari S, Smeland S, Mercuri M, Bertoni F, Longhi A, Ruggieri P, Alvegard TA, Picci P, Capanna R, Bernini G *et al*: Neoadjuvant chemotherapy with high-dose Ifosfamide, high-dose methotrexate, cisplatin, and doxorubicin for patients with localized osteosarcoma of the extremity: a joint study by the Italian and Scandinavian Sarcoma Groups. *J Clin Oncol* 2005, 23(34):8845-8852.
37. Oeffinger KC, Hudson MM: Long-term complications following childhood and adolescent cancer: foundations for providing risk-based health care for survivors. *CA: a cancer journal for clinicians* 2004, 54(4):208-236.
38. Kremer LC, van Dalen EC, Offringa M, Voute PA: Frequency and risk factors of anthracycline-induced clinical heart failure in children: a systematic review. *Ann Oncol* 2002, 13(4):503-512.
39. Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G: Primary bone osteosarcoma in the pediatric age: state of the art. *Cancer treatment reviews* 2006, 32(6):423-436.
40. McTiernan A, Meyer T, Michelagnoli MP, Lewis I, Whelan JS: A phase I/II study of doxorubicin, ifosfamide, etoposide and interval methotrexate in patients with poor prognosis osteosarcoma. *Pediatric blood & cancer* 2006, 46(3):345-350.
41. Patel SJ, Lynch JW, Jr., Johnson T, Carroll RR, Schumacher C, Spanier S, Scarborough M: Dose-intense ifosfamide/doxorubicin/cisplatin based chemotherapy for osteosarcoma in adults. *Am J Clin Oncol* 2002, 25(5):489-495.
42. Chou AJ, Gorlick R: Chemotherapy resistance in osteosarcoma: current challenges and future directions. *Expert review of anticancer therapy* 2006, 6(7):1075-1085.
43. Lamoureux F, Trichet V, Chipoy C, Blanchard F, Gouin F, Redini F: Recent advances in the management of osteosarcoma and forthcoming therapeutic strategies. *Expert review of anticancer therapy* 2007, 7(2):169-181.

44. Mertens F, Mandahl N, Orndal C, Baldetorp B, Bauer HC, Rydholm A, Wiebe T, Willen H, Akerman M, Heim S *et al*: Cytogenetic findings in 33 osteosarcomas. *International journal of cancer* 1993, 55(1):44-50.
45. Fletcher JA, Gebhardt MC, Kozakewich HP: Cytogenetic aberrations in osteosarcomas. Nonrandom deletions, rings, and double-minute chromosomes. *Cancer genetics and cytogenetics* 1994, 77(1):81-88.
46. Hoogerwerf WA, Hawkins AL, Perlman EJ, Griffin CA: Chromosome analysis of nine osteosarcomas. *Genes, chromosomes & cancer* 1994, 9(2):88-92.
47. Lopez-Gines C, Carda-Batalla C, Lopez-Terrada L, Llombart-Bosch A: Presence of double minutes and monosomy 17p in xenografted human osteosarcomas. *Cancer genetics and cytogenetics* 1996, 90(1):57-62.
48. Tarkkanen M, Karhu R, Kallioniemi A, Elomaa I, Kivioja AH, Nevalainen J, Bohling T, Karaharju E, Hyytinen E, Knuutila S *et al*: Gains and losses of DNA sequences in osteosarcomas by comparative genomic hybridization. *Cancer research* 1995, 55(6):1334-1338.
49. Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, Maale GE, Spanier S, Neff JR: Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. *Cancer genetics and cytogenetics* 1997, 95(1):74-87.
50. Boehm A, Squire, J. A., Bayani, J., Nelson, M., Neff, J., and Bridge, J. A. : Cytogenetic Findings in 36 Osteosarcomas Specimns and Review of the Literature. *Pediatric Pathology and Molecular Medicine* 2000, 19:359-376.
51. Al-Romaih K, Bayani J, Vorobyova J, Karaskova J, Park PC, Zielenska M, Squire JA: Chromosomal instability in osteosarcoma and its association with centrosome abnormalities. *Cancer genetics and cytogenetics* 2003, 144(2):91-99.
52. Zielenska M, Marrano P, Thorner P, Pei J, Beheshti B, Ho M, Bayani J, Liu Y, Sun BC, Squire JA *et al*: High-resolution cDNA microarray CGH mapping of genomic imbalances in osteosarcoma using formalin-fixed paraffin-embedded tissue. *Cytogenetic and genome research* 2004, 107(1-2):77-82.
53. Squire JA, Pei J, Marrano P, Beheshti B, Bayani J, Lim G, Moldovan L, Zielenska M: High-resolution mapping of amplifications and deletions in pediatric osteosarcoma by use of CGH analysis of cDNA microarrays. *Genes, chromosomes & cancer* 2003, 38(3):215-225.
54. Lim G, Karaskova J, Vukovic B, Bayani J, Beheshti B, Bernardini M, Squire JA, Zielenska M: Combined spectral karyotyping, multicolor banding, and microarray comparative genomic hybridization analysis provides a detailed characterization of complex structural chromosomal rearrangements associated with gene amplification in the osteosarcoma cell line MG-63. *Cancer genetics and cytogenetics* 2004, 153(2):158-164.
55. Zielenska M, Bayani J, Pandita A, Toledo S, Marrano P, Andrade J, Petrilli A, Thorner P, Sorensen P, Squire JA: Comparative genomic hybridization

analysis identifies gains of 1p35 approximately p36 and chromosome 19 in osteosarcoma. *Cancer genetics and cytogenetics* 2001, 130(1):14-21.

56. Bayani J, Zielenska M, Pandita A, Al-Romaih K, Karaskova J, Harrison K, Bridge JA, Sorensen P, Thorner P, Squire JA: Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. *Genes, chromosomes & cancer* 2003, 36(1):7-16.
57. Dutrillaux B: Chromosome amd Karyotype Instability in Human Cancers. In. Natwick, MA, USA: Eaton; 2000: 369-382.
58. Bayani J, Selvarajah S, Maire G, Vukovic B, Al-Romaih K, Zielenska M, Squire JA: Genomic mechanisms and measurement of structural and numerical instability in cancer cells. *Seminars in cancer biology* 2007, 17(1):5-18.
59. Lengauer C, Kinzler KW, Vogelstein B: Genetic instability in colorectal cancers. *Nature* 1997, 386(6625):623-627.
60. Venkitaraman AR: Chromosomal instability in cancer: causality and interdependence. *Cell cycle (Georgetown, Tex* 2007, 6(19):2341-2343.
61. Wilson AS, Power BE, Molloy PL: DNA hypomethylation and human diseases. *Biochimica et biophysica acta* 2007, 1775(1):138-162.
62. Bonin G, Scamps C, Turc-Carel C, Lipinski M: Chimeric EWS-FLI1 transcript in a Ewing cell line with a complex t(11;22;14) translocation. *Cancer research* 1993, 53(16):3655-3657.
63. Zucman J, Delattre O, Desmaze C, Epstein AL, Stenman G, Speleman F, Fletchers CD, Aurias A, Thomas G: EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. *Nature genetics* 1993, 4(4):341-345.
64. Rauscher FJ, 3rd, Benjamin LE, Fredericks WJ, Morris JF: Novel oncogenic mutations in the WT1 Wilms' tumor suppressor gene: a t(11;22) fuses the Ewing's sarcoma gene, EWS1, to WT1 in desmoplastic small round cell tumor. *Cold Spring Harbor symposia on quantitative biology* 1994, 59:137-146.
65. Gill S, McManus AP, Crew AJ, Benjamin H, Sheer D, Gusterson BA, Pinkerton CR, Patel K, Cooper CS, Shipley JM: Fusion of the EWS gene to a DNA segment from 9q22-31 in a human myxoid chondrosarcoma. *Genes, chromosomes & cancer* 1995, 12(4):307-310.
66. Kleinfinger P, Labelle Y, Melot T, Thomas G, Delattre O, Aurias A: Localization of TEC to 9q22.3-q31 by fluorescence in situ hybridization. *Annales de genetique* 1996, 39(4):233-235.
67. Biegel JA, Nycum LM, Valentine V, Barr FG, Shapiro DN: Detection of the t(2;13)(q35;q14) and PAX3-FKHR fusion in alveolar rhabdomyosarcoma by fluorescence in situ hybridization. *Genes, chromosomes & cancer* 1995, 12(3):186-192.
68. Besnard-Guerin C, Newsham I, Winqvist R, Cavenee WK: A common region of loss of heterozygosity in Wilms' tumor and embryonal rhabdomyosarcoma distal to the D11S988 locus on chromosome 11p15.5. *Human genetics* 1996, 97(2):163-170.

69. Zhan S, Shapiro DN, Helman LJ: Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. *The Journal of clinical investigation* 1994, 94(1):445-448.
70. Papachristou DJ, Papavassiliou AG: Osteosarcoma and chondrosarcoma: new signaling pathways as targets for novel therapeutic interventions. *The international journal of biochemistry & cell biology* 2007, 39(5):857-862.
71. Gokgoz N, Wunder JS, Mousses S, Eskandarian S, Bell RS, Andrulis IL: Comparison of p53 mutations in patients with localized osteosarcoma and metastatic osteosarcoma. *Cancer* 2001, 92(8):2181-2189.
72. Harvey M, Vogel H, Morris D, Bradley A, Bernstein A, Donehower LA: A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. *Nature genetics* 1995, 9(3):305-311.
73. Wilkie TM, Schmidt RA, Baetscher M, Messing A: Smooth muscle and bone neoplasms in transgenic mice expressing SV40 T antigen. *Oncogene* 1994, 9(10):2889-2895.
74. Liu G, McDonnell TJ, Montes de Oca Luna R, Kapoor M, Mims B, El-Naggar AK, Lozano G: High metastatic potential in mice inheriting a targeted p53 missense mutation. *Proceedings of the National Academy of Sciences of the United States of America* 2000, 97(8):4174-4179.
75. Malkin D, Li FP, Strong LC, Fraumeni JF, Jr., Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA *et al*: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science (New York, NY* 1990, 250(4985):1233-1238.
76. Iwakuma T, Lozano G, Flores ER: Li-Fraumeni syndrome: a p53 family affair. *Cell cycle (Georgetown, Tex* 2005, 4(7):865-867.
77. Tsuchiya T, Sekine K, Hinohara S, Namiki T, Nobori T, Kaneko Y: Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. *Cancer genetics and cytogenetics* 2000, 120(2):91-98.
78. Momand J, Jung D, Wilczynski S, Niland J: The MDM2 gene amplification database. *Nucleic acids research* 1998, 26(15):3453-3459.
79. Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH: MDM2 gene amplification in metastatic osteosarcoma. *Cancer research* 1993, 53(1):16-18.
80. Gil J, Peters G: Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nature reviews* 2006, 7(9):667-677.
81. Gallagher SJ, Kefford RF, Rizos H: The ARF tumour suppressor. *The international journal of biochemistry & cell biology* 2006, 38(10):1637-1641.
82. Wei G, Lonardo F, Ueda T, Kim T, Huvos AG, Healey JH, Ladanyi M: CDK4 gene amplification in osteosarcoma: reciprocal relationship with INK4A gene alterations and mapping of 12q13 amplicons. *International journal of cancer* 1999, 80(2):199-204.
83. Giacinti C, Giordano A: RB and cell cycle progression. *Oncogene* 2006, 25(38):5220-5227.

84. Entz-Werle N, Schneider A, Kalifa C, Voegeli AC, Tabone MD, Marec-Berard P, Marcellin L, Pacquement H, Terrier P, Boutard P *et al*: Genetic alterations in primary osteosarcoma from 54 children and adolescents by targeted allelotyping. *British journal of cancer* 2003, 88(12):1925-1931.
85. Belchis DA, Meece CA, Benko FA, Rogan PK, Williams RA, Gocke CD: Loss of heterozygosity and microsatellite instability at the retinoblastoma locus in osteosarcomas. *Diagn Mol Pathol* 1996, 5(3):214-219.
86. Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, Yamamuro T: Mutation spectrum of the retinoblastoma gene in osteosarcomas. *Cancer research* 1994, 54(11):3042-3048.
87. Wong FL, Boice JD, Jr., Abramson DH, Tarone RE, Kleinerman RA, Stovall M, Goldman MB, Seddon JM, Tarbell N, Fraumeni JF, Jr. *et al*: Cancer incidence after retinoblastoma. Radiation dose and sarcoma risk. *Jama* 1997, 278(15):1262-1267.
88. Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP: Alterations of the p53, Rb and MDM2 genes in osteosarcoma. *Journal of cancer research and clinical oncology* 1996, 122(9):559-565.
89. Miller CW, Aslo A, Campbell MJ, Kawamata N, Lampkin BC, Koeffler HP: Alterations of the p15, p16,and p18 genes in osteosarcoma. *Cancer genetics and cytogenetics* 1996, 86(2):136-142.
90. Kanoe H, Nakayama T, Murakami H, Hosaka T, Yamamoto H, Nakashima Y, Tsuboyama T, Nakamura T, Sasaki MS, Toguchida J: Amplification of the CDK4 gene in sarcomas: tumor specificity and relationship with the RB gene mutation. *Anticancer research* 1998, 18(4A):2317-2321.
91. Nilsson JA, Cleveland JL: Myc pathways provoking cell suicide and cancer. *Oncogene* 2003, 22(56):9007-9021.
92. Nesbit CE, Tersak JM, Prochownik EV: MYC oncogenes and human neoplastic disease. *Oncogene* 1999, 18(19):3004-3016.
93. Gomez-Roman N, Felton-Edkins ZA, Kenneth NS, Goodfellow SJ, Athineos D, Zhang J, Ramsbottom BA, Innes F, Kantidakis T, Kerr ER *et al*: Activation by c-Myc of transcription by RNA polymerases I, II and III. *Biochemical Society symposium* 2006(73):141-154.
94. Pelengaris S, Khan M: The many faces of c-MYC. *Archives of biochemistry and biophysics* 2003, 416(2):129-136.
95. Ladanyi M, Park CK, Lewis R, Jhanwar SC, Healey JH, Huvos AG: Sporadic amplification of the MYC gene in human osteosarcomas. *Diagn Mol Pathol* 1993, 2(3):163-167.
96. Stock C, Kager L, Fink FM, Gadner H, Ambros PF: Chromosomal regions involved in the pathogenesis of osteosarcomas. *Genes, chromosomes & cancer* 2000, 28(3):329-336.
97. Gamberi G, Benassi MS, Bohling T, Ragazzini P, Molendini L, Sollazzo MR, Pompetti F, Merli M, Magagnoli G, Balladelli A *et al*: C-myc and c-fos in human osteosarcoma: prognostic value of mRNA and protein expression. *Oncology* 1998, 55(6):556-563.

98. Curran T, MacConnell WP, van Straaten F, Verma IM: Structure of the FBJ murine osteosarcoma virus genome: molecular cloning of its associated helper virus and the cellular homolog of the v-fos gene from mouse and human cells. *Molecular and cellular biology* 1983, 3(5):914-921.
99. Miller AD, Curran T, Verma IM: c-fos protein can induce cellular transformation: a novel mechanism of activation of a cellular oncogene. *Cell* 1984, 36(1):51-60.
100. Price CH, Moore M, Jones DB: FBJ virus-induced tumours in mice. A histopathological study of FBJ virus tumours and their relevance to murine and human osteosarcoma arising in bone. *British journal of cancer* 1972, 26(1):15-27.
101. Wu JX, Carpenter PM, Gresens C, Keh R, Niman H, Morris JW, Mercola D: The proto-oncogene c-fos is over-expressed in the majority of human osteosarcomas. *Oncogene* 1990, 5(7):989-1000.
102. Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD *et al*: Genomic instability in Gadd45a-deficient mice. *Nature genetics* 1999, 23(2):176-184.
103. Hollander MC, Fornace AJ, Jr.: Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. *Oncogene* 2002, 21(40):6228-6233.
104. Fayolle C, Pourchet J, Cohen A, Pedeux R, Puisieux A, de Fromentel CC, Dore JF, Voeltzel T: UVB-induced G2 arrest of human melanocytes involves Cdc2 sequestration by Gadd45a in nuclear speckles. *Cell cycle (Georgetown, Tex* 2006, 5(16):1859-1864.
105. Zhan Q: Gadd45a, a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage. *Mutation research* 2005, 569(1-2):133-143.
106. Tong T, Ji J, Jin S, Li X, Fan W, Song Y, Wang M, Liu Z, Wu M, Zhan Q: Gadd45a expression induces Bim dissociation from the cytoskeleton and translocation to mitochondria. *Molecular and cellular biology* 2005, 25(11):4488-4500.
107. Kenny PA, Lee GY, Bissell MJ: Targeting the tumor microenvironment. *Front Biosci* 2007, 12:3468-3474.
108. Yamaguchi Y: Microenvironmental regulation of estrogen signals in breast cancer. *Breast cancer (Tokyo, Japan)* 2007, 14(2):175-181.
109. Morris DS, Tomlins SA, Rhodes DR, Mehra R, Shah RB, Chinnaiyan AM: Integrating biomedical knowledge to model pathways of prostate cancer progression. *Cell cycle (Georgetown, Tex* 2007, 6(10):1177-1187.
110. Sung SY, Hsieh CL, Wu D, Chung LW, Johnstone PA: Tumor microenvironment promotes cancer progression, metastasis, and therapeutic resistance. *Current problems in cancer* 2007, 31(2):36-100.
111. Sharma SM, Hu R, Bronisz A, Meadows N, Lusby T, Fletcher B, Hume DA, Cassady AI, Ostrowski MC: Genetics and genomics of osteoclast differentiation: integrating cell signaling pathways and gene networks. *Critical reviews in eukaryotic gene expression* 2006, 16(3):253-277.

112. Westendorf JJ, Kahler RA, Schroeder TM: Wnt signaling in osteoblasts and bone diseases. *Gene* 2004, 341:19-39.
113. Vaes BL, Dechering KJ, van Someren EP, Hendriks JM, van de Ven CJ, Feijen A, Mummery CL, Reinders MJ, Olijve W, van Zoelen EJ *et al*: Microarray analysis reveals expression regulation of Wnt antagonists in differentiating osteoblasts. *Bone* 2005, 36(5):803-811.
114. Cremer T, Cremer M, Dietzel S, Muller S, Solovei I, Fakan S: Chromosome territories--a functional nuclear landscape. *Current opinion in cell biology* 2006, 18(3):307-316.
115. Zamurovic N, Cappellen D, Rohner D, Susa M: Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. *The Journal of biological chemistry* 2004, 279(36):37704-37715.
116. Gobbi G, Sangiorgi L, Lenzi L, Casadei R, Canaider S, Strippoli P, Lucarelli E, Ghedini I, Donati D, Fabbri N *et al*: Seven BMPs and all their receptors are simultaneously expressed in osteosarcoma cells. *International journal of oncology* 2002, 20(1):143-147.
117. Dass CR, Ek ET, Choong PF: Human xenograft osteosarcoma models with spontaneous metastasis in mice: clinical relevance and applicability for drug testing. *Journal of cancer research and clinical oncology* 2007, 133(3):193-198.
118. Bruheim S, Bruland OS, Breistol K, Maelandsmo GM, Fodstad O: Human osteosarcoma xenografts and their sensitivity to chemotherapy. *Pathol Oncol Res* 2004, 10(3):133-141.
119. Ek ET, Dass CR, Choong PF: Commonly used mouse models of osteosarcoma. *Critical reviews in oncology/hematology* 2006, 60(1):1-8.
120. Al-Mohanna MA, Manogaran PS, Al-Mukhalafi Z, K AA-H, Aboussekhra A: The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. *Oncogene* 2004, 23(1):201-212.
121. Robson H, Meyer S, Shalet SM, Anderson E, Roberts S, Eden OB: Platinum agents in the treatment of osteosarcoma: efficacy of cisplatin vs. carboplatin in human osteosarcoma cell lines. *Med Pediatr Oncol* 2002, 39(6):573-580.
122. Allan LA, Fried M: p53-dependent apoptosis or growth arrest induced by different forms of radiation in U2OS cells: p21WAF1/CIP1 repression in UV induced apoptosis. *Oncogene* 1999, 18(39):5403-5412.
123. Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, Schneider-Stock R, Gali-Muhtasib H: Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Cancer biology & therapy* 2007, 6(2):160-169.
124. Matsui TA, Murata H, Sakabe T, Sowa Y, Horie N, Nakanishi R, Sakai T, Kubo T: Sulforaphane induces cell cycle arrest and apoptosis in murine osteosarcoma cells in vitro and inhibits tumor growth in vivo. *Oncology reports* 2007, 18(5):1263-1268.

125. Waddington CH: The Strategy of the Genes In.: (Geo Allen & Unwin, London); 1957.
126. Slack JM: Conrad Hal Waddington: the last Renaissance biologist? *Nat Rev Genet* 2002, 3(11):889-895.
127. Egger G, Liang G, Aparicio A, Jones PA: Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004, 429(6990):457-463.
128. Kiefer JC: Epigenetics in development. *Dev Dyn* 2007, 236(4):1144-1156.
129. Laird PW: Cancer epigenetics. *Human molecular genetics* 2005, 14 Spec No 1:R65-76.
130. Esteller M: Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *British journal of cancer* 2007, 96 Suppl:R26-30.
131. Rando OJ: Global patterns of histone modifications. *Current opinion in genetics & development* 2007, 17(2):94-99.
132. Jones PA: Overview of cancer epigenetics. *Seminars in hematology* 2005, 42(3 Suppl 2):S3-8.
133. Rodenhiser D, Mann M: Epigenetics and human disease: translating basic biology into clinical applications. *Cmaj* 2006, 174(3):341-348.
134. Sumner AT: Chromosomes Organization and Function, 1st edn: Blackwell Publishing; 2003.
135. Kouzarides T: Chromatin modifications and their function. *Cell* 2007, 128(4):693-705.
136. Bernstein BE, Meissner A, Lander ES: The mammalian epigenome. *Cell* 2007, 128(4):669-681.
137. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD *et al*: Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 2000, 406(6796):593-599.
138. Gruenbaum Y, Stein R, Cedar H, Razin A: Methylation of CpG sequences in eukaryotic DNA. *FEBS letters* 1981, 124(1):67-71.
139. Sinsheimer RL: The action of pancreatic deoxyribonuclease. II. Isomeric dinucleotides. *The Journal of biological chemistry* 1955, 215(2):579-583.
140. Cross SH, Bird AP: CpG islands and genes. *Current opinion in genetics & development* 1995, 5(3):309-314.
141. Yoder JA, Walsh CP, Bestor TH: Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 1997, 13(8):335-340.
142. Smith AF: Interspersed repeats and other mementos of transposable elements in mammalian genome. *Current opinion in genetics & development* 1999, 9:657-663.
143. Riggs AD: X inactivation, differentiation, and DNA methylation. *Cytogenetics and cell genetics* 1975, 14(1):9-25.
144. Holliday R, Pugh JE: DNA modification mechanisms and gene activity during development. *Science (New York, NY* 1975, 187(4173):226-232.
145. Kaneda M, Sado T, Hata K, Okano M, Tsujimoto N, Li E, Sasaki H: Role of de novo DNA methyltransferases in initiation of genomic imprinting and X-

chromosome inactivation. *Cold Spring Harbor symposia on quantitative biology* 2004, 69:125-129.

146. Turek-Plewa J, Jagodzinski PP: The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cellular & molecular biology letters* 2005, 10(4):631-647.
147. Bestor TH: The DNA methyltransferases of mammals. *Human molecular genetics* 2000, 9(16):2395-2402.
148. Bestor T, Laudano A, Mattaliano R, Ingram V: Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *Journal of molecular biology* 1988, 203(4):971-983.
149. Bestor TH: Cloning of a mammalian DNA methyltransferase. *Gene* 1988, 74(1):9-12.
150. Posfai J, Bhagwat AS, Posfai G, Roberts RJ: Predictive motifs derived from cytosine methyltransferases. *Nucleic acids research* 1989, 17(7):2421-2435.
151. Lauster R, Trautner TA, Noyer-Weidner M: Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. *Journal of molecular biology* 1989, 206(2):305-312.
152. Yoder JA, Soman NS, Verdine GL, Bestor TH: DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *Journal of molecular biology* 1997, 270(3):385-395.
153. Grunwald S, Drahovsky D: DNA-cytosine-5-methyltransferase from P815 mouse mastocytoma cells: "maintenance" and "de novo" activities are carried out by the same enzyme molecule. *The International journal of biochemistry* 1984, 16(8):883-888.
154. Hitt MM, Wu TL, Cohen G, Linn S: De novo and maintenance DNA methylation by a mouse plasmacytoma cell DNA methyltransferase. *The Journal of biological chemistry* 1988, 263(9):4392-4399.
155. Okano M, Xie S, Li E: Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature genetics* 1998, 19(3):219-220.
156. Pradhan S, Bacolla A, Wells RD, Roberts RJ: Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *The Journal of biological chemistry* 1999, 274(46):33002-33010.
157. Li E, Bestor TH, Jaenisch R: Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992, 69(6):915-926.
158. Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E: De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development (Cambridge, England)* 1996, 122(10):3195-3205.

159. Aoki A, Suetake I, Miyagawa J, Fujio T, Chijiwa T, Sasaki H, Tajima S: Enzymatic properties of de novo-type mouse DNA (cytosine-5) methyltransferases. *Nucleic acids research* 2001, 29(17):3506-3512.
160. Okano M, Bell DW, Haber DA, Li E: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999, 99(3):247-257.
161. Dong A, Yoder JA, Zhang X, Zhou L, Bestor TH, Cheng X: Structure of human DNMT2, an enigmatic DNA methyltransferase homolog that displays denaturant-resistant binding to DNA. *Nucleic acids research* 2001, 29(2):439-448.
162. Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH: Dnmt3L and the establishment of maternal genomic imprints. *Science (New York, NY* 2001, 294(5551):2536-2539.
163. Hata K, Okano M, Lei H, Li E: Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development (Cambridge, England)* 2002, 129(8):1983-1993.
164. Huang LH, Wang R, Gama-Sosa MA, Shenoy S, Ehrlich M: A protein from human placental nuclei binds preferentially to 5-methylcytosine-rich DNA. *Nature* 1984, 308(5956):293-295.
165. Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, Bird A: Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 1992, 69(6):905-914.
166. Meehan RR, Lewis JD, Bird AP: Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic acids research* 1992, 20(19):5085-5092.
167. Nan X, Campoy FJ, Bird A: MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 1997, 88(4):471-481.
168. Nan X, Tate P, Li E, Bird A: DNA methylation specifies chromosomal localization of MeCP2. *Molecular and cellular biology* 1996, 16(1):414-421.
169. Cross SH, Charlton JA, Nan X, Bird AP: Purification of CpG islands using a methylated DNA binding column. *Nature genetics* 1994, 6(3):236-244.
170. Nan X, Meehan RR, Bird A: Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic acids research* 1993, 21(21):4886-4892.
171. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A: Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 1998, 393(6683):386-389.
172. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP: Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature genetics* 1998, 19(2):187-191.
173. Kaludov NK, Wolffe AP: MeCP2 driven transcriptional repression in vitro: selectivity for methylated DNA, action at a distance and contacts with the basal transcription machinery. *Nucleic acids research* 2000, 28(9):1921-1928.

174. Bird AP, Wolffe AP: Methylation-induced repression--belts, braces, and chromatin. *Cell* 1999, 99(5):451-454.
175. Wade PA: Methyl CpG-binding proteins and transcriptional repression. *Bioessays* 2001, 23(12):1131-1137.
176. Yu F, Thiesen J, Stratling WH: Histone deacetylase-independent transcriptional repression by methyl-CpG-binding protein 2. *Nucleic acids research* 2000, 28(10):2201-2206.
177. Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D, Bird A: MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nature genetics* 1999, 23(1):58-61.
178. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature genetics* 1999, 21(1):103-107.
179. Cross SH, Meehan RR, Nan X, Bird A: A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nature genetics* 1997, 16(3):256-259.
180. Hendrich B, Bird A: Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Molecular and cellular biology* 1998, 18(11):6538-6547.
181. Boeke J, Ammerpohl O, Kegel S, Moehren U, Renkawitz R: The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. *The Journal of biological chemistry* 2000, 275(45):34963-34967.
182. Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP: Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nature genetics* 1999, 23(1):62-66.
183. Hendrich B, Hardeland U, Ng HH, Jiricny J, Bird A: The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature* 1999, 401(6750):301-304.
184. Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Stoerker J, Genuardi M, Yeung AT, Matsumoto Y, Bellacosa A: Biphasic kinetics of the human DNA repair protein MED1 (MBD4), a mismatch-specific DNA N-glycosylase. *The Journal of biological chemistry* 2000, 275(42):32422-32429.
185. Esteller M, Herman JG: Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *The Journal of pathology* 2002, 196(1):1-7.
186. Ordway JM, Curran T: Methylation matters: modeling a manageable genome. *Cell Growth Differ* 2002, 13(4):149-162.
187. Attwood JT, Yung RL, Richardson BC: DNA methylation and the regulation of gene transcription. *Cell Mol Life Sci* 2002, 59(2):241-257.
188. Tuck-Muller CM, Narayan A, Tsien F, Smeets DF, Sawyer J, Fiala ES, Sohn OS, Ehrlich M: DNA hypomethylation and unusual chromosome instability

in cell lines from ICF syndrome patients. *Cytogenetics and cell genetics* 2000, 89(1-2):121-128.
189. Santos F, Hendrich B, Reik W, Dean W: Dynamic reprogramming of DNA methylation in the early mouse embryo. *Developmental biology* 2002, 241(1):172-182.
190. Kang YK, Park JS, Koo DB, Choi YH, Kim SU, Lee KK, Han YM: Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. *The EMBO journal* 2002, 21(5):1092-1100.
191. Smith AC, Choufani S, Ferreira JC, Weksberg R: Growth Regulation, Imprinted Genes, and Chromosome 11p15.5. *Pediatric research* 2007.
192. Glaser RL, Ramsay JP, Morison IM: The imprinted gene and parent-of-origin effect database now includes parental origin of de novo mutations. *Nucleic acids research* 2006, 34(Database issue):D29-31.
193. Brannan CI, Bartolomei MS: Mechanisms of genomic imprinting. *Current opinion in genetics & development* 1999, 9(2):164-170.
194. Bartolomei MS, Tilghman SM: Genomic imprinting in mammals. *Annual review of genetics* 1997, 31:493-525.
195. Teshima I, Chadwick D, Chitayat D, Kobayashi J, Ray P, Shuman C, Siegel-Bartelt J, Strasberg P, Weksberg R: FISH detection of chromosome 15 deletions in Prader-Willi and Angelman syndromes. *American journal of medical genetics* 1996, 62(3):217-223.
196. Glenn CC, Saitoh S, Jong MT, Filbrandt MM, Surti U, Driscoll DJ, Nicholls RD: Gene structure, DNA methylation, and imprinted expression of the human SNRPN gene. *American journal of human genetics* 1996, 58(2):335-346.
197. Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP: Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell* 1993, 73(1):61-71.
198. Feil R, Walter J, Allen ND, Reik W: Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. *Development (Cambridge, England)* 1994, 120(10):2933-2943.
199. Bartolomei MS, Webber AL, Brunkow ME, Tilghman SM: Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. *Genes & development* 1993, 7(9):1663-1673.
200. Chang SC, Tucker T, Thorogood NP, Brown CJ: Mechanisms of X-chromosome inactivation. *Front Biosci* 2006, 11:852-866.
201. Heard E, Disteche CM: Dosage compensation in mammals: fine-tuning the expression of the X chromosome. *Genes & development* 2006, 20(14):1848-1867.
202. Kramerov DA, Vassetzky NS: Short retroposons in eukaryotic genomes. *International review of cytology* 2005, 247:165-221.
203. Dewannieux M, Heidmann T: LINEs, SINEs and processed pseudogenes: parasitic strategies for genome modeling. *Cytogenetic and genome research* 2005, 110(1-4):35-48.

204. Kazazian HH, Jr.: Mobile elements: drivers of genome evolution. *Science (New York, NY* 2004, 303(5664):1626-1632.
205. Enklaar T, Zabel BU, Prawitt D: Beckwith-Wiedemann syndrome: multiple molecular mechanisms. *Expert reviews in molecular medicine* 2006, 8(17):1-19.
206. Weksberg R, Shuman C, Smith AC: Beckwith-Wiedemann syndrome. *Am J Med Genet C Semin Med Genet* 2005, 137(1):12-23.
207. Cooper WN, Luharia A, Evans GA, Raza H, Haire AC, Grundy R, Bowdin SC, Riccio A, Sebastio G, Bliek J *et al*: Molecular subtypes and phenotypic expression of Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 2005, 13(9):1025-1032.
208. Horsthemke B, Buiting K: Imprinting defects on human chromosome 15. *Cytogenetic and genome research* 2006, 113(1-4):292-299.
209. Kubota T, Sutcliffe JS, Aradhya S, Gillessen-Kaesbach G, Christian SL, Horsthemke B, Beaudet AL, Ledbetter DH: Validation studies of SNRPN methylation as a diagnostic test for Prader-Willi syndrome. *American journal of medical genetics* 1996, 66(1):77-80.
210. Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC *et al*: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nature genetics* 2000, 24(2):132-138.
211. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007, 8(4):286-298.
212. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R: Induction of tumors in mice by genomic hypomethylation. *Science (New York, NY* 2003, 300(5618):489-492.
213. Hoffmann MJ, Schulz WA: Causes and consequences of DNA hypomethylation in human cancer. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 2005, 83(3):296-321.
214. Corn PG, Summers MK, Fogt F, Virmani AK, Gazdar AF, Halazonetis TD, El-Deiry WS: Frequent hypermethylation of the 5' CpG island of the mitotic stress checkpoint gene Chfr in colorectal and non-small cell lung cancer. *Carcinogenesis* 2003, 24(1):47-51.
215. Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, Frommer M, Clark SJ: Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. *Cancer research* 1997, 57(11):2229-2237.
216. Gopisetty G, Ramachandran K, Singal R: DNA methylation and apoptosis. *Mol Immunol* 2006, 43(11):1729-1740.
217. Martinez R, Schackert G, Yaya-Tur R, Rojas-Marcos I, Herman JG, Esteller M: Frequent hypermethylation of the DNA repair gene MGMT in long-term survivors of glioblastoma multiforme. *J Neurooncol* 2006.
218. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA *et al*: Promoter

hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *Journal of the National Cancer Institute* 2000, 92(7):564-569.

219. Zerbini LF, Libermann TA: GADD45 deregulation in cancer: frequently methylated tumor suppressors and potential therapeutic targets. *Clin Cancer Res* 2005, 11(18):6409-6413.
220. Akama TO, Okazaki Y, Ito M, Okuizumi H, Konno H, Muramatsu M, Plass C, Held WA, Hayashizaki Y: Restriction landmark genomic scanning (RLGS-M)-based genome-wide scanning of mouse liver tumors for alterations in DNA methylation status. *Cancer research* 1997, 57(15):3294-3299.
221. Zardo G, Tiirikainen MI, Hong C, Misra A, Feuerstein BG, Volik S, Collins CC, Lamborn KR, Bollen A, Pinkel D *et al*: Integrated genomic and epigenomic analyses pinpoint biallelic gene inactivation in tumors. *Nature genetics* 2002, 32(3):453-458.
222. Novak P, Jensen T, Oshiro MM, Wozniak RJ, Nouzova M, Watts GS, Klimecki WT, Kim C, Futscher BW: Epigenetic inactivation of the HOXA gene cluster in breast cancer. *Cancer research* 2006, 66(22):10664-10670.
223. Frigola J, Song J, Stirzaker C, Hinshelwood RA, Peinado MA, Clark SJ: Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. *Nature genetics* 2006, 38(5):540-549.
224. Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, Weinberg RA, Jaenisch R: Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* 1995, 81(2):197-205.
225. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM *et al*: Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* 1994, 91(21):9700-9704.
226. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW *et al*: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* 1998, 95(12):6870-6875.
227. Myohanen SK, Baylin SB, Herman JG: Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. *Cancer research* 1998, 58(4):591-593.
228. Jones PA, Laird PW: Cancer epigenetics comes of age. *Nature genetics* 1999, 21(2):163-167.
229. Feinberg AP, Vogelstein B: Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983, 301(5895):89-92.
230. Feinberg AP, Vogelstein B: Hypomethylation of ras oncogenes in primary human cancers. *Biochemical and biophysical research communications* 1983, 111(1):47-54.

231. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M: The 5-methylcytosine content of DNA from human tumors. *Nucleic acids research* 1983, 11(19):6883-6894.
232. Gama-Sosa MA, Wang RY, Kuo KC, Gehrke CW, Ehrlich M: The 5-methylcytosine content of highly repeated sequences in human DNA. *Nucleic acids research* 1983, 11(10):3087-3095.
233. Feinberg AP, Tycko B: The history of cancer epigenetics. *Nat Rev Cancer* 2004, 4(2):143-153.
234. Eden A, Gaudet F, Waghmare A, Jaenisch R: Chromosomal instability and tumors promoted by DNA hypomethylation. *Science (New York, NY* 2003, 300(5618):455.
235. Choi IS, Estecio MR, Nagano Y, Kim do H, White JA, Yao JC, Issa JP, Rashid A: Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol* 2007, 20(7):802-810.
236. Ji W, Hernandez R, Zhang XY, Qu GZ, Frady A, Varela M, Ehrlich M: DNA demethylation and pericentromeric rearrangements of chromosome 1. *Mutation research* 1997, 379(1):33-41.
237. Schulz WA, Elo JP, Florl AR, Pennanen S, Santourlidis S, Engers R, Buchardt M, Seifert HH, Visakorpi T: Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. *Genes, chromosomes & cancer* 2002, 35(1):58-65.
238. Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M: DNA hypomethylation is prevalent even in low-grade breast cancers. *Cancer biology & therapy* 2004, 3(12):1225-1231.
239. Agapova LS, Ilyinskaya GV, Turovets NA, Ivanov AV, Chumakov PM, Kopnin BP: Chromosome changes caused by alterations of p53 expression. *Mutation research* 1996, 354(1):129-138.
240. Florl AR, Steinhoff C, Muller M, Seifert HH, Hader C, Engers R, Ackermann R, Schulz WA: Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *British journal of cancer* 2004, 91(5):985-994.
241. Baylin SB, Ohm JE: Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006, 6(2):107-116.
242. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. *The New England journal of medicine* 2003, 349(21):2042-2054.
243. Issa JP, Kantarjian H: Azacitidine. *Nat Rev Drug Discov* 2005, Suppl:S6-7.
244. Toyota M, Issa JP: Epigenetic changes in solid and hematopoietic tumors. *Seminars in oncology* 2005, 32(5):521-530.
245. Toyota M, Shen L, Ohe-Toyota M, Hamilton SR, Sinicrope FA, Issa JP: Aberrant methylation of the Cyclooxygenase 2 CpG island in colorectal tumors. *Cancer research* 2000, 60(15):4044-4048.

246. Issa JP: DNA methylation as a therapeutic target in cancer. *Clin Cancer Res* 2007, 13(6):1634-1637.
247. Qiu W, Zhou B, Zou H, Liu X, Chu PG, Lopez R, Shih J, Chung C, Yen Y: Hypermethylation of growth arrest DNA damage-inducible gene 45 beta promoter in human hepatocellular carcinoma. *The American journal of pathology* 2004, 165(5):1689-1699.
248. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J *et al*: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* 2004, 103(5):1635-1640.
249. Lindberg EH: Strategies for biology- and molecular-based treatment of myelodysplastic syndromes. *Curr Drug Targets* 2005, 6(6):713-725.
250. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F *et al*: Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* 2006, 106(8):1794-1803.
251. Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP: Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *Journal of molecular biology* 2002, 321(4):591-599.
252. Gowher H, Jeltsch A: Mechanism of inhibition of DNA methyltransferases by cytidine analogs in cancer therapy. *Cancer biology & therapy* 2004, 3(11):1062-1068.
253. Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B: Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J Immunol* 1988, 140(7):2197-2200.
254. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA: Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Molecular cancer therapeutics* 2005, 4(10):1515-1520.
255. Siedlecki P, Boy RG, Musch T, Brueckner B, Suhai S, Lyko F, Zielenkiewicz P: Discovery of two novel, small-molecule inhibitors of DNA methylation. *Journal of medicinal chemistry* 2006, 49(2):678-683.
256. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, Garcia-Manero G, Rios MB, Shan J, Andreeff M *et al*: Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 2003, 98(3):522-528.
257. Abele R, Clavel M, Dodion P, Bruntsch U, Gundersen S, Smyth J, Renard J, van Glabbeke M, Pinedo HM: The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *European journal of cancer & clinical oncology* 1987, 23(12):1921-1924.

258. Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP: Phase I trial of sequential low-dose 5-aza-2'-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. *Clin Cancer Res* 2006, 12(15):4619-4627.
259. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM *et al*: Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol* 2002, 20(10):2429-2440.
260. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z *et al*: Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood* 2007, 109(1):52-57.
261. Yang AS, Estecio MR, Garcia-Manero G, Kantarjian HM, Issa JP: Comment on "Chromosomal instability and tumors promoted by DNA hypomethylation" and "Induction of tumors in nice by genomic hypomethylation". *Science (New York, NY* 2003, 302(5648):1153; author reply 1153.
262. Yang AS, Doshi KD, Choi SW, Mason JB, Mannari RK, Gharybian V, Luna R, Rashid A, Shen L, Estecio MR *et al*: DNA methylation changes after 5-aza-2'-deoxycytidine therapy in patients with leukemia. *Cancer research* 2006, 66(10):5495-5503.
263. Mund C, Hackanson B, Stresemann C, Lubbert M, Lyko F: Characterization of DNA demethylation effects induced by 5-Aza-2'-deoxycytidine in patients with myelodysplastic syndrome. *Cancer research* 2005, 65(16):7086-7090.
264. Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000, 100(1):57-70.
265. Arney KL, Fisher AG: Epigenetic aspects of differentiation. *Journal of cell science* 2004, 117(Pt 19):4355-4363.
266. Ryhanen S, Pirskanen A, Jaaskelainen T, Maenpaa PH: State of methylation of the human osteocalcin gene in bone-derived and other types of cells. *Journal of cellular biochemistry* 1997, 66(3):404-412.
267. Locklin RM, Oreffo RO, Triffitt JT: Modulation of osteogenic differentiation in human skeletal cells in Vitro by 5-azacytidine. *Cell biology international* 1998, 22(3):207-215.
268. Villagra A, Gutierrez J, Paredes R, Sierra J, Puchi M, Imschenetzky M, Wijnen Av A, Lian J, Stein G, Stein J *et al*: Reduced CpG methylation is associated with transcriptional activation of the bone-specific rat osteocalcin gene in osteoblasts. *Journal of cellular biochemistry* 2002, 85(1):112-122.
269. Ulaner GA, Vu TH, Li T, Hu JF, Yao XM, Yang Y, Gorlick R, Meyers P, Healey J, Ladanyi M *et al*: Loss of imprinting of IGF2 and H19 in

osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. *Human molecular genetics* 2003, 12(5):535-549.

270. Harada K, Toyooka S, Maitra A, Maruyama R, Toyooka KO, Timmons CF, Tomlinson GE, Mastrangelo D, Hay RJ, Minna JD *et al*: Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. *Oncogene* 2002, 21(27):4345-4349.
271. Thomas D, Kansara M: Epigenetic modifications in osteogenic differentiation and transformation. *Journal of cellular biochemistry* 2006, 98(4):757-769.
272. Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, Moreno V, Esteller M, Capella G, Ribas M *et al*: Chromosomal Instability Correlates with Genome-wide DNA Demethylation in Human Primary Colorectal Cancers. *Cancer research* 2006, 66(17):8462-9468.
273. Dass CR, Ek ET, Contreras KG, Choong PF: A novel orthotopic murine model provides insights into cellular and molecular characteristics contributing to human osteosarcoma. *Clinical & experimental metastasis* 2006, 23(7-8):367-380.
274. Navaraj A, Mori T, El-Deiry WS: Cooperation between BRCA1 and p53 in repair of cyclobutane pyrimidine dimers. *Cancer biology & therapy* 2005, 4(12):1409-1414.
275. Park YB, Park MJ, Kimura K, Shimizu K, Lee SH, Yokota J: Alterations in the INK4a/ARF locus and their effects on the growth of human osteosarcoma cell lines. *Cancer genetics and cytogenetics* 2002, 133(2):105-111.
276. Raile K, Hoflich A, Kessler U, Yang Y, Pfuender M, Blum WF, Kolb H, Schwarz HP, Kiess W: Human osteosarcoma (U-2 OS) cells express both insulin-like growth factor-I (IGF-I) receptors and insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptors and synthesize IGF-II: autocrine growth stimulation by IGF-II via the IGF-I receptor. *Journal of cellular physiology* 1994, 159(3):531-541.
277. Bogden AE, Haskell PM, LePage DJ, Kelton DE, Cobb WR, Esber HJ: Growth of human tumor xenografts implanted under the renal capsule of normal immunocompetent mice. *Exp Cell Biol* 1979, 47(4):281-293.
278. Ian F Tannock RPH, Robert G Bristow and Lea Harrington: The Basic Science of Oncology. Third Edition. , 4th edn. Toronto, Canada: McGraw-Hill , Medical Publishing Division.; 2004.
279. Manara MC, Baldini N, Serra M, Lollini PL, De Giovanni C, Vaccari M, Argnani A, Benini S, Maurici D, Picci P *et al*: Reversal of malignant phenotype in human osteosarcoma cells transduced with the alkaline phosphatase gene. *Bone* 2000, 26(3):215-220.
280. Wang Y, Revelo MP, Sudilovsky D, Cao M, Chen WG, Goetz L, Xue H, Sadar M, Shappell SB, Cunha GR *et al*: Development and characterization of efficient xenograft models for benign and malignant human prostate tissue. *Prostate* 2005, 64(2):149-159.

281. Cutz JC, Guan J, Bayani J, Yoshimoto M, Xue H, Sutcliffe M, English J, Flint J, LeRiche J, Yee J *et al*: Establishment in severe combined immunodeficiency mice of subrenal capsule xenografts and transplantable tumor lines from a variety of primary human lung cancers: potential models for studying tumor progression-related changes. *Clin Cancer Res* 2006, 12(13):4043-4054.
282. Doxsee DW, Gout PW, Kurita T, Lo M, Buckley AR, Wang Y, Xue H, Karp CM, Cutz JC, Cunha GR *et al*: Sulfasalazine-induced cystine starvation: potential use for prostate cancer therapy. *Prostate* 2007, 67(2):162-171.
283. Zhang X, Yashiro M, Ohira M, Ren J, Hirakawa K: Synergic antiproliferative effect of DNA methyltransferase inhibitor in combination with anticancer drugs in gastric carcinoma. *Cancer Sci* 2006, 97(9):938-944.
284. Reu FJ, Bae SI, Cherkassky L, Leaman DW, Lindner D, Beaulieu N, MacLeod AR, Borden EC: Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. *J Clin Oncol* 2006, 24(23):3771-3779.
285. Reu FJ, Leaman DW, Maitra RR, Bae SI, Cherkassky L, Fox MW, Rempinski DR, Beaulieu N, MacLeod AR, Borden EC: Expression of RASSF1A, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. *Cancer research* 2006, 66(5):2785-2793.
286. Bender CM, Pao MM, Jones PA: Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer research* 1998, 58(1):95-101.
287. Liang G, Gonzales FA, Jones PA, Orntoft TF, Thykjaer T: Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2'-deoxycytidine. *Cancer research* 2002, 62(4):961-966.
288. Covey JM, Zaharko DS: Comparison of the in vitro cytotoxicity (L1210) of 5-aza-2'-deoxycytidine with its therapeutic and toxic effects in mice. *European journal of cancer & clinical oncology* 1985, 21(1):109-117.
289. Momparler RL: Molecular, cellular and animal pharmacology of 5-aza-2'-deoxycytidine. *Pharmacol Ther* 1985, 30(3):287-299.
290. Lantry LE, Zhang Z, Crist KA, Wang Y, Kelloff GJ, Lubet RA, You M: 5-Aza-2'-deoxycytidine is chemopreventive in a 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone-induced primary mouse lung tumor model. *Carcinogenesis* 1999, 20(2):343-346.
291. Alleman WG, Tabios RL, Chandramouli GV, Aprelikova ON, Torres-Cabala C, Mendoza A, Rogers C, Sopko NA, Linehan WM, Vasselli JR: The in vitro and in vivo effects of re-expressing methylated von Hippel-Lindau tumor suppressor gene in clear cell renal carcinoma with 5-aza-2'-deoxycytidine. *Clin Cancer Res* 2004, 10(20):7011-7021.
292. Dotan ZA, Dotan A, Ramon J, Avivi L: Altered mode of allelic replication accompanied by aneuploidy in peripheral blood lymphocytes of prostate cancer patients. *International journal of cancer* 2004, 111(1):60-66.

293. Uchida A, Myoui A, Araki N, Yoshikawa H, Shinto Y, Ueda T: Neoadjuvant chemotherapy for pediatric osteosarcoma patients. *Cancer* 1997, 79(2):411-415.
294. Bielack SS, Erttmann R, Looft G, Purfurst C, Delling G, Winkler K, Landbeck G: Platinum disposition after intraarterial and intravenous infusion of cisplatin for osteosarcoma. Cooperative Osteosarcoma Study Group COSS. *Cancer Chemother Pharmacol* 1989, 24(6):376-380.
295. Meyers PA, Heller G, Healey J, Huvos A, Lane J, Marcove R, Applewhite A, Vlamis V, Rosen G: Chemotherapy for nonmetastatic osteogenic sarcoma: the Memorial Sloan-Kettering experience. *J Clin Oncol* 1992, 10(1):5-15.
296. Munoz Villa A, Ocete G, Aymerich ML, Maldonado S, Otheo E, Calvo M, Amaya J: [Preoperative and postoperative chemotherapy of osteogenic sarcoma of the limbs in children]. *Med Clin (Barc)* 1996, 107(5):161-164.
297. Lopez-Guerrero JA, Lopez-Gines C, Pellin A, Carda C, Llombart-Bosch A: Deregulation of the G1 to S-phase cell cycle checkpoint is involved in the pathogenesis of human osteosarcoma. *Diagn Mol Pathol* 2004, 13(2):81-91.
298. Oh JH, Kim HS, Kim HH, Kim WH, Lee SH: Aberrant methylation of p14ARF gene correlates with poor survival in osteosarcoma. *Clinical orthopaedics and related research* 2006, 442:216-222.
299. Lim S, Yang MH, Park JH, Nojima T, Hashimoto H, Unni KK, Park YK: Inactivation of the RASSF1A in osteosarcoma. *Oncology reports* 2003, 10(4):897-901.
300. Hou P, Ji M, Yang B, Chen Z, Qiu J, Shi X, Lu Z: Quantitative analysis of promoter hypermethylation in multiple genes in osteosarcoma. *Cancer* 2006, 106(7):1602-1609.
301. Muthusamy V, Duraisamy S, Bradbury CM, Hobbs C, Curley DP, Nelson B, Bosenberg M: Epigenetic silencing of novel tumor suppressors in malignant melanoma. *Cancer research* 2006, 66(23):11187-11193.
302. Cantor JP, Iliopoulos D, Rao AS, Druck T, Semba S, Han SY, McCorkell KA, Lakshman TV, Collins JE, Wachsberger P *et al*: Epigenetic modulation of endogenous tumor suppressor expression in lung cancer xenografts suppresses tumorigenicity. *International journal of cancer* 2007, 120(1):24-31.
303. Shi H, Wei SH, Leu YW, Rahmatpanah F, Liu JC, Yan PS, Nephew KP, Huang TH: Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation. *Cancer research* 2003, 63(9):2164-2171.
304. Kim TY, Zhong S, Fields CR, Kim JH, Robertson KD: Epigenomic profiling reveals novel and frequent targets of aberrant DNA methylation-mediated silencing in malignant glioma. *Cancer research* 2006, 66(15):7490-7501.
305. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA: Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. *Mol Pharmacol* 2004, 65(1):18-27.

306. Karpf AR, Jones DA: Reactivating the expression of methylation silenced genes in human cancer. *Oncogene* 2002, 21(35):5496-5503.
307. Schmelz K, Sattler N, Wagner M, Lubbert M, Dorken B, Tamm I: Induction of gene expression by 5-Aza-2'-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. *Leukemia* 2005, 19(1):103-111.
308. Eramo A, Pallini R, Lotti F, Sette G, Patti M, Bartucci M, Ricci-Vitiani L, Signore M, Stassi G, Larocca LM *et al*: Inhibition of DNA methylation sensitizes glioblastoma for tumor necrosis factor-related apoptosis-inducing ligand-mediated destruction. *Cancer research* 2005, 65(24):11469-11477.
309. Xu J, Zhou JY, Tainsky MA, Wu GS: Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2'-deoxycytidine sensitizes human breast cancer cells to adriamycin. *Cancer research* 2007, 67(3):1203-1211.
310. Larsen F, Gundersen G, Lopez R, Prydz H: CpG islands as gene markers in the human genome. *Genomics* 1992, 13(4):1095-1107.
311. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D: Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics* 2007, 39(4):457-466.
312. Zhu WG, Dai Z, Ding H, Srinivasan K, Hall J, Duan W, Villalona-Calero MA, Plass C, Otterson GA: Increased expression of unmethylated CDKN2D by 5-aza-2'-deoxycytidine in human lung cancer cells. *Oncogene* 2001, 20(53):7787-7796.
313. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA: Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer research* 2002, 62(22):6456-6461.
314. Lavelle D, DeSimone J, Hankewych M, Kousnetzova T, Chen YH: Decitabine induces cell cycle arrest at the G1 phase via p21(WAF1) and the G2/M phase via the p38 MAP kinase pathway. *Leuk Res* 2003, 27(11):999-1007.
315. Gallicchio MA, Kneen M, Hall C, Scott AM, Bach LA: Overexpression of insulin-like growth factor binding protein-6 inhibits rhabdomyosarcoma growth in vivo. *International journal of cancer* 2001, 94(5):645-651.
316. Yan T, Wergedal J, Zhou Y, Mohan S, Baylink DJ, Strong DD: Inhibition of human osteoblast marker gene expression by retinoids is mediated in part by insulin-like growth factor binding protein-6. *Growth Horm IGF Res* 2001, 11(6):368-377.
317. Gallicchio MA, van Sinderen M, Bach LA: Insulin-like growth factor binding protein-6 and CCI-779, an ester analogue of rapamycin, additively inhibit rhabdomyosarcoma growth. *Horm Metab Res* 2003, 35(11-12):822-827.

318. Liao B, Hu Y, Herrick DJ, Brewer G: The RNA-binding protein IMP-3 is a translational activator of insulin-like growth factor II leader-3 mRNA during proliferation of human K562 leukemia cells. *The Journal of biological chemistry* 2005, 280(18):18517-18524.
319. Zumkeller W, Groth O, Commentz J: Regulation of insulin-like growth factors and IGF-binding proteins in bone tumours. *Growth regulation* 1996, 6(1):10-15.
320. Savage SA, Woodson K, Walk E, Modi W, Liao J, Douglass C, Hoover RN, Chanock SJ: Analysis of genes critical for growth regulation identifies Insulin-like Growth Factor 2 Receptor variations with possible functional significance as risk factors for osteosarcoma. *Cancer Epidemiol Biomarkers Prev* 2007, 16(8):1667-1674.
321. Campbell PG, Novak JF: Insulin-like growth factor binding protein (IGFBP) inhibits IGF action on human osteosarcoma cells. *Journal of cellular physiology* 1991, 149(2):293-300.
322. Slootweg MC, Ohlsson C, Salles JP, de Vries CP, Netelenbos JC: Insulin-like growth factor binding proteins-2 and -3 stimulate growth hormone receptor binding and mitogenesis in rat osteosarcoma cells. *Endocrinology* 1995, 136(10):4210-4217.
323. Richter TM, Tong BD, Scholnick SB: Epigenetic inactivation and aberrant transcription of CSMD1 in squamous cell carcinoma cell lines. *Cancer Cell Int* 2005, 5:29.
324. Jin S, Mazzacurati L, Zhu X, Tong T, Song Y, Shujuan S, Petrik KL, Rajasekaran B, Wu M, Zhan Q: Gadd45a contributes to p53 stabilization in response to DNA damage. *Oncogene* 2003, 22(52):8536-8540.
325. Takekawa M, Saito H: A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* 1998, 95(4):521-530.
326. Sheikh MS, Hollander MC, Fornance AJ, Jr.: Role of Gadd45 in apoptosis. *Biochemical pharmacology* 2000, 59(1):43-45.
327. Daino K, Ichimura S, Nenoi M: Comprehensive search for X-ray-responsive elements and binding factors in the regulatory region of the GADD45a gene. *Journal of radiation research* 2003, 44(4):311-318.
328. Barsyte-Lovejoy D, Mao DY, Penn LZ: c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism. *Oncogene* 2004, 23(19):3481-3486.
329. Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, Fornace AJ, Jr.: Dimethylbenzanthracene carcinogenesis in Gadd45a-null mice is associated with decreased DNA repair and increased mutation frequency. *Cancer research* 2001, 61(6):2487-2491.
330. Ying J, Srivastava G, Hsieh WS, Gao Z, Murray P, Liao SK, Ambinder R, Tao Q: The stress-responsive gene GADD45G is a functional tumor suppressor, with its response to environmental stresses frequently disrupted epigenetically in multiple tumors. *Clin Cancer Res* 2005, 11(18):6442-6449.

331. Wang W, Huper G, Guo Y, Murphy SK, Olson JA, Jr., Marks JR: Analysis of methylation-sensitive transcriptome identifies GADD45a as a frequently methylated gene in breast cancer. *Oncogene* 2005, 24(16):2705-2714.
332. Al-Romaih KI, Somers GR, Bayani J, Hughes S, Prasad M, Cutz JC, Xue H, Zielenska M, Wang Y, Squire JA: Modulation by decitabine of gene expression and growth of osteosarcoma U2OS cells in vitro and in xenografts: Identification of apoptotic genes as targets for demethylation. *Cancer Cell Int* 2007, 7(1):14.
333. Billiau A, Edy VG, Heremans H, Van Damme J, Desmyter J, Georgiades JA, De Somer P: Human interferon: mass production in a newly established cell line, MG-63. *Antimicrobial agents and chemotherapy* 1977, 12(1):11-15.
334. Meyers PA, Heller G, Healey J: Retrospective review of neoadjuvant chemotherapy for osteogenic sarcoma. *Journal of the National Cancer Institute* 1992, 84(3):202-204.
335. Ferrari S, Briccoli A, Mercuri M, Bertoni F, Cesari M, Longhi A, Bacci G: Late relapse in osteosarcoma. *J Pediatr Hematol Oncol* 2006, 28(7):418-422.
336. Jung HJ, Kim EH, Mun JY, Park S, Smith ML, Han SS, Seo YR: Base excision DNA repair defect in Gadd45a-deficient cells. *Oncogene* 2007.
337. Barreto G, Schafer A, Marhold J, Stach D, Swaminathan SK, Handa V, Doderlein G, Maltry N, Wu W, Lyko F *et al*: Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* 2007, 445(7128):671-675.
338. Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ, Jr., Harris CC: GADD45 induction of a G2/M cell cycle checkpoint. *Proceedings of the National Academy of Sciences of the United States of America* 1999, 96(7):3706-3711.
339. Daino K, Ichimura S, Nenoi M: Both the basal transcriptional activity of the GADD45A gene and its enhancement after ionizing irradiation are mediated by AP-1 element. *Biochimica et biophysica acta* 2006, 1759(10):458-469.
340. Kansara M, Thomas DM: Molecular pathogenesis of osteosarcoma. *DNA and cell biology* 2007, 26(1):1-18.
341. Mita H, Tsutsui J, Takekawa M, Witten EA, Saito H: Regulation of MTK1/MEKK4 kinase activity by its N-terminal autoinhibitory domain and GADD45 binding. *Molecular and cellular biology* 2002, 22(13):4544-4555.
342. Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ, Jr.: Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. *Cancer research* 2002, 62(24):7305-7315.
343. Zerbini LF, Wang Y, Czibere A, Correa RG, Cho JY, Ijiri K, Wei W, Joseph M, Gu X, Grall F *et al*: NF-kappa B-mediated repression of growth arrest- and DNA-damage-inducible proteins 45alpha and gamma is essential for cancer cell survival. *Proceedings of the National Academy of Sciences of the United States of America* 2004, 101(37):13618-13623.

344. Zhang D, Song L, Li J, Wu K, Huang C: Coordination of JNK1 and JNK2 is critical for GADD45alpha induction and its mediated cell apoptosis in arsenite responses. *The Journal of biological chemistry* 2006, 281(45):34113-34123.
345. Yamauchi J, Miyamoto Y, Murabe M, Fujiwara Y, Sanbe A, Fujita Y, Murase S, Tanoue A: Gadd45a, the gene induced by the mood stabilizer valproic acid, regulates neurite outgrowth through JNK and the substrate paxillin in N1E-115 neuroblastoma cells. *Experimental cell research* 2007, 313(9):1886-1896.
346. Smith LT, Lin M, Brena RM, Lang JC, Schuller DE, Otterson GA, Morrison CD, Smiraglia DJ, Plass C: Epigenetic regulation of the tumor suppressor gene TCF21 on 6q23-q24 in lung and head and neck cancer. *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103(4):982-987.
347. Affymetrix: NetAffx. *(http://wwwaffymetrixcom)*.
348. UCSC: Genome Browser Data Base. *(http://genomeucscedu/)*.
349. Xie H, Hu Z, Chyna B, Horrigan SK, Westbrook CA: Human mortalin (HSPA9): a candidate for the myeloid leukemia tumor suppressor gene on 5q31. *Leukemia* 2000, 14(12):2128-2134.
350. Takai D, Jones PA: The CpG island searcher: a new WWW resource. *In Silico Biol* 2003, 3(3):235-240.
351. Rubinstein R, Simon I: MILANO--custom annotation of microarray results using automatic literature searches. *BMC Bioinformatics* 2005, 6:12.
352. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003, 4(5):P3.