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Histone H2A exogène induit la différenciation et la sénescence des cellules cancéreuses

par
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Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de Maître ès sciences (M.Sc.)
en Pathologie et Biologie cellulaire
option Biologie du cancer

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Ce mémoire intitulé :

Histone H2A exogène induit la différenciation et la sénescence des cellules cancéreuses

Présenté par
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RÉSUMÉ

Malgré les progrès récents dans le traitement contre le cancer, il existe encore beaucoup de défis à relever dans ce domaine. La présente étude visait d'abord à évaluer le potentiel de l'histone exogène H2A du thymus de veau à inhiber la prolifération des cellules cancéreuses, puis, à en disséquer le mécanisme d'action. Bien que les histones participent principalement à l'organisation de la chromatine, dans le compartiment intranucléaire, ces protéines exercent aussi d'importantes fonctions hors du noyau. Dans cette étude, nous démontrons que l'histone H2A inhibe la croissance des cellules cancéreuses, réduit la capacité des cellules à croître dans un milieu semi-solide d'agarose (« anchorage-independent growth ») et provoque l'arrêt du cycle cellulaire. Nous démontrons également que l'histone H2A induit la différenciation et la sénescence des cellules du cancer du sein. Le mécanisme d'action de l'histone H2A implique l'augmentation de l'expression de p21, un inhibiteur du cycle cellulaire ou de p53, un gène suppresseur de tumeurs, dépendamment de la lignée cellulaire analysée. Les modifications post-traductionnelles de l'histone H2A conditionnent ses effets anti-prolifératifs. Tandis que l'histone H2A du thymus de veau contient autant la forme acétylée que non-acétylée de l'histone H2A, l'histone humaine recombinante H2A exprimée dans les bactéries ne contient que la forme non-acétylée et n'exerce aucun effet sur la prolifération des cellules MCF-7. Somme toute, ces résultats suggèrent que l'histone H2A pourrait devenir un agent anti-prolifératif ayant des applications dans la thérapie anti-tumorale.

Mots clés : histone H2A, cancer du sein, sénescence, différenciation, épigénétique, inhibiteurs de l'histone déacétylase (HDACi), p21, p53.

ABSTRACT

Despite the recent progress made in cancer therapy, there remain outstanding challenges in cancer therapeutics. The present study was designed to evaluate whether exogenous calf thymus histone H2A inhibits the proliferation of cancer cells, and to dissect out its mechanism of action. While the physiological compartment of histones is within the nucleus, where they participate in chromatin organization, extranuclear functions are also being characterized. In this study we show that exogenous calf thymus histone H2A arrests cell growth, reduces anchorage-independent growth capacity and induces cell cycle arrest in the MCF-7 human breast adenocarcinoma cell line. Moreover, we show for the first time that exogenous calf thymus histone H2A induces cellular differentiation and senescence in breast cancer cells. The mechanism underlying histone H2A effects involved cell cycle inhibitor p21 and tumor suppressor gene p53 in a cell line-dependent manner. While calf thymus histone H2A, which contains both acetylated and unacetylated histones, inhibits the proliferation of MCF-7 cells, recombinant human histone H2A expressed in bacteria is ineffective under the same conditions. Thus, we suggest that the anti-proliferative effects of histone H2A require the presence of post-translational modifications. In conclusion, these results suggest that histone H2A may be useful as an anti-proliferative agent in cancer therapy.

Keywords: histone H2A, breast cancer, senescence, differentiation, epigenetics, histone deacetylases inhibitors (HDACi), p21, p53.

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LISTE DES ABRÉVIATIONS

5-Aza-CR : 5-azacytidine

5-Aza-CdR : 5-aza-2-deoxycytidine

ABC transporters : ATP-binding cassette transporters

AchE : acétylcholinestérase

ALT : alternative lengthening of telomeres

APL : acute promyelocytic leukemia

ATM : ataxia telangiectasia mutated

ATR : ATM and Rad3-related

ATRA : all-trans-retinoic acid

BCRP : breast cancer resistance protein

CIPA : comité institutionnel de protection des animaux du CHUM

Chk2 : checkpoint kinase 2

CPP : cell-penetrating proteins

CRCHUM: Centre hospitalier de l'Université de Montréal

CTA : cancer/testis antigenes

CTCL : cutaneous T-cell lymphoma

DNA-PK : DNA-dependent protein kinase

DNMT : DNA methyltransferase

DNMTi : DNA methyltransferase inhibitors

DTNB : dithiobisnitrobenzoate

ER : estrogen receptor

FDA : food and drug administration

HDAC : histone deacetylases

HDACi : histone deacetylases inhibitors

hTERT: human telomerase reverse transcriptase

MDR1 : multidrug resistance transporter 1

MDS : myelodysplastic syndrome

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC : N-acetyl-L-cysteine

P-gp : p-glycoprotein

PI : propidium iodide

PI3-K : phosphatidyl-inositol-3-kinase

PTD : protein transduction domain

RAR : retinoic acid receptor

ROS : reactive oxygen species

SAHA : suberoxylanilide hydroxamic acid

SA-β-gal : senescence-associated β-galactosidase

Smc 1 : structural maintenance of chromosomes

SP : side population

TSA : trichostatin A

X-gal : 5-bromo-4-chloro-3-indoyl β -galactoside

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1. INTRODUCTION

Le cancer est la première cause de décès au Québec, selon la Société canadienne du cancer (http://www.cancer.ca/ccs/internet/niw_splash/0%2C%2C3172%2C00.html). La mise au point d'un traitement efficace contre le cancer qui ne comporterait que très peu d'effets secondaires demeure un objectif important pour la communauté scientifique.

L'épigénétique a pour objet l'étude des changements transmissibles de l'expression des gènes indépendants des changements de la séquence d'ADN (Goldberg et al. 2007;Yoo and Jones 2006).

Le cancer est une maladie associée à des altérations à la fois génétiques et épigénétiques. Les altérations génétiques, telles que les modifications dans la séquence des gènes, sont difficiles à renverser, en revanche les altérations épigénétiques, comme l'acétylation des histones et la méthylation de l'ADN, sont beaucoup plus accessibles à la réparation. C'est pourquoi des médicaments épigénétiques ont été développés afin de corriger les altérations épigénétiques qui caractérisent le développement et la progression du cancer.

Récemment, plusieurs études ont rapporté un rôle des histones dans la croissance des cellules cancéreuses. Nos propres résultats suggèrent que l'histone H2A exogène purifiée à partir de thymus de veau inhibe la prolifération des cellules cancéreuses.

La revue de la littérature de ce mémoire de maîtrise a été présentée sous forme d'un article de revue. Cet article expose les fonctions classiques des histones ainsi que de nouvelles fonctions qui commencent à être décrites. Cette revue présente

aussi un aperçu de la thérapie épigénétique. Enfin, un nouveau modèle pour la thérapie épigénétique y est décrit.

2. ARTICLE #1 : HISTONE TAIL MODIFICATIONS AND NON-CANONICAL FUNCTIONS OF HISTONES: PERSPECTIVES IN CANCER EPIGENETICS

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ARTICLE PUBLIÉ DANS

"MOLECULAR CANCER THERAPEUTICS"

2.1. CONTRIBUTION PERSONNELLE :

Pour cet article j'évalue ma contribution à 80%.

Histone tail modifications and non-canonical functions of histones: perspectives in cancer epigenetics

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Research Centre and Department of Medicine§, Hôtel-Dieu du Centre hospitalier de l'Université de Montréal (CHUM) and Department of Medicine† and Biology and Cellular Pathology‡, Université de Montréal, Montréal, Québec, Canada

Running Title: Histones and cancer therapy

Keywords: epigenetics, histones, HDAC inhibitors, methylation, acetylation

Abbreviation List: HDAC (histone deacetylases), HDACi (histone deacetylase inhibitors), DNMT (DNA methyltransferases), DNMTi (DNA methyltransferase inhibitors).

[1] D.B. is a scholar of Fonds de la recherche en santé du Québec (FRSQ). A.H. is a recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC) graduate scholarship. This study was also made possible through a Canadian Institutes of Health Research (CIHR) operating grant to D.B..
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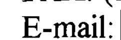
E-mail: 

2.2. ABSTRACT:

Over the past few years histone deacetylase (HDAC) inhibitors have come to occupy an important place in the effort to develop novel, but less toxic anti-cancer therapy. HDAC inhibitors block HDAC which are the enzymes responsible for histone deacetylation and therefore, they modulate gene expression. The cellular effects of HDAC inhibitors include growth arrest and the induction of differentiation. Early successes in cancer therapeutics obtained using these drugs alone or in combination with other anti-cancer drugs emphasize the important place of post-translational modifications of histones in cancer therapy. Histone tail modifications along with DNA methylation are the most studied epigenetic events related to cancer progression. Moreover, extranuclear functions of histones have also been described. Since HDAC inhibitors block HDACs and thereby increase histone acetylation, we propose a model wherein exogenous acetylated histones or other related acetylated proteins that are introduced into the nucleus become HDAC substrates, and thereby compete with endogenous histones for HDACs. This competition may lead to the increased acetylation of the endogenous histones, as in the case of HDAC inhibitor therapy. Moreover, other mechanisms of action, such as binding to chromatin and modulating gene expression is also possible for exogenously introduced histones.

2.3. HISTONES

Histones are the most abundant proteins bound to DNA in eukaryotic cells and amongst the most evolutionary conserved proteins known (1). They are small basic proteins with a molecular weight between 11 and 20 kilodalton (Kda) and they contain a high percentage of positively charged amino acids (approximately 20%), such as lysine and arginine. Eukaryotic cells contain mainly five types of histones: histone linker H1 and core histones H2A, H2B, H3 and H4. All four core histones, i.e. histone H2A, H2B, H3 and H4, share a similar structure, with a central "fold domain" and terminal "tails", N-terminal and C-terminal (2). The fold domain contributes to octamer histone assembly and terminal tails are crucial for the normal functioning of cellular processes, including replication and transcription (3) as they are targets for post-translational modifications: acetylation, methylation, phosphorylation and ubiquitination (4). Linker histone H1 binds nucleosomes together and thus participates in a higher order compaction of chromatin (5).

Beside these canonical histones, several histone variants have been described such as histone H2A.X implicated in DNA repair and genomic stability (6) and histone H1.2, a pro-apoptotic protein that is translocated from the nucleus to mitochondria (7) .

2.4. EPIGENETICS IN CANCER

The term "epigenetics" was introduced by Conrad Waddington (Waddington, 1942 cited by (8)). Epigenetics is the study of heritable changes in gene expression that are not related to changes in DNA sequence (4;8).

In eukaryotic cells, DNA is wrapped around histones and thus, is limited in its accessibility in biological processes such as replication, transcription, and DNA repair. Therefore, cellular mechanisms of chromatin modulation were identified in cells, including recruitment of nucleosome remodeling factors, such as SWI/SNF (9) and modulation of the contact between DNA and histones through post-translational modifications of histones (3).

Epigenetics changes include DNA methylation, histone modifications, chromatin remodeling and expression of microRNAs (10). These processes may be deregulated in several diseases, including cancer, neurological and cardiovascular disease.

Cancer is characterized by a deregulation in normal cell proliferation caused by genetic or epigenetic alterations. Genetic alterations of various genes implicated in normal cell proliferation such as tumor suppression genes lead to abnormal protein expression encoded by these genes and thus to loss of normal functioning of these proteins. Histone tail modifications, such as acetylation, methylation, phosphorylation and ubiquitination along with DNA methylation are the most studied epigenetic events related to cancer progression (4). Epigenetic events modulate gene expression without modification of primary gene sequence. For example, hypermethylation of DNA promoter regions of genes that control normal cell proliferation, such as tumor suppression genes, is associated with gene silencing and thus, with tumor progression (11). It was suggested that the DNA hypermethylome can be associated with tumor aggressiveness and it may be used as a clinical marker in cancer cell characterization. Fiegl et al. (12) suggested that HER-2/neu positive

aggressive breast cancer cells are characterized by a specific DNA methylation profile. They identified 3 genes: PGR (coding for the progesterone receptor), HSD17B4 (coding for type 4 17-beta-hydroxysteroid dehydrogenase, an enzyme involved in estrogen metabolism), and CDH13 (coding for H-cadherin) whose DNA methylation correlates with Her2/neu status and, subsequently they suggested that this methylation profile may explain the aggressiveness and reduced responsiveness to antiestrogen treatment.

Thus, epigenetic events play an important role in cancer development and progression and an understanding of these events has recent led to advances in epigenetic therapy. Moreover, epigenetic modifications may also be implicated in prognosis and drug response of the patients. Thus, Esteller et al. (13) have demonstrated that methylation of the DNA-repair enzyme O^6-methyl-guanine-DNA methyltransferase (MGMT) promoter, an enzyme involved in resistance to alkylating agents, can be used as a predictor of responsiveness to treatment with these drugs. Moreover, they suggested that MGMT methylation may be a better prognostic factor than those used classically, such as tumor grade and patient age.

2.4.1. HISTONE ACETYLATION

Histone acetylation and deacetylation play an important role in chromatin remodeling and, thus, in gene expression. There is a fine balance between acetylation and deacetylation of histones in normal cells, and the enzymes catalyzing these modifications are histone acetyltransferases (HAT) and histone deacetylases (HDAC) respectively (14). While histone acetylation is associated with an open chromatin and

enhanced transcription, histone deacetylation is associated with closed chromatin and transcriptional repression. For example, acetylation of N-terminal core histones facilitate the recruitment of transcription factors, such as TFIIIA (15) and histone H3 and H4 acetylation is associated with an open $(H3\text{-}H4)_2$ tetrameric particle allowing access of transcriptional machinery to DNA (3).

Along with transcriptional modulation, histone acetylation plays an important role in other biological processes such as replication and DNA repair, as histone acetylation facilitates the movement of the replication machinery along the DNA strand (16) and creates a favorable environment for DNA repair (17).

2.4.2. DNA METHYLATION

Methylation of CpG islands (DNA regions characterized by a high incidence of the nucleotide pair C followed by G) is an epigenetic event characterized by the transfer of a methyl group to the C-5 position of cytosine. This process is catalyzed by DNA methyltransferases (DNMT) (18).

Changes in DNA methylation can result in either DNA hypermethylation or DNA hypomethylation. Both modifications have been identified in cancer cells. Gene promoter hypermethylation and global gene hypomethylation play important roles in tumorigenesis (4). Gene promoter hypermethylation was associated with the inhibition of cancer-related genes such as tumor suppressor genes and DNA mismatch repair genes (4;11). DNA methylation is associated with chromatin compaction as the loss of DNA methylation alters the binding of the linker histone H1 (19). DNA hypomethylation, the first epigenetic event identified in cancer cells

(11), can lead to gene activation, such as oncogenes (11) and to genomic instability (4).

Following the completion of the human genome project which was designed to identify all human genes, a human epigenome project has been launched to identify DNA methylation sites in human genes in major tissues (10). This project acknowledges the role of epigenetic modifications in human diseases such as cancer. Epigenetic modifications play an integral role in cancer. For example, Espada et al. (18) have demonstrated that the loss of DNA methylation in cells lacking DNMT1 is associated with an increase of the acetylation and decrease of the methylation of histone H3. These results are not surprising since a specific interaction between DNMT1 and HDAC has also been reported (20). Moreover, DNMT1 itself is associated with deacetylase activity (20).

2.5. EPIGENETIC DRUGS

The implications of epigenetic events in gene expression and DNA repair, and therefore in the tumorigenesis process, make them a valuable target for cancer therapy. Tumorigenesis is associated with genetic and epigenetic alterations. While genetic alterations such as gene deletions cannot be reversed, certain epigenetic alterations can. Thus, the rationale for epigenetic therapy is reactivation of the expression of several genes silenced by epigenetic events during tumorigenesis. As we mentioned previously, abnormal histone acetylation and DNA methylation were identified in cancer cells. Thus, drugs that target these epigenetic alterations are being studied, and are termed epigenetic drugs. There are two classes of epigenetic

drugs that are currently being investigated: histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi).

2.5.1. HISTONE DEACETYLASE INHIBITORS (HDACI)

Alterations of histone acetylation are reported in cancers (21). As mentioned previously, two types of enzymes, HDAC and HAT, modulate histone acetylation. The deregulation of HDAC functions have been associated with hematological cancers, e.g. HDACs are recruited by the acute promyelocytic leukemia fusion protein (PML-RARα) (22). They have also been associated with solid tumors, e.g. the breast and ovarian cancer susceptibility gene BRCA1 is associated with HDACs, as BRCA1 interacts with components of the HDAC complex (23).

The mechanism of action of HDACi is not entirely understood. The rationale behind the development of HDACis was that these inhibitors lead to an increased acetylation of histones and thus, might reactivate genes, such as the cell cycle inhibitor p21, that are silenced during carcinogenesis (24). However, recently other mechanisms of action of HDACi have been identified such as the generation of oxidative stress (25) and induction of premature sister chromatid separation that renders the mitotic spindle assembly checkpoint ineffective (26).

Several HDACis are currently being investigated, including suberoylanilide hydroxamic acid (SAHA) (27), suberoyl-3-aminopyridineamide hydroxamic acid (pyroxamide) (28) and the benzamide derivate MS-275 (29). SAHA obtained U.S. Food and Drug Administration (FDA) approval for clinical use for the treatment of

cutaneous T-cell lymphoma (CTCL)[2] and, currently, it is in a phase III clinical trial for the treatment of advanced mesothelioma[3].

2.5.2. DNA METHYLTRANSFERASE INHIBITORS (DNMTI)

Although the global low level of gene methylation is associated with cancer, hypermethylation was observed in the promoter region of several genes implicated in carcinogenesis, which correlates with gene silencing (4). DNMTi, nucleoside and non-nucleoside analogues, are epigenetic drugs that target DNA hypermethylation. While nucleoside analogues require DNA incorporation for DNMT inhibition, non-nucleoside analogues can block DNMT directly without DNA incorporation (4). Inhibition of enzymes responsible for DNA methylation during the process of tumorigenesis results in reactivation of previously silenced cancer related genes, such as tumor suppression genes, DNA mismatch repair genes and cell cycle related genes (30).

Recently, it has been suggested that epigenetic modifications, such as methylation can regulate the expression of microRNAs (31). They are small RNA molecules encoded in the genome and they control expression of several genes by translational repression. For example, Saito et al. (31), have demonstrated that DNMTi treatment increases the expression of microRNA-127, a member of the microRNA family. The target of this microRNA is a proto-oncogene and therefore, the authors suggested that up-regulation of microRNA, by epigenetic therapy may be a novel strategy in cancer treatment.

[2] U.S. Food and Drug Administration (FDA) http://www.fda.gov/

There are several DNMTi under preclinical and clinical investigation, two of them, members of nucleoside analogues family, 5-azacytidine (5-Aza-CR) and 5-aza-2̀-deoxycytidine (5-Aza-CdR) obtained Food and Drug Administation (FDA) approval for clinical use in the treatment of myelodysplastic syndrome (MDS)[1]. Zebularine (1-[β-D-ribofuranosil]-1,2-dihydropyrimidin-2-1) is a 5-Aza-CR derivative that has been recently described as a novel DNMTi which is more stable and less toxic compared with 5-Aza-CR and 5-Aza-CdR (32). As these drugs are incorporated into DNA, they are associated with cytotoxicity (33).

Nonnucleoside DNMTi are also under investigation. For example, MG98, a phosphorothioate antisense oligodeoxynucleotide that is a specific inhibitor of mRNA for human DNA methyltransferase 1 (DNMT1) is currently being tested in clinical trials (34). Fini et al. (35) have evaluated the anticancer effect of Annurca polyphenol extract (APE) in sporadic colorectal cancers (CRC). They demonstrated that APE functions as DNMTi with comparable effects to those obtained with 5-Aza-CdR, but with no side effects, including myelossupression as reported for 5-Aza-CdR.

Although epigenetic drugs can be used as monotherapy, their effects can be optimized by combination therapies, such as combinations of demethylating agents and HDACi (30;36). Mongan et al. (36) have demonstrated that the combination of valproic acid, a short-chain fatty acid structurally related to the butyrate class of HDACi with 5-Aza-CdR, a DNMTi and with retinoic acid lead to reactivation of the tumor suppression gene RARβ2 that is epigenetically silenced in breast cancer cells.

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[3] U.S. National Institutes of Health http://clinicaltrials.gov/

Epigenetic drugs can be used in combination with each other, but also with chemotherapeutic agents. For example, it has been demonstrated in a phase I trial that the administration of valproic acid, an HDACi, with epirubicin, an anthracycline antitumor antibiotic, can improve the patients' response to epirubicin. Moreover, an antitumor response was obtained in patients with anthracycline-resistant tumors (37). A promising application of combination therapy is the reactivation of the estrogen receptor (ER) in breast cancer, followed by antiestrogen treatment (38). In general, ER+ tumors are characterized by a better prognosis and treatment outcome compared to ER- tumors. Loss of ER expression in breast cancer is caused by genetic events, such as DNA sequence mutation (30), but also by epigenetic events, such as hypermethylation of ER promoter (38). Sharma et al. (38) have demonstrated that treatment of the MDA-MB-231 ER- breast cancer cell line with a combination of trichostatin, an HDACi and 5-aza-CdR, a DNMTi can restore ER expression, and thus became sensitive to hormonal therapy, such as tamoxifen, an antiestrogen agent. An interesting combination therapy includes epigenetic drugs and cancer immunotherapy. Development of tumor-associated antigen-directed vaccines is one strategy involved in cancer immunotherapy but, tumor-associated antigens are characterized by a limited and heterogeneous expression in tumors. Sigalotti et al. (39) have demonstrated that the expression of cancer antigens is related to methylation status of their promoter. Therefore, they have shown that DNMTi treatment induces expression of cancer/testis antigenes (CTA) and thus, it may improve CTA-directed immunotherapy. Thus, it is hoped that combination therapy

including epigenetic drugs will overcome some of the resistance to therapy that we currently face.

Most conventional cancer therapies, such as chemotherapy and radiation therapy are highly toxic and non-specific. Advances made in our understanding of the molecular basis of cancer facilitated the development of novel drugs, directed more specifically to the cancer cell. This is the case of imatinib mesylate, an ABL kinase inhibitor effective against chronic myeloid leukemia and trastuzumab, a monoclonal antibody against HER-2 receptor, which is effective against breast cancer cells that overexpress HER-2 receptor. It has been suggested that cancer epigenetic therapy may also be specific to cancer cells. Thus, Ungerstedt et al. (40) have shown that HDACi induce cell death in transformed cells, while normal cells are relatively resistant. One possible explanation for this resistance is the increased expression of thioredoxin, a protein implicated in cell protection against oxidative stress. Thus, increased expression of thireodoxin in normal cells, but not in cancer cells, may protect cells against the cytotoxic effects of HDACi.

The induction of DNA damage is the mechanism underlying cancer cell death following chemotherapy or radiation therapy, which will activate cellular DNA damage response and trigger apoptosis (7). Besides apoptosis, other strategies may be used to induce cancer cell growth arrest, including cellular differentiation and senescence. This review will focus on differentiation and senescence, as these two mechanisms were often reported to be associated with epigenetic drugs.

2.5.3. DIFFERENTIATION THERAPY

The absence of the cellular differentiation, or anaplasia, is a hallmark of malignant tumors and it is associated with morphological and functional changes. The morphological changes of anaplastic cells include alterations in the size and cellular morphology (pleomorphism) and a higher nucleus-cytoplasmic ratio as compared with normal cells. A highly transformed cell is undifferentiated, and loses the functional characteristics of the normal cell of origin. In contrast, differentiated cells preserve the functional properties of normal cells.

As it is well accepted that undifferentiated tumors are associated with a poor prognosis, and hence the induction of differentiation is a promising strategy in cancer therapy. The rationale behind differentiation therapy lies in the finding that undifferentiated cancer cells are characterized by an unlimited potential to proliferate and, thus, the induction of differentiation will halt their proliferative capacity. The underlying molecular mechanisms include the induction of specific gene expression, e.g. p21 a cell cycle inhibitor (29).

One of the most successful differentiation agents tested so far is all-trans-retinoic acid (ATRA) used in the treatment of acute promyelocytic leukemia (APL) (22). Under normal physiological conditions retinoic acid binds to the retinoic acid receptor (RARα) and releases the histone deacetylase complex leading to transcriptional activation and hematopoietic cell maturation. While this pathway is disrupted in APL, high levels of retinoic acid can restore this pathway and allow the maturation arrest (22). However, after promising initial remissions obtained in the patients treated with retinoic acid, many of these patients acquire retinoid resistance. Lin et al. (41) made the assumption that this resistance is the result of the constitutive

activation of histone deacetylases and subsequently, they demonstrate that the association of Thichostatin A (TSA), an HDACi with retinoic acid enhances the differentiating effect of retinoic acid and may overcome retinoic acid resistance.

Induction of differentiation by HDACi is not limited to hematopoietic tumors, but can also be used in the treatment of solid tumors. For example, Munster et al. (27) have shown that SAHA induces growth arrest and differentiation in human breast cancer cells.

2.5.4. INDUCTION OF CELLULAR SENESCENCE

Cellular senescence is a state of irreversible growth arrest associated with morphological and functional changes. Senescent cells are characterized by the presence of the senescence-associated β-galactosidase marker (SA-β-gal). This enzyme is a lysosomal hydrolase active at pH 4 in normal cells, but also active at pH 6 in senescent cells. The increase in SA-β-gal activity at pH 6 is a result of the increased lysosomal content in senescent cells (42). Hayflick et al. (43) made the initial suggestion that cells can divide in culture a finite number of times, and beyond that they will stop dividing. This phenomenon is called replicative senescence. Two signals can trigger senescence: telomeres shortening which is associated with replicative senescence and cellular stress exposure associated with stress or aberrant signaling-induced senescence (STASIS) (44).

One important step in the carcinogenesis process is overcoming normal cellular senescence and acquiring limitless replicative potential. Maintenance of telomere length is mandatory for a cell to acquire the immortal phenotype. Two

mechanisms are implicated in this process. The first one is the reactivation of telomerase, the enzyme responsible for the maintenance of telomere length in most cancer cells and the second is the alternative lengthening of telomeres (ALT) by intra-telomeric recombination mechanism in cancer cells that do not express the telomerase. Epigenetic mechanisms appear to be involved in both processes. While, telomerase reactivation is associated with histone H3 and H4 hyperacetylation, lack of telomerase expression in ALT cells is associated with histone H3 and H4 hypoacetylation (45).

It has been suggested that since cellular senescence is associated with growth arrest, the induction of senescence can be applied to cancer therapeutics (46). It has been demonstrated that chemotherapeutic drugs, such as etoposide (47) and differentiation agents, such as retinoic acid (48) can trigger cellular senescence. The induction of cellular senescence has been also reported for epigenetic drugs, i.e. HDACi (49) and DNMTi (50).

2.6. "NON-CANONICAL" FUNCTIONS OF HISTONES: PERSPECTIVES IN CANCER THERAPEUTICS

While most of the focus on histones is classically involved in chromatin organization in the nucleus, the extracellular localization of histones has also been identified. For example, extracellular histones associated with DNA, i.e. nucleosomes, are found in the circulation under various pathological conditions, such as autoimmune disease (51). Patients with malignant diseases undergoing chemotherapy also have higher levels of circulating nucleosomes, which correlates with a higher rate of cellular death caused by chemotherapy (52).

Extracellular histones have been also located on the surface of the human T HPB-ALL cell line and phytohemaglutinin (PHA)-activated human peripheral blood lymphocytes (53). Rose et al. (54) have shown that ileal epithelial cells release histone H1, while undergoing apoptosis, and that histone H1 possesses antimicrobial activity.

Histone participation in chromatin organization, gene expression and DNA repair is well documented. For example, histone H2B participates in post-replicational DNA repair (PRR) (55) and in the cellular response to double-stranded DNA breaks (DSB) (56). It was also demonstrated that histone H2B is phosphorylated in apoptotic cells and, thus, it may be used as a hallmark of apoptotic cells (57). The increased acetylation of histones H3 and H4 has been systematically reported after treatment with HDACi (24;28) and it was associated with transcriptional activation of several genes implicated in tumor growth suppression (24).

By "non-canonical" functions of histones we understand those functions that are not related to chromatin organization. It has been demonstrated that linker histone H1, as well as core histones H2A, H2B, H3 and H4, can be used as transfection agents to deliver DNA into various cells (reviewed in (58). In addition to their functions in DNA metabolism, histones H1 and H2A and histone variants H1.2 and H2A.X display additional activities that may have important repercussions in cancer therapy.

2.6.1. HISTONE H1

Besides its participation in chromatin organization, linker histone H1 possesses other functions. Widlak et al. (59) have demonstrated that the C-terminal tail of histone H1 (CTD) activates the apoptotic nuclease DNA fragmentation factor DFF40/CAD via protein-protein interactions. It has also been demonstrated that histone H1 suppresses tumor cell growth in vitro in the Burkitt's lymphoma Daudi cell line and the IM-9 lymphoblastic leukemia cell line, as well as in vivo in a mouse xenograft model (60). Histone H1 inhibits cellular proliferation and induces apoptosis in the human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 (61). Class et al. (60) suggested that the mechanism underlying histone H1 cytotoxicity is the presence of histone H1-binding proteins on the cell surface that will trigger cellular responses such as apoptosis. Moreover, the entire histone H1 molecule may be required to obtain the observed cellular response since different peptides derived from histone H1 have no inhibitory effect (60).

2.6.2. HISTONE H1.2

Histone H1.2 is a member of the histone H1 family (5). Pohlmeyer et al. (62) have shown that histone H1 derived from calf thymus possesses cytotoxic effects on leukemia cells. The authors identified histone H1.2 as a major component of the histone H1 preparation and thus, they concluded that histone H1.2 is responsible for the cytotoxic effects observed. Moreover, exogenous recombinant histone H1.2 triggers apoptosis in human HeLa cervical carcinoma cells[4].

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[4] Hadnagy A., Beaulieu R., Balicki D. – unpublished data.

Recently, it has been demonstrated that histone H1.2 is involved in X-ray-induced apoptosis (7). Among all histones, histone H1.2 along with histone H1.1 bind with the lowest affinity to chromatin and thus, it was suggested that histone H1.2 may be a very sensitive DNA double break sensor (5). Indeed, Konishi et al. (7) have shown that after X-ray irradiation, histone H1.2 translocates from the nucleus to the cytoplasm. The mechanism underlying this histone H1.2 translocation and the activation of apoptosis is not completely understood. However, they suggest that histone H1.2 triggers apoptosis by the release of cytochrome c from mitochondria following activation of Bak, a member of Bcl-2 family. Thus, histone H1.2 becomes a pro-apoptotic protein once it reaches the cytoplasmic compartment and translocates to mitochondria.

Based on these reports, Gine et al. (63) analyzed the cytosolic release of histone H1.2 in primary tumoral chronic lymphocytic leukemia cells after treatment with genotoxic and non-genotoxic agents. They have shown that resistance to treatment with genotoxic agents is associated with the lack of histone H1.2 release. Therefore, the release of histone H1.2 may indicate the treatment outcome.

Another interesting function of histone H1.2 is its antimicrobial activity. Jacobsen et al. (64) have demonstrated in vitro and in vivo the efficacy of histone H1.2 against burn wound infection pathogens. Moreover, because of its low hemolytic effect, it has been suggested that histone H1.2 may be used as a systemic antimicrobial agent (64).

2.6.3. HISTONE H2A

Histone H2A contains a histone fold domain and a N and C-terminal tail, just like other core histones. In contrast to other core histones, histone H2A has the largest family of variants (65). It contains a cluster of DNA binding sites localized near the N-terminal tail (66) and it possesses the largest consensus C-terminal tail (67).

Exogenous histone H2A inhibits cellular proliferation in several cancer cell lines, including the human MCF-7 non-invasive adenocarcinoma cell line and the human MDA-MB-231 invasive adenocarcinoma cell line. Histone H2A blocks cell cycle progression and induces differentiation and cellular senescence in these cells. The mechanism of action underlying these effects is the increase of the cell cycle inhibitor p21 [5,6].

2.6.4. HISTONE H2A.X

Histone H2A.X represents 2-25 % of the histone H2A family (68) and its C-terminal motif SQ(D/E)(I/L/Y) distinguishes it from other H2A variants (68). The serine (S) in this motif is serine 139, and is the site of a γ-phosphorylation. Recently, Celeste et al. (6) have demonstrated that the loss of one H2A.X allele compromises genomic integrity and increases cancer incidence in the absence of the tumor suppresor gene p53.

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[5] Kaouass M., Hadnagy A., Mansour S., Beaulieu R., Balicki D. " Post-translational modifications of histone H2A are pivotal in its inhibition of human breast cancer cell proliferation via senescence. Poster presentation at San Antonio Breast Cancer Symposium, 2006

[6] Hadnagy A., Kaouass M., Mansour S., Beaulieu R., Balicki D. – manuscript in preparation

Phosphorylation of histone H2A.X is catalyzed by members of the phosphatidyl-inositol-3-kinase (PI3-K) family, including ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related) and DNA-PK (DNA-dependent protein kinase) (69). However, ATM may be the major kinase that contributes to H2A.X phosphorylation in response to DNA double-strand breaks (69). This phosphorylation occurs rapidly in response to DNA double strand breaks induced by ionizing radiation (IR) (68). IR is used in radiotherapy of cancer and it has been demonstrated that it induces histone H2A.X phosphorylation (70). Taneja et al. (70) have demonstrated that the level of the phosphorylated form of histone H2A.X (γH2A.X) after IR exposure may predict tumor response to radiotherapy. Moreover, they suggested that histone γH2A.X might represent a biological target in therapy of radioresistant tumors, since blocking histone H2A.X phosphorylation increases IR-induce apoptosis in cancer cells.

Liu et al. (71) have demonstrated that imatinib mesylate treatment of gastrointestinal stromal tumor cells (GIST) induces an increase in histone H2A.X level. Moreover, this up-regulation is critical for imatinib mesylate efficiency and thus, novel therapeutic strategies designed to increase histone H2A.X levels, such as proteasome inhibition, might prevent imatinib resistance of GIST cells.

2.7. CONCLUSION AND FUTURE GOALS

Epigenetic drugs represent a promising strategy in cancer therapy, as monotherapy as well as combination therapies. The intense focus on HDACi was rewarded by the recent introduction of SAHA into clinical settings. While the

classical substrates of HDAC are acetylated histones, it has been suggested that not only histone proteins, but acetylated non-histone proteins may also be HDAC substrates (72).

2.7.1. EXOGENOUS PROTEINS ENTER THE NUCLEUS

It has been demonstrated that exogenous proteins may be taken up by Physarum cells, an eukaryotic organism implicated in cellular metabolism. This is the case of exogenous H2A/H2B dimers. Thus, Thiriet et al. (73) have demonstrated that these exogenous dimers are assembled into nucleosomes. Moreover, it has been shown that the amino-terminal tails of H2A/H2B dimers are not required for their nuclear import, but that they are pivotal for efficient chromatin assembly.

Balicki et al. have demonstrated that (66) exogenous histone H2A enters the cytoplasm and nucleus within 24 hours. Subsequently, Hariton-Gazal et al. (74) demonstrated that core histones translocate directly across mammalian cell membranes and that this translocation is temperature- and energy- independent and uninhibited by endocytosis inhibitors, such as colchicine, nocodazole, cytochalasin D, brefeldin A, chlorochine and nystatin. Rosenbluh et al. (75) have demonstrated that core histones penetrate lipid bilayers and Mycoplasma membranes. As several proteins have the ability to penetrate cellular membranes, this property was related to the presence of a specific domain rich in arginine and lysine residues termed the protein transduction domain (PTD). Proteins containing this domain were termed cell-penetrating proteins (CPP). It has been suggested that histones may be

considered CPPs (75). Thus, all these studies suggested that exogenous histone H2A penetrates the nuclear compartment of the cell.

2.7.2. HDAC COMPETITION MODEL

We propose a model (Figure 1) where once exogenous acetylated histone H2A enters the nuclear compartment, it becomes a substrate for HDAC and thereby competes with endogenous nuclear histones for HDAC. The consequence of this competition will be an increase in the acetylation status of endogenous histones, analogous to treatment with HDACi. Our unpublished data regarding the effects of exogenous histone H2A on cancer cells support this model, as we have observed similarities between HDACi and histone H2A effects. The inhibition of cellular proliferation, induction of cell cycle arrest, increase of p21 expression, initiation of cellular differentiation and senescence are the common effects of HDACi (24;28;49) and exogenous histone H2A that we have observed, and which support this model[7]. However, we do not exclude the possibility that exogenously introduced histones may bind directly to chromatin and modulate gene expression.

In conclusion, the acetylation status of histones plays an important role in cancer progression and treatment. Thereby, epigenetic drugs, such as HDACi, have been studied and they are being introduced into clinical cancer therapy, e.g. SAHA. We propose a model whereby the histone acetylation status can be modulated not only by HDAC inhibition, but also by competition for HDAC. As HDAC substrates may potentially be histone and non-histone proteins, the development of acetylated

[7] Hadnagy A., Kaouass M., Mansour S., Beaulieu R., Balicki D. – manuscript in preparation

substrates of HDACs capable of reaching the nuclear compartment holds promise as an alternative strategy in epigenetic therapy.

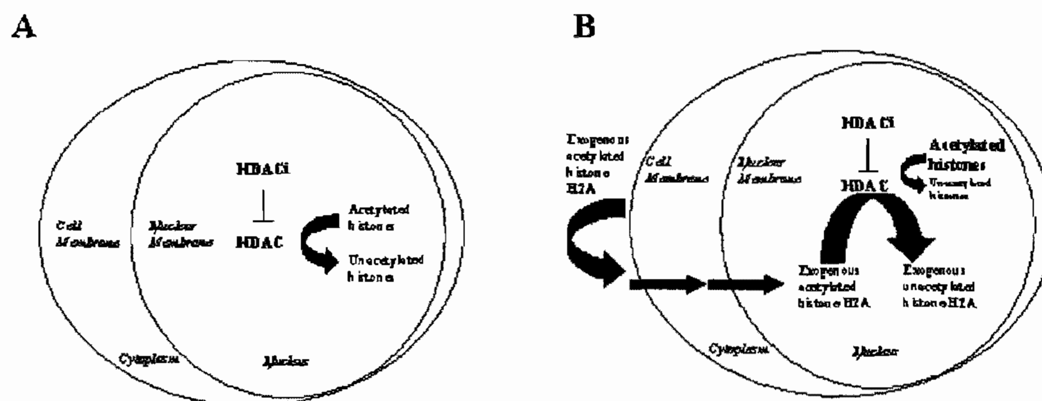

Figure 1 (introduction): HDAC competition model.

Figure 1A demonstrates normal homeostasis where deacetylation of endogenous histones is accomplished by HDAC and inhibited by HDACi. Figure 1B demonstrates that exogenous histone H2A translocates into the nucleus and disrupts the normal activities of HDAC and HDACi by serving as an additional substrate for HDAC. As a result of this competition for HDAC, less HDAC is available to deacetylate histones H3 and H4, leading to an accumulation of the acetylated forms of these histones, as is also described with HDACi. The increased acetylation of histones H3 and H4 has been associated with the transcriptional activation of several genes involved in the suppression of tumor growth.

2.8. ACKNOWLEDGEMENTS

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3. ARTICLE #2: EXOGENOUS HISTONE H2A INDUCES p21-DEPENDENT GROWTH ARREST AND SENESCENCE IN HUMAN BREAST CANCER CELLS

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ARTICLE SOUMIS

"EXPERIMENTAL CELL RESEARCH"

3.1. CONTRIBUTION PERSONNELLE:

Au niveau de la planification et du design des expériences, j'évalue ma contribution à 40%. Au niveau de la réalisation des expériences, ma contribution a été 60%. Au niveau de la rédaction de l'article ma contribution a été de 60%.

Exogenous histone H2A induces p21-dependent growth arrest and senescence in human breast cancer cells

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3.2. ABSTRACT

Epigenetic therapy has emerged as a novel field in cancer treatment. Post-translational modifications of histones play an important role in cancer progression and thus, they are being currently targeted. While the physiological compartment of histones is within the nucleus, where they participate in chromatin organization, extranuclear functions are also being characterized. The present study was designed to assess whether exogenous histone H2A can arrest cancer cell growth, and to subsequently determine its mechanism of action. Utilizing the MCF-7 human breast adenocarcinoma cell line, we demonstrate that exogenous histone H2A decreases the proliferation of MCF-7 cells and reduces its anchorage-independent growth capacity. Histone H2A treatment of MCF-7 cells leads to G2/M phase growth arrest, induces cellular senescence, and upregulates the cell cycle inhibitor p21WAF1/CIP1/Sdi1, while p53 RNA and protein levels were unchanged. Interestingly, recombinant human non-acetylated histone H2A has no effect on MCF-7 cell proliferation. While the acetylation of endogenous histones H3 and H4 play an important role in anti-cancer effects of histone deacetylase (HDAC) inhibitors, we speculate that acetylation of exogenous histone H2A is pivotal for its suppressing effects. These are the first data to demonstrate that exogenous histone H2A inhibits cancer cell growth via p21-dependent senescence.

Keywords: histone H2A, histone deacetylase (HDAC) inhibitors, epigenetics, MCF-7 breast cancer cells, senescence, p21, p53.

3.3. INTRODUCTION

Histones are the most abundant proteins bound to DNA in eukaryotic cells. They are small proteins with a molecular weight between 11 and 20 Kda with a high percentage (~20%) of basic amino acids. Eukaryotic cells contain 5 principal types of histones: H1, H2A, H2B, H3, and H4. Histones assemble into a nucleosome octamer core consisting of 2 molecules each of histone H2A, H2B, H3, and H4. Histone H1 is localized in the linker region between nucleosomes [1-4]. The core histones have long N-terminal tails that may undergo a series of post-translational modifications, including acetylation, phosphorylation, ubiquitination, and methylation [5;6]. These modifications are pivotal in the regulation of DNA replication and transcription, critical to gene expression, and include both gene repression and the facilitation of gene activation [6-8].

While histones are classically involved in DNA condensation and differential gene expression, their extranuclear role is also being recognized. Indeed, there is growing evidence that histone proteins display hormone-like functions. For instance, histones have been detected in biological fluids such as plasma and milk [9]. Goya *et al.* and Reichhart *et al.* have reported structural similarity between homeostatic thymus hormone and histones H2A and H2B, and they suggested that histones may have hormone-like activities [10;11]. Several studies indicate that histones are involved in a variety of biological functions, including the hormonal coordination of immune responses, as well as the proliferation of lymphocyte cells [12], the processing of antigenic peptide-major histocompatibility complexes [13], the intracellular and extracellular lipopolysaccharide sensors [14], the insulin-like effects on adipocytes [15], and the control of hormone release [16].

Recent results demonstrate that histones H1, H2A, H2B, H3, and H4 added exogenously to cells directly cross the plasma membrane via translocation and not endocytosis, and are subsequently imported into the nucleus. Histones have been demonstrated to mediate the penetration of macromolecules covalently attached to them [17]. Their nuclear transport is facilitated via nuclear localization signals (NLS) present within histone molecules [18]. The presence of NLS and basic amino acids as well as their capacity to condense DNA, has made histones candidate vectors for non-viral gene transfer [19] and facilitators of viral gene transfer [20].

Histones have also been shown to be important players in cancer biology. For example, Bassing *et al.* [21] have found that inactivation of the histone H2A.X gene, a histone H2A subtype, leads to increased genomic instability and tumor formation. Histone H2A.X is a minor variant of histone H2A. The 9 C-terminal amino acids of the 129-amino acid human histone H2A are replaced by a 22-amino acid tail in histone H2A.X [22]. Another group has reported that the combined deficiency of histone H2A.X and the tumor suppressor gene, p53, in mice results in lymphoid and solid tumors [23]. Recent studies in signal transduction pathways resulting from double-stranded DNA damage have demonstrated that proteins synthesized immediately after this event play a critical role in DNA repair, including the maintenance of genomic integrity and tumor suppression. The principal proteins involved in this process are p53, histone H2A.X, Smc1 (structural maintenance of chromosomes) and Chk2 (checkpoint kinase 2) [24]. In addition, variants of histone H2A participate in transcriptional activation, DNA repair, meiosis, and apoptosis [25].

Additional studies have demonstrated that histone H1 has an indirect cytotoxic effect on leukemia and lymphoma cells [26]. Histone H1 has also been shown to have an anti-tumor effect in breast cancer associated with its activation of the immune system [27]. Konishi *et al.* [28] have reported an unexpected role of histone H1.2 in triggering apoptosis in response to cell damage *in vitro* and *in vivo*. Activation of the pro-apoptotic Bak and cytochrome C release from mitochondria are required for H1.2-induced apoptosis [28].

In this study, we investigated the ability of exogenous histone H2A to halt the proliferation of tumor cells *in vitro*. We demonstrate that histone H2A suppresses the growth of human cancer cells. The suppressing effect of histone H2A is rapid, irreversible, and mediated via p21-dependent senescence.

3.4. MATERIALS AND METHODS

3.4.1. CELL CULTURE AND HISTONE

All cells were purchased form American Type Culture Collection, USA and were routinely maintained as previously described. The MCF-7 human breast adenocarcinoma cell lines were routinely maintained in RPMI-1640 medium, 1 mM pyruvate and MEM non-essential amino acids. Human breast fibrocystic MCF-10A cells were maintained in DMEM/F12 medium supplemented with epidermal growth factor (EGF) (20 ng/ml), hydrocortisone (500 ng/ml), insulin (10 μg/ml) and cholera toxin (100 ng/ml). Human embryonic kidney epithelial HEK293 cells, human HeLa cervical carcinoma cells, and human HepG2 hepatocellular carcinoma cells were routinely maintained in DMEM medium. The EBV-transformed IM-9 B

lymphoblastoid cell line derived from the blood of a patient with multiple myeloma were maintained in RPMI1640 medium. Normal human foreskin fibroblasts (BJ) cells were maintained in DMEM supplemented with 1 mM pyruvate and MEM non-essential amino acids. The media were supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotics, and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Histones H2A, H2B, H3, and H4 were purchased from Roche Diagnostics, Canada. The recombinant human histone H2A and its H2A.X variant were purchased from Upstate Inc., USA.

3.4.2. CELLULAR PROLIFERATION ASSAY

Exponentially growing cells were seeded in 24-well plates in the absence or presence of calf thymus histones H2A, H2B, H3 and H4, recombinant human H2A and H2A.X for 3 to 6 days. At the end of treatment, cell numbers were determined by hemacytometer counts after testing the cell viability by Trypan Blue exclusion.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to estimate the number of metabolically active cells by measuring the conversion of MTT to its formazan derivative. Exponentially-growing cells were plated in 96-well plates in the absence or presence of calf thymus histones H2A for 24 and 48 hours. At the end of treatment, the cells were washed with PBS and incubated with MTT (0.5 mg/ml in culture medium) for 5 hours at 37°C. The formazan crystals formed were dissolved in 200 µl DMSO and the absorbance of formazan was measured at 575 nm.

3.4.3. ANCHORAGE-INDEPENDENT GROWTH IN SOFT AGAR

MCF-7 cells were trypsinized, counted, and $2x10^4$ cells were mixed with 1.5 ml of 0.35% agarose-DMEM (top layer) containing 0-50 μg/ml of H2A, and then poured on top of 1.5 ml of solidified 0.5% agarose-DMEM (bottom layer) in 6-well plates. After 4 weeks of culture at 37°C, colonies were stained with MTT (0.5 mg/ml in RPMI medium), counted and photographed.

3.4.4. FLOW CYTOMETRIC ANALYSIS OF DNA CONTENT

DNA content of cells was measured by FACScan using the propidium iodide (PI) staining method [29], and quantified using ModFit LT™ software (Verity House Software, Topsham, ME). Cells were treated with histone H2A (0-100 μg/ml) for 48 hours. They were then washed with PBS and incubated with PI in Krishan buffer (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% NP-40 and 50 μg/ml PI) at a pH of 7.4 for 30 minutes on ice. The cell suspension was analyzed by fluorescence-activated cell sorting (FACS; Becton-Dickinson, Franklin Lakes, NJ).

3.4.5. SENESCENCE-ASSOCIATED B -GALACTOSIDASE STAINING

Senescence-associated β-galactosidase (SA-β-gal) staining was performed as described previously [30]. Control and treated MCF-7 cells were cultured on coverslips in 6-well chamber slides and fixed with 4% formaldehyde in PBS for 10 minutes at room temperature. The cells were then rinsed twice with PBS and incubated for 6 hours with a SA β -gal staining solution containing 40 mM sodium citrate (pH 6.0), 150 mM NaCl, 5 mM potassium ferricyanide, 2 mM MgCl2, and 1

mg/ml of 5-bromo-4-chloro-3-indoyl β -galactoside (X-GAL) (Sigma-Aldrich, Canada Ltd).

3.4.6. IMMUNOBLOT ANALYSIS

MCF-7 cells were tested with or without histone H2A at various concentrations ranging from 0 to 50 μg/ml. At the end of treatment, the cells were rinsed in PBS, and proteins were extracted in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin). Proteins (50 μg/lane) were loaded on 10% polyacrylamide gels and blotted onto PVDF membranes. Primary rabbit monoclonal anti-p21 (Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-p53 (1:500) (Sigma-Aldrich Canada Ltd) and mouse monoclonal anti-β-actin (1/10,000) (Sigma-Aldrich Canada Ltd). Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories Canada Ltd) were employed at a 1:3,000 dilution. Detection was carried out using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

To determine whether histone H2A contained both non-acetylated and acetylated (Ac-H2A), we undertook immunoblotting to detect Ac-H2A in the histone H2A preparation. Serial dilutions of histone H2A representing 0 to 16 μg of histone H2A were subjected to SDS-PAGE electrophoresis and blotted against the acetylated form of histone H2A using rabbit polyclonal antibody to histone H2A (acetyl K5) (Abcam Inc., USA).

3.4.7. RT-PCR

Total cellular RNA was isolated using the TRIzol® reagent (Invitrogen Canada Inc.). Three μg of total RNA were reverse-transcribed into cDNA by reverse transcriptase, and p53 and actin transcripts were amplified by PCR. The primers used for PCR were: GCGCACAGAGGAAGAGAATC (p53 sense), CAGTCTGAGTCAGGCCCTTC (p53 antisense), TCTCACTTCTTGAAGGAACG (actin sense) and CCCCATTCTGTTTACACAGC (actin antisense).

3.4.8. ROS ANALYSIS

Exponentially-growing cells were plated in 96-well plates and pre-treated with various concentration of N-acetyl-L-cysteine (NAC). Cells were treated with 0 to 100 μg/ml histones H2A for 72 hours. At the end of treatment, the cells were washed with PBS and incubated with MTT (0.5 mg/ml in culture medium) for 5 hours at 37°C. The formazan crystals formed were dissolved in 200 μl DMSO and the absorbance of formazan was measured at 575 nm.

3.5. RESULTS

3.5.1. EXOGENOUS HISTONE H2A INHIBITS CELL PROLIFERATION

To demonstrate the inhibitory effect of histone H2A on cancer cell proliferation, we first examined its effect on cell growth in the MCF-7 human breast adenocarcinoma cell line. MCF-7 cells were treated with 0-50 μg/ml of histone H2A for 6 days. The effect of histone on cell viability was assessed by Trypan Blue

exclusion method (Fig. 1A). The effect of histone H2A was also assessed by MTT assay (Fig. 1B). Histone H2A treatment of MCF-7 cells resulted in a time- and dose-dependent decrease in cell proliferation. We also tested the effect of cell density on the growth suppressing effect of histone H2A. MCF-7 cells were plated in 96-well plates at initial densities of 2,500, 5,000, or 10,000 cells/well. The cells were cultured for 48 hours, and the number of metabolically active cells was determined by the MTT assay (Fig. 1C). Our results demonstrate that the growth suppressing action of histone H2A was unrelated to the density of MCF-7 cells. In addition, the growth inhibitory effect of histone H2A was irreversible since washing out histone H2A from the culture medium did not allow MCF-7 cells to divide and continue to proliferate unlike control cells (data not shown).

To assess whether histone H2A has differential effects in normal and cancer cells, various types of tumors and normal fibroblasts cells were seeded in 24-well plate and cultured with various concentrations of histone H2A for 72 hours. Individual cell line sensitivity was assessed in a comparative fashion by estimating the histone H2A concentration that resulted in 50 percent of control growth arrest (IC50). Histone H2A inhibited the number of viable cells (as determined by Trypan Blue exclusion) in a dose-dependent manner in all cell lines tested (Fig. 2). The IM-9 cells are the most sensitive cells with IC50 estimated at 25 μg/ml of H2A. HeLa, HEK293, HepG2 and MCF-10A as well as the BJ foreskin fibroblasts have intermediate sensitivity with IC50 $\geq$ 50μM. The MCF-7 cell line has an IC50 >75 μg/ml.

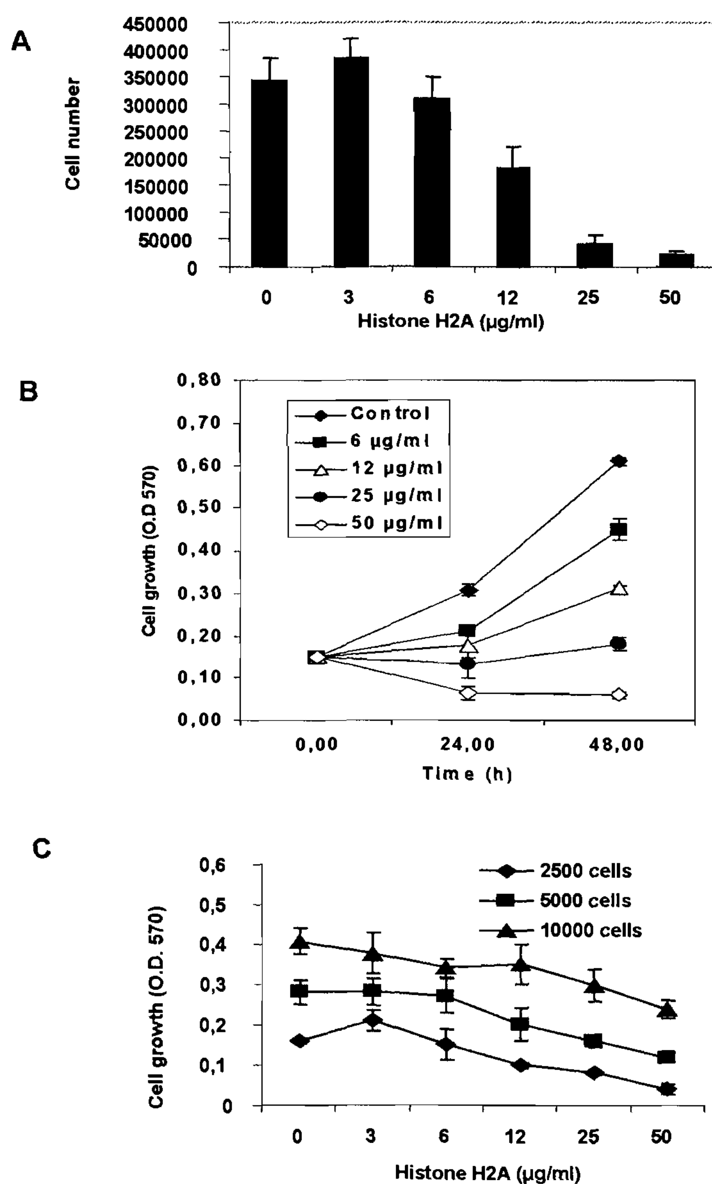


Figure 1. Effects of histone H2A on MCF-7 cell proliferation.

(A) The growth inhibitory effect of histone H2A on MCF-7 cells as determined by Trypan Blue dye exclusion test. Cells were incubated with increasing concentrations of histone H2A, and the viable cells were counted after 6 days of treatment.

(B) MTT quantification of MCF-7 cells after treatment with 0 to 50 μg/ml of histone H2A for 24 and 48 hours. Data points represent the mean of triplicate measurements ± SE. These experiments were performed at least three times.

(C) Effect of cell density on the growth suppressing effect of histone H2A. MCF-7 cells were plated in 96-well pates at an initial density of 2500, 5000, and 10000 cells/well. Cells were cultured for 48 hours, and the number of metabolically active cells was determined by MTT assay. Data points represent the mean of triplicate measurements ± SE.

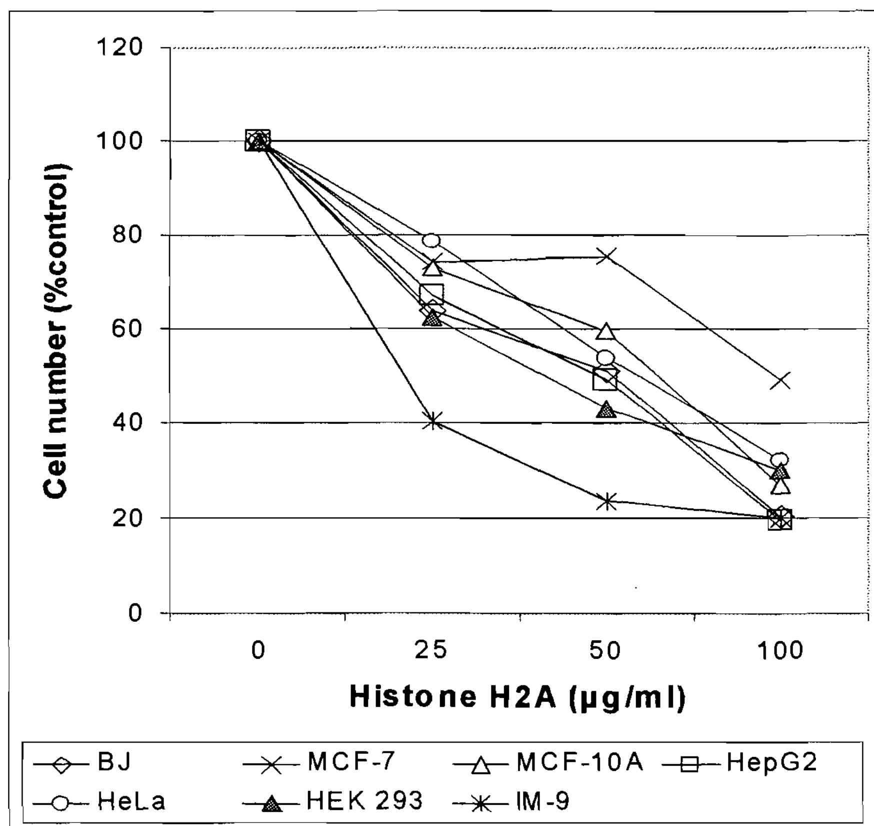


Figure 2. Histone H2A inhibits the growth of several cell types.

Immortalized MCF-10A (human fibrocystic breast cells), MCF-7 (human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma), HeLa (human cervical carcinoma), HEK293 (human embryonic kidney epithelial cells), IM-9 (B

lymphoblastoid cell line), and normal foreskin fibroblasts BJ were cultured in 24-well plates with histone H2A (0 to 100 μg/ml) for 72 hours. Cell numbers were counted using a hemocytometer and cell viability was determined by Trypan Blue dye exclusion test. The effect of histone H2A on cell proliferation was statistically significant for MCF-7, HepG2, HEK 293, IM9, and BJ cell line ($p<0.05$ based on Student's t-test). This experiment was repeated at least twice.

3.5.2. HISTONE-INDUCED CELL GROWTH ARREST IS SPECIFIC TO HISTONE H2A

The 4 core histones H2A, H2B, H3 and H4 are basic proteins that are highly conserved throughout evolution. We investigated whether each of the core histones have potential anti-tumor activity. MCF-7 cells were treated with increasing doses of histones H2A, H2B, H3, H4 and 5 days later, the number of cells was determined by Trypan Blue exclusion method. As depicted in Fig. 3A, histones H2B, H3 and H4, derived from calf thymus, had no effect on MCF-7 cell growth rate in contrast to histone H2A also derived from calf thymus. These findings suggest that the anti-tumor activity of histone H2A is not shared by the other core histones. We next tested human recombinant histone H2A.X (H2A.X Rec), and we did not observe any anti-proliferative effect for this histone. (Fig. 3B). Surprisingly, human recombinant histone H2A (H2A Rec) expressed in bacteria, that is almost identical in sequence to bovine histone H2A, shown no effect on cellular proliferation (Fig. 3C). The difference between calf thymus histone H2A and the recombinant histone H2A tested consists of the presence of post-translational modifications in calf thymus histone H2A which are absent in the recombinant human histone H2A. Thus, it appears that

at least some (if not all) of these post-translational modifications are pivotal in the arrest of cell growth.

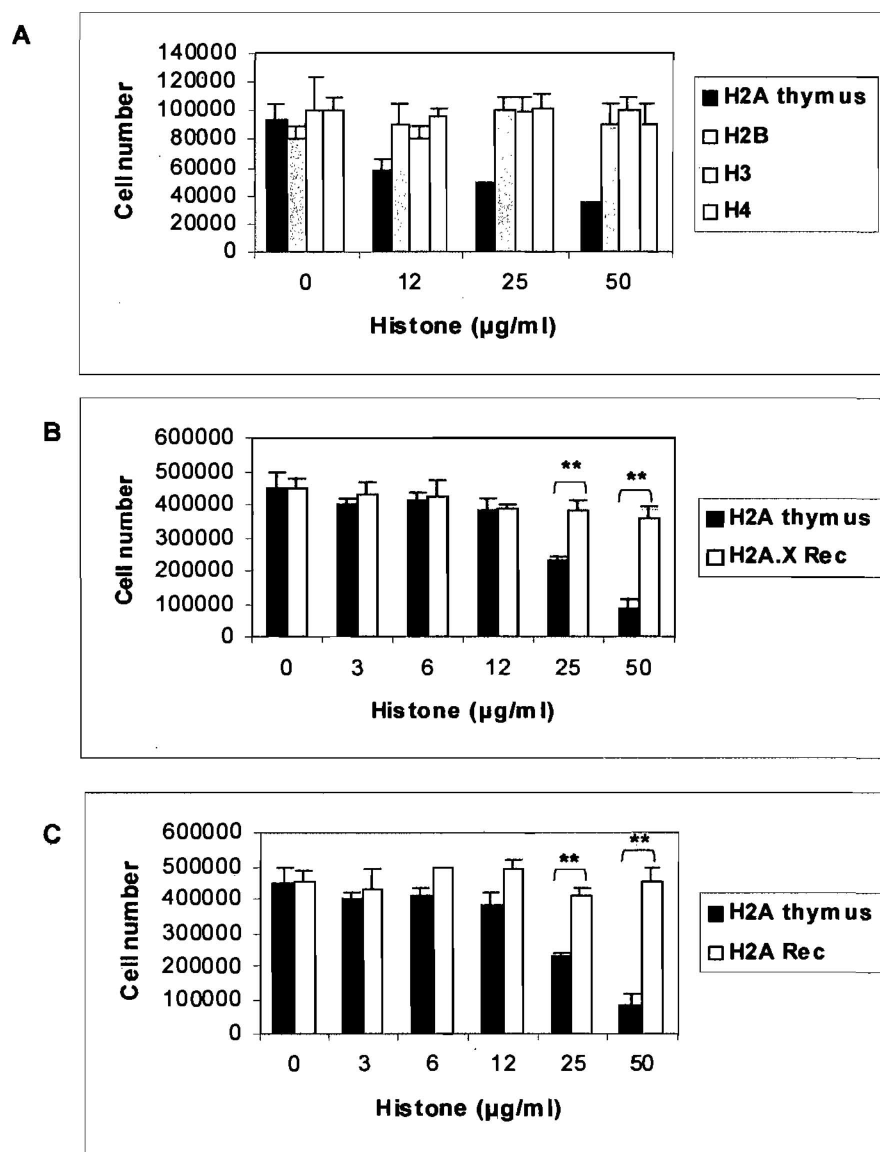


Figure 3. (A) Effect of the core histones H2A, H2B, H3 and H4 extracted from calf thymus on MCF-7 cell proliferation.

MCF-7 cells were treated with histone H2A, H2B, H3 or H4 (0 to 50 µg/ml) for 5 days, and the cell viability was assessed by Trypan Blue dye exclusion test.

Figure 3. (B) The effect of calf thymus histone H2A was compared with human recombinant histone H2A.X on MCF-7 cell proliferation.

MCF-7 cells were treated with calf thymus histone H2A and recombinant histone H2A.X (0 to 50 μg/ml) for 5 days, and the cell viability was assessed by Trypan Blue dye exclusion test. The differences between the calf thymus histone H2A-treated cells and human recombinant H2A.X histone variant-treated cells were statistically significant (** $p<0.01$ based on Student's t-test).

Figure 3. (C) Effect of calf thymus histone H2A was compared with human recombinant histone H2A on MCF-7 cell proliferation.

MCF-7 cells were treated with calf thymus histone H2A and recombinant histone H2A (0 to 50 μg/ml) for 5 days, and the cell viability was assessed by Trypan Blue dye exclusion test. The differences between the calf thymus histone H2A-treated cells and human recombinant histone H2A-treated cells were statistically significant (** $p<0.01$ based on Student's t-test). Data points represent the mean of triplicate measurements ± SE. Experiments were performed twice.

3.5.3. HISTONE H2A INHIBITS ANCHORAGE-INDEPENDENCE OF MCF-7 CELLS

We subsequently tested whether exogenous histone H2A could influence the ability of MCF-7 cells to form colonies in soft agar. The cells were cultured for 4 weeks in 0.35% agar and RPMI medium containing 0, 12, 25 or 50 μg/ml of calf thymus histone H2A. Cell colonies were stained with MTT and colonies with a diameter greater than 100 μm were counted. As illustrated in Fig. 4A, histone H2A

suppressed the anchorage-independent growth of MCF-7 cells, with a maximum effect of histone H2A observed at a concentration of 50 μg/ml. The colony size of MCF-7-treated cells was also significantly smaller than that of untreated cells (Fig. 4B).

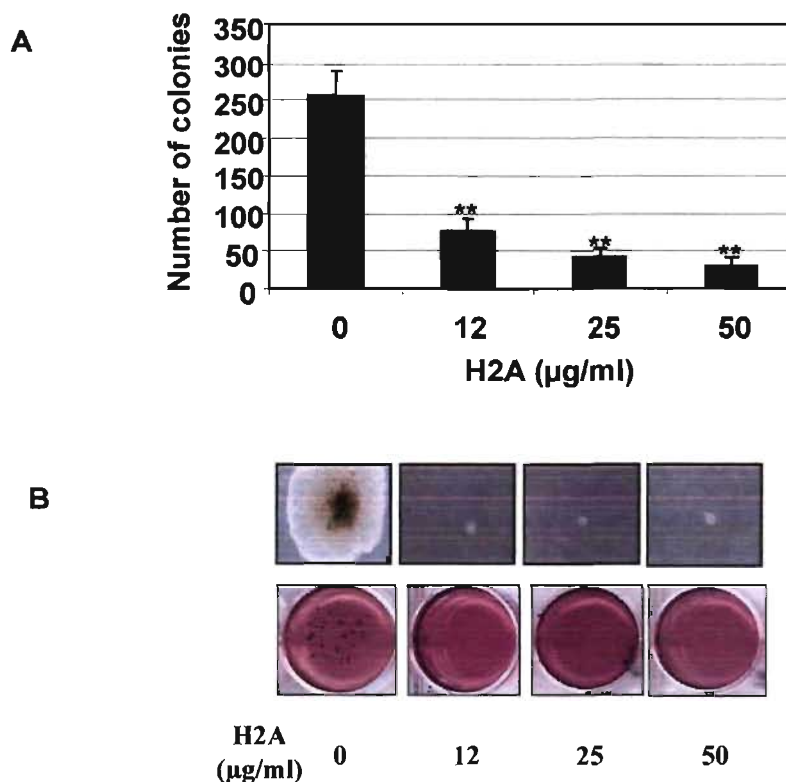


Figure 4. (A,B) Histone H2A reduces the anchorage-independent growth of MCF-7 cells.

(A) The ability of MCF-7 cells to proliferate and to form colonies in soft agar were examined. MCF-7 cells (20,000 cells/well) were cultured in 0.35% agarose containing RPMI medium in the presence of 0, 12, 25, or 50 μg/ml histone H2A at 37°C for 4 weeks. The colonies were stained with MTT and counted. Data points represent the mean of triplicate measurements ± SE. Experiments were performed twice. The differences between the experimental groups and control groups were statistically significant (** $p<0.01$ based on Student's t-test). **(B)** Representative

culture wells were scanned with Duoscan T1200 (AGFA), and typical individual colonies from each culture condition were photographed at 100X magnification.

3.5.4. HISTONE H2A BLOCKS THE CELL CYCLE

As the suppression of cell growth and colony formation seen in histone H2A treated MCF-7 cells could be due to cell cycle blockade, the cell cycle profile was analyzed by flow cytometry in MCF-7 cells treated with histone H2A, using concentrations ranging from 0 to 100 μg/ml. When compared to untreated control cells, histone H2A treatment of MCF-7 cells resulted in a dose-dependent decrease of G1 cell populations and accumulation of cells in the G2/M phase of the cell cycle. Thus, histone H2A induces inhibition of cellular proliferation through cell cycle arrest (Fig. 4C).

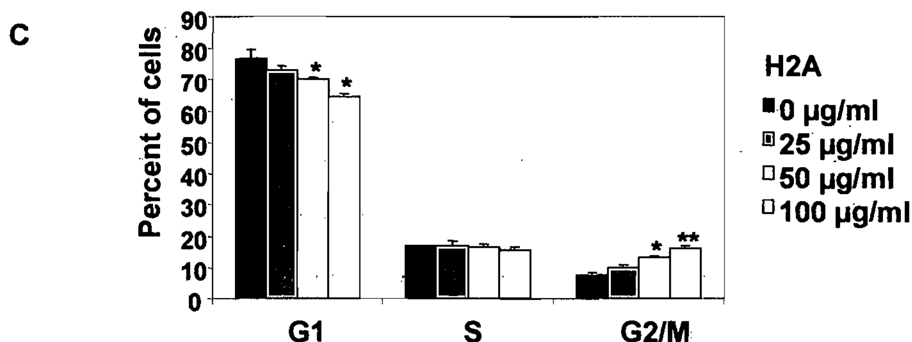


Figure 4. (C) Histone H2A induces cell cycle arrest.

MCF-7 cells were cultured in the presence of 0, 25, 50 and 100 μg/ml of histone H2A for 48 hours. Cellular DNA content was analyzed by flow cytometry. The percent of cells in the G1, S and G2/M phases are shown. The differences between

the untreated control cells and histone H2A-treated cells were statistically significant (*$p<0.05$, ** $p<0.01$ based on Student's t-test). The experiment was repeated three times.

3.5.5. HISTONE H2A ELICITS SENESCENCE-LIKE GROWTH ARREST IN MCF-7 CELLS

Growth arrest and blockade of the cell cycle are the main characteristics of cells undergoing senescence. Other characteristics of senescent cells are their flat and enlarged morphology, and increase in SA-β-galactosidase [30]. Thus, we tested the effect of histone H2A on the morphology and β-galactosidase activity of MCF-7 cells. In the presence of histone H2A, MCF-7 cells became flat, increased in diameter, and developed cellular extensions (Fig. 5B) as compared to untreated MCF-7 cells (Fig. 5A). Histone H2A-treated MCF-7 cells also exhibited a phenotype that is typical of cellular senescence, i.e. increased SA-β-galactosidase activity. This enzymatic activity was determined by staining cells with the chromogenic substrate X-GAL at pH 6.0. Small numbers of untreated MCF-7 cells expressed background levels of SA-β-galactosidase (Fig. 5C). However, this expression increased dramatically in MCF-7 cells after 5 days (Fig. 5D) of exposure to histone H2A. The intensity of cellular staining for SA-β-galactosidase was also enhanced in a time- and dose-dependent manner (data not shown).

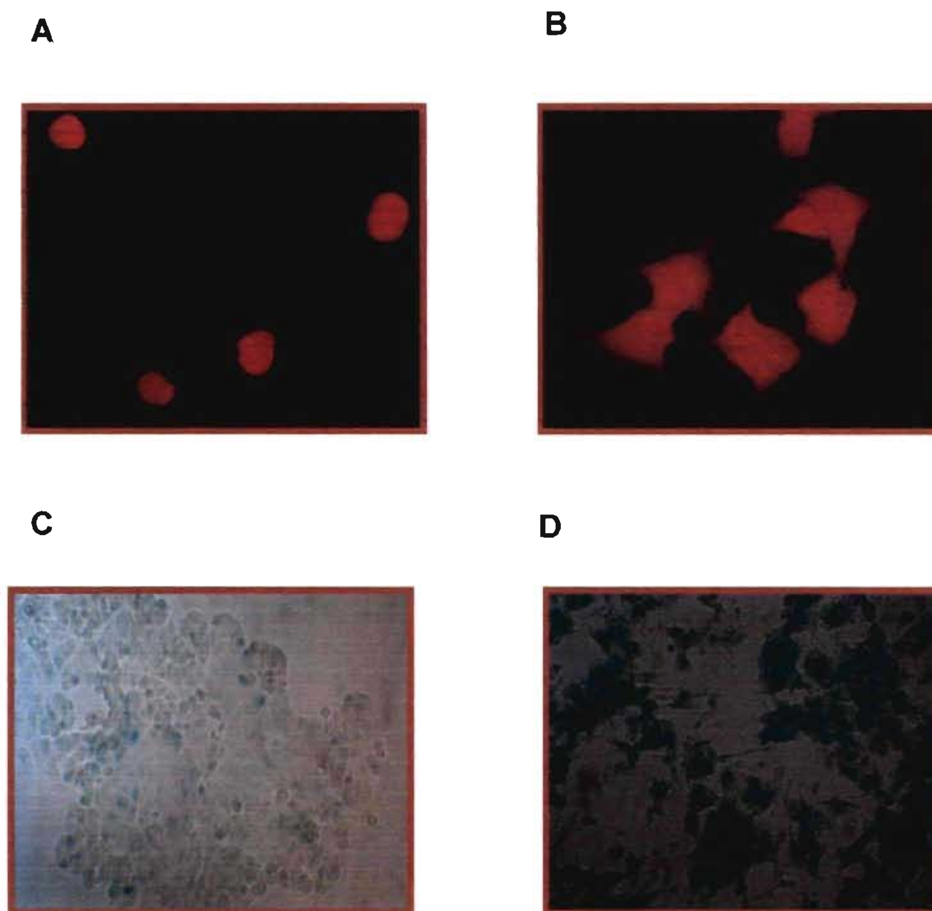


Figure 5. (A,B) Histone H2A-treated MCF-7 cells exhibited enlarged and flattened cell morphology.

MCF-7 cells (100,000) were cultured on coverslips in the presence of 0 μg/ml **(A)** or 25 μg/ml **(B)** of histone H2A and stained 3 days later with crystal violet. Representative fields were photographed at 400-fold magnification. The experiment was repeated 3 times.

Figure 5. (C,D) Histone H2A treatment resulted in the appearance of SA-β-galactosidase-positive cells.

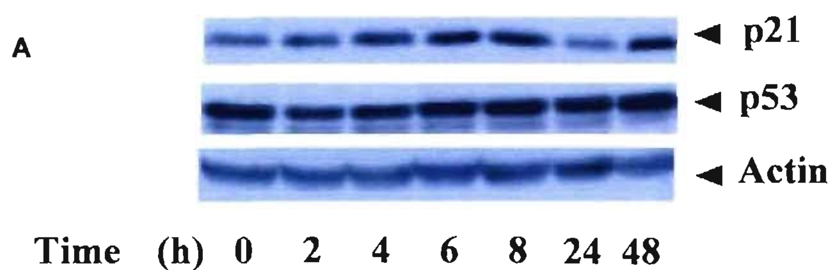
MCF-7 cells were left untreated **(C)** or treated with 25 μg/ml of histone H2A **(D)** for 5 days. Cells were fixed and stained with X-GAL, a substrate of β-galactosidase. Blue staining corresponds to cells, which express SA-β-galactosidase activity.

Representative fields were photographed at 200-fold magnification. This experiment was repeated at least three times.

3.5.6. P21 AND P53 EXPRESSION IN H2A-TREATED MCF-7

As p21, and in some cases, p53 are necessary for induction of irreversible senescence, we analyzed the changes in p21 and p53 protein expression levels in response to histone H2A treatment of MCF-7 and HeLa cells. Immunoblot analysis revealed that the exposure of MCF-7 cells to 50 μg/ml (Fig. 6A) of exogenous histone H2A transiently increased the expression of p21 protein, a cyclin-dependent kinase inhibitor that mediates cellular senescence. Time course experiments demonstrated rapid p21 induction in response to histone H2A (occurring after 4 hours) and a return of baseline level after 24 hours. No significant changes in p53 protein level occurred in the first 24 hours when MCF-7 cells were treated with 50 μg/ml of histone H2A. After 48 hours both p21 and p53 protein expression increased. Fig. 6B shows the densitometry analysis of the immunoblot presented in Fig. 6A. RT-PCR analyses were unable to detect changes in p53 RNA expression (Fig. 7), suggesting a p21-dependent and p53-independent histone H2A induction of cell growth arrest.

A



B

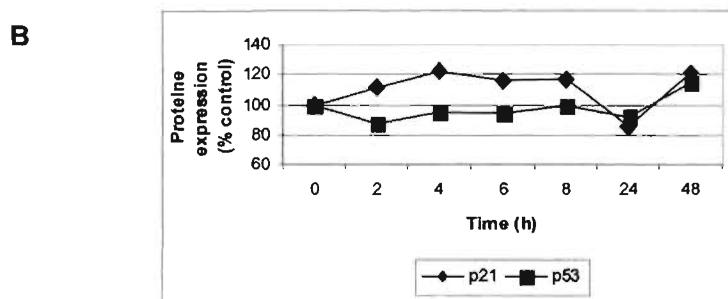


Figure 6. Immunoblot analysis of p21 and p53 proteins in MCF-7 cells exposed to histone H2A.

(A) MCF-7 cells were treated with 50 μg/ml of histone H2A for the time indicated, and 50 μg proteins/lane were analyzed by Western blotting using anti-p21 and anti-p53 antibodies. **(B)** Intensity of bands was determined by densitometry and normalized to those of corresponding actin bands. This experiment was repeated three times.

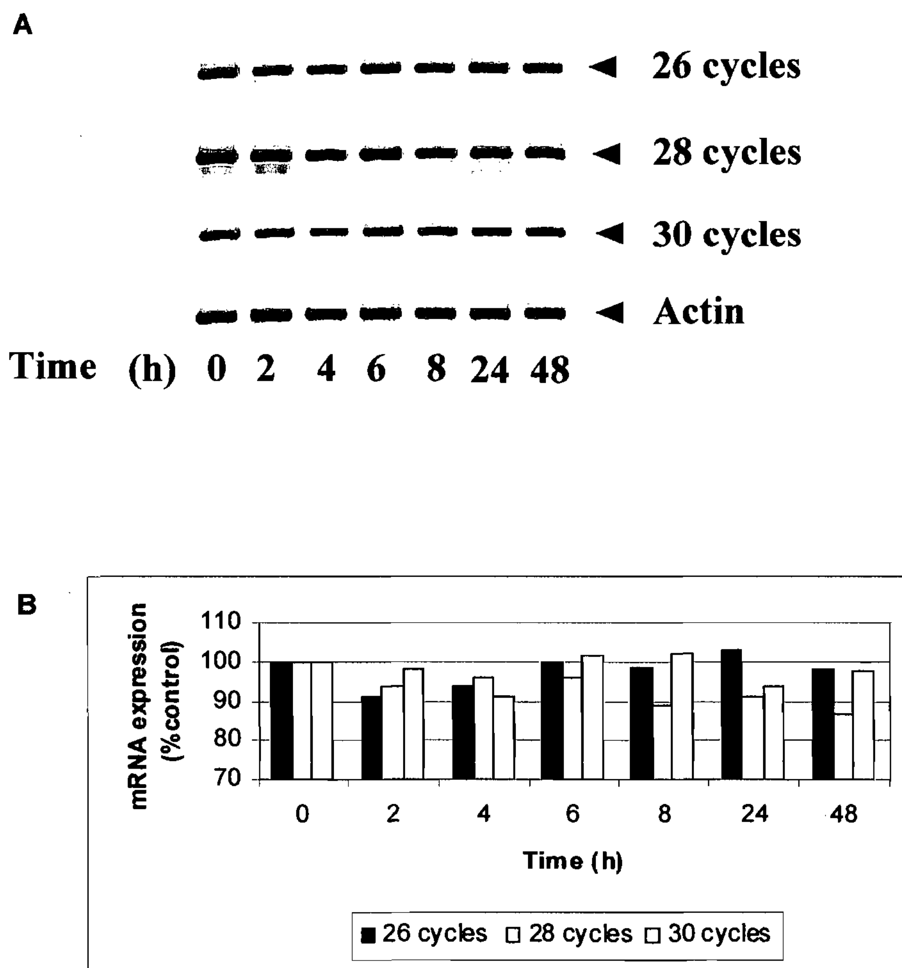


Figure 7. Treatment with histone H2A did not change p53 RNA expression.

(A) MCF-7 cells were treated with histone H2A for up to 48 hours and p53 RNA expression was assessed by RT-PCR using various amounts of cycles (26, 28 and 30 cycles) **(B)** Intensity of bands was determined by densitometry and normalized to those of corresponding actin bands.

A

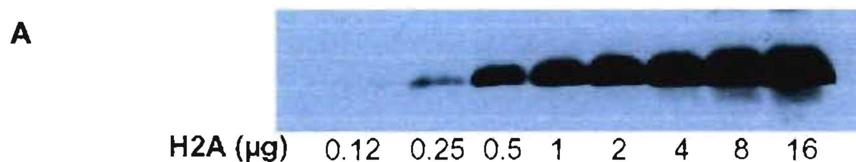


Figure 8. (A) Acetylated histones H2A are present in calf thymus histone H2A preparation.

Serial dilutions of calf thymus histone H2A protein were subjected to SDS-PAGE, and the presence of the acetylated form of histone H2A was analyzed by Western blotting with a polyclonal antibody against acetylated histone H2A.

B

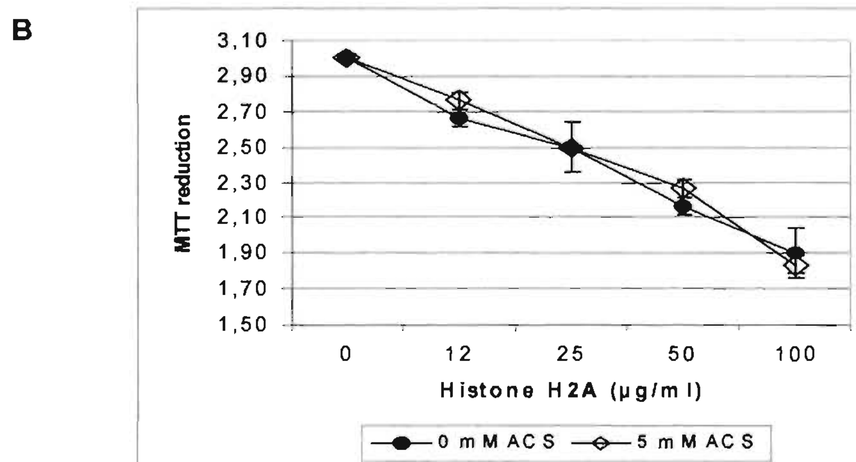


Figure 8. (B) Reactive oxygen species (ROS) generation is not involved in growth arrest effect of histone H2A.

MCF-7 cells were cultured in 96-well plates and were pre-treated with 5 and 10 mM N-acetyl-L-cysteine (NAC), a general ROS scavenger. After 72 hours treatment with

histone H2A (0-100 μg/ml), the number of metabolically active cells was determined by MTT assay. Data points represent the mean of triplicate measurements ± SE.

3.6. DISCUSSION

The present study demonstrates that exogenous histone H2A from calf thymus inhibits the proliferation of MCF-7 human breast cancer cells in a dose- and time-dependent fashion (Fig. 1). We have also demonstrated the sensitivity of several cell lines to histone H2A (Fig. 2). Among the tested cell lines, the IM-9 B lymphoblastoid cell line appears to be the most sensitive. The other transformed cell lines tested i.e. HeLa, HepG2, and HEK293 shown similar sensitivity. We also tested MCF-10A cells, a breast fibrocystic cell line which showed no difference in its sensitivity to other cell lines tested. To assess the difference between sensitivity of normal and cancer cell lines to histone H2A, we examined the normal fibroblast BJ cell line. These cells were also sensitive to histone H2A, as were the cancer cell lines tested. Thus, histone H2A affects both normal and tumor cell lines, as is the case for several chemotherapeutic agents (Fig. 2). This is an expected result as the underlying mechanism of action targets cellular proliferation, common to all cells.

We next tested whether the anti-proliferative effect observed was specific to histone H2A, or applicable to all members of the histone family. Histones H2B, H3 and H4 have similar structural features present in histone H2A [3], but have no anti-tumor activity on MCF-7 cells (Fig. 3A). Histone H2A.X contains 142 amino acid residues, 13 more than human histone H2A. The sequence of the first 120 residues of H2A.X is almost identical to that of human histone H2A [22]. Our results show that human recombinant histone H2A.X has no effect on cellular proliferation (Fig. 3B).

Unless otherwise specified, the histone H2A used is this study was extracted from calf thymus, which contains both acetylated and unacetylated histones (Fig. 8A). While calf thymus histone H2A inhibits cellular proliferation, human recombinant histone H2A was unable to do so (Fig. 3C). The primary structures of these proteins are very close related. However, recombinant human histone H2A produced in bacteria is unacetylated since post-translational protein modifications are not present in bacteria [31]. Histones undergo additional post-translational modifications which alter their interaction with DNA and nuclear proteins [32]. Such modifications include ubiquitination, methylation and phosphorylation. Consequently, we cannot exclude the involvement of one of these additional types of post-translational modifications in the growth-suppressing effect of histone H2A. Interestingly, Huyen *et al.* [33] have demonstrated that calf thymus histone H3 and not recombinant histone H3 (expressed in bacteria) interact with 53BP1, a checkpoint protein in DNA double-strand breaks signaling pathway. This suggests that post-translational modifications are required for histone H3 binding. These results highlight the importance of post-translational modifications in histone functions.

Modulation of post-translational modifications of histones, particularly acetylation and methylation, gave rise to a new field in cancer therapeutics, i.e. epigenetic targeted therapy. The extent of histone acetylation is a fine balance between the activities of its acetylases and deacetylases. As such, histone deacetylase (HDAC) inhibitors emerged as a new class of anti-cancer drugs. Inhibitors of HDAC activity induce cell cycle arrest, differentiation or apoptosis in tumor cells, and suppress tumor growth in various cancer models [34]. The mechanisms of action of

HDAC inhibition have been studied extensively. HDAC inhibition results in the accumulation of acetylated histones H2A, H2B, H3 and H4, which affects gene transcription and induces cancer cell growth arrest [35-38].

Although histones acetylation is a common modification of all 4 core histones, acetylation of histone H3 and H4 correlates most strongly with an open and active chromatin [39]. Accordingly, Richon *et al. [40]* have shown that cancer cells treatment with suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, is associated with an accumulation of acetylated histones H3 and H4 and transcriptional activation of genes, such as p21. Thus, acetylation of endogenous histones H3 and H4 play an important role in anti-cancer effects of HDAC inhibitors. Our results suggest that post-translational modifications of exogenous histone H2A are pivotal for its anti-cancer properties.

Another interesting finding of this study was that histone H2A treatment reverses the anchorage-independence of MCF-7 cells. We clearly demonstrate that exogenous treatment of MCF-7 cells with histone H2A leads to a significant decrease in the number (Fig. 4A) and size of colonies (Fig. 4B) in semi-solid agarose culture. Since anchorage-independence correlates with regulatory mechanisms that restrict cell division and are lost in transformed cells [41], our results suggest that histone H2A alters regulatory mechanisms that allow MCF-7 cells to lose their proliferative potential.

Senescence is a state of irreversible cellular growth arrest initiated by the shortening of telomeres (replicative senescence) or by other endogenous or exogenous signals [30;42-44]. As a result of our discovery that exogenous histone

H2A induces senescence, we analyzed the distribution of cells through the cell cycle phases. We demonstrate that exogenous histone H2A induces G2/M cell cycle arrest (Fig. 4C). Senescence is characterized by various phenotypic changes, such as a flattened and enlarged morphology, an increased in senescence-associated β-galactosidase (SA β-gal) activity, and an accumulation of lipofuscin [30;42-44]. Since cancer cells acquire the ability to escape normal senescence, induction of senescence in cancer cells is a promising strategy in cancer therapy. For example, Poele *et al.* [45] have shown that etoposide, an anticancer drug, induces senescence in cancer cell lines, such as the MCF-7 breast adenocarcinoma, A2780 ovarian carcinoma and LS174T colon carcinoma.

In this study, we demonstrate that exogenous histone H2A induces senescence in MCF-7 cells. Histone H2A-treated MCF-7 cells have an altered morphology as compared with untreated cells. They flatten, increase in diameter, and develop cellular extensions (Fig. 5B). Histone H2A treatment also increases the expression of β -galactosidase, a biochemical marker of senescence (Fig. 5D).

Senescent cell death occurs via p53 and cyclin-dependent kinase inhibitors, including p21 and p16 [46]. We have demonstrated that exogenous histone H2A transiently increases p21 protein levels in MCF-7 cells (Fig. 6). A transient increase in p21 expression has also been reported by Robles *et al.* [47] when human foreskin NHF3 fibroblasts and lung MRC 5 fibroblasts were driven to senescence by treatment with bleomycin and actinomycin, two anticancer drugs.

The p53 protein is a transcription factor that controls the cell cycle, apoptosis and senescence via the transactivation of several genes, such as p21, 14-3-3σ,

Gadd45 and Bax [48-51]. During the first 24 hours, no significant changes in p53 protein (Fig. 6) and RNA (Fig. 7) level occurred when MCF-7 cells were treated with histone H2A. However, p53 is not essential for the induction of cell growth arrest by all anticancer drugs. For example, treatment of breast cancer cells with 6-anilino-5,8-quinolinequinone (LY83583), an inhibitor of guanylate cyclase, induces cellular senescence in a p53-independent manner [52].

Our results suggest that histone H2A-activated p21 protein expression is a p53-independent process. Our findings are consistent with the results reported by Atadja *et al.* [53]. This group has shown that p21 expression increases in senescent fibroblasts in the absence of p53.

The functional and morphological changes observed in histone H2A-treated MCF-7 cells are similar to the growth suppressing effects of some HDAC inhibitors [38]. It has been reported that SAHA blocked cell proliferation, induced G1 and G2-M growth arrest, and up-regulated the p21 protein via a p53-independent mechanism [38;54].

The effect of histone H2A we observed is irreversible, since washing out histone H2A from the culture does not allow MCF-7 cells to divide and to continue to proliferate (data not shown), a phenomenon which resembles replicative senescence [30;43]. The mechanism and intracellular signaling pathways by which histone H2A induces senescence of breast cancer cells remain unknown. Our preliminary investigations demonstrate that oxidative stress, as measured by the generation of reactive oxygen species (ROS), may not be responsible for the H2A-induced senescence. Inhibition of oxidative burst by N-acetyl-L-cysteine (NAC), a

glutathione precursor and a general ROS scavenger [55] showed no effect on MTT reduction assay, suggesting that ROS production is not involved in inhibitory growth effect of H2A (Fig. 8B).

Previous studies linking exogenous histones and cancer therapy have been limited to experiments with histone H1 in the setting of breast cancer therapy [27] and growth inhibition of leukemia and lymphoma cells [26] However, the underlying molecular mechanism was not elucidated. Vani et al. injected histone H1 intratumoraly as a single injection in tumor-bearing animals [27]and found that histone H1 treatment significantly inhibited tumor growth, enhanced mean survival time and significantly improved the immune response and status. Purified histone H1 has also been shown to exert growth inhibition of leukemia cells independent of lineage, stage, and maturation [26] Interestingly, synthetic H1-peptides as well as peptides and proteins with biochemical properties similar to H1 had no inhibitory growth effect at equimolar concentrations [26].

In conclusion, we demonstrate that exogenous histone H2A inhibits cellular proliferation, and induces growth arrest and cellular senescence in *vitro* in a p21-dependent and p53-independent manner. This effect is histone H2A-specific. In addition, since recombinant histone H2A does not elicit the same anti-proliferative effect as calf thymus-extracted histone H2A and the difference between these 2 histones consists of the presence of the post-translational modifications in calf thymus-extracted histone H2A, we speculate that these modifications are pivotal to the histone H2A effects observed. Additional studies will be required to confirm this hypothesis.

3.7. ACKNOWLEDGEMENTS

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4. METHODES EXPÉRIMENTALES:

Les méthodes expérimentales qui suivent n'ont pas été décrites dans l'article #2. La majorité des expériences ont été réalisées avec l'histone H2A purifiée à partir de thymus de veau. Pour éviter des répétitions, nous nous référerons à cette histone comme étant l'histone H2A. Lorsque des histones recombinantes seront analysées, nous le spécifierons.

4.1. CULTURE CELLULAIRE ET HISTONES

En plus des lignées cellulaires décrites dans l'article #2, nous ferons référence à d'autres lignées cellulaires. Il s'agit de cellules humaines MDA-MB-231 d'adénocarcinome du sein et de cellules humaines Hs578T d'adénocarcinome du sein. Nous nous sommes procurés ces lignées auprès de Dr. Edward Bradley. Les cellules de neuroblastome murin NXS2 proviennent de Scripps Research Institute (É.U.) et les cellules humaines de neuroblastome SH-SY5Y ont été achetées chez ATCC. Les quatre lignées ont été cultivées dans le milieu DMEM avec du glucose, de la L-glutamine, de l'hydrochlorure de pyridoxine et un supplément de 10% du serum de veau fœtal (FBS) et de 100U/ml pénicilline/streptomycine.
L'histone H1.2 recombinante a été achetée chez Axxora Platform.

4.2. INCORPORATION DE LA THYMIDINE

Les cellules MCF-7 et MDA-MB-231 ont été ensemencées dans des plaques de 96 puits à une densité allant de 5000 jusqu'à 10 000 cellules par puits. Le lendemain, les cellules ont été traitées avec l'histone H2A (50 et 100 µg/ml). Vingt-

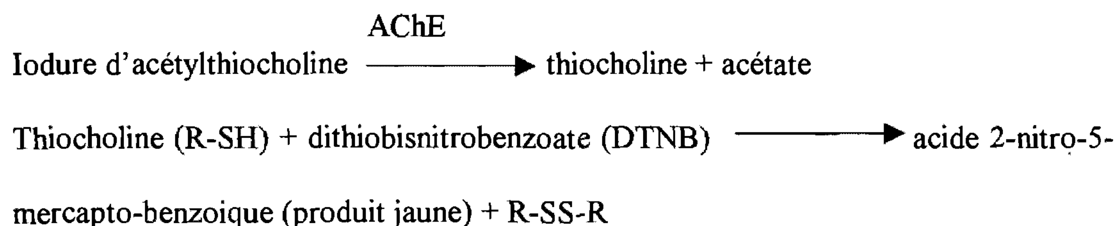
quatre heures avant la fin du traitement, les cellules ont été marquées par l'ajout de 0.5 µCi/puits de thymidine tritiée ([^{3}H] thymidine). À la fin du traitement, les cellules ont été fixées avec l'acide trichloroacétique 5% et l'ADN marqué à la thymidine tritiée a été extrait dans du NaOH 0.1N. La quantité de radioactivité incorporée a été mesurée à l'aide d'un compteur de radioactivité (Tri-Carb 1600TR).

4.3. ACCUMULATION DES LIPIDES

Les cellules MCF-7 ont été ensemencées dans des plaques de 12 puits à la densité de 500 000 cellules par puits. Le lendemain, elles ont été traitées avec 25 et 50 µg/ml d'histone H2A. Après 3 jours, les cellules ont été récoltées et traitées avec le rouge de Nil (Red Nile) à une concentration finale de 100 ng/ml (Greenspan et al. 1985). Les échantillons ont été analysés par cytométrie en flux (FACS-calibur).

4.4. DOSAGE DE L'ACTIVITÉ DE L'ACÉTYLCHOLINESTÉRASE (TEST ELLMAN)

Le test d'Ellman a été développé en 1961 par Ellman *et al.(Ellman et al. 1961)* pour déterminer l'activité de l'enzyme acétylcholinestérase (AChE). Le principe du test peut être résumé par les deux réactions suivantes :



L'acétylcholine, le substrat physiologique de l'AChE, a été remplacée par l'iodure d'acétylthiocholine. Suite à l'action de l'AchE, des groupements thiol sont

libérés. Ces thiols libres (R-SH) réagissent avec le DTNB pour donner un produit coloré jaune et qui peut être dosé par spectrophotométrie.

Les cellules SH-SY5Y ont été ensemencées dans des plaques de 6 puits à une densité de 1 million de cellules par puits. Le lendemain, elles ont été traitées avec l'histone H2A (50 µg/ml). À la fin du traitement, les cellules ont été récoltées et les protéines extraites dans le tampon RIPA (150 mM NaCl, 10 mM Tris, pH7.2, 0.1 % SDS, 1 % Triton X-100, 5 mM EDTA) avec un supplément d'inhibiteurs de protéases : 1µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin et 1 mM PMSF. Après l'ajout de 50 µl RIPA, les cellules ont été laissées sur la glace pendant 30 minutes. Par la suite, elles ont été centrifugées et le surnageant a été récupéré.

Le dosage des protéines a été fait en utilisant la trousse BCA Protein Assay. Il s'agit d'une méthode qui allie la réduction du Cu^{+2} en Cu^{+1} par les protéines en milieu alcalin avec la détection du Cu^{+1} avec l'acide bicinchonique (BCA). Le complexe pourpre formé a été dosé par spectrophotométrie. Bref, 25 µl d'échantillon dilué a été traité avec 200 µl de solution A : B (50 : 1). Après une période d'incubation à 37° C, les absorbances ont été lues à 575 nm (Molecular devices Vmax kinetic microplate reader). Également, une courbe standard à l'aide de l'albumine (BSA) a été préparée suivant la même technique.

La procédure expérimentale utilisée pour déterminer l'activité de l'AchE a été déjà décrite dans la littérature (Ellman et al. 1961;Boone et al. 2001;2007;Quantitation of sulfhydryls DTNB 2007). 15 µl d'extrait protéique a été traité avec 85 µl de Tris HCl 0.05 M, pH 7.5 et 2 µl iodure d'acétylthiocholine 50 mM. Après l'incubation à 37° C pendant 15 minutes, la réaction enzymatique a été

interrompue avec 44 μl SDS 1%. Les produits de la réaction enzymatique ont été mis en évidence par l'ajout de 2 μl de DTNB. Les absorbances ont été lues à 450 nm (Molecular devices kinetic microplate reader). La concentration molaire en thiocholine libérée a été calculée selon la formule suivante :

Concentration molaire = DO/coefficient d'extinction du produit jaune (13.600 $M^{-1}cm^{-1}$) X volume total/volume échantillon.

Après 15 minutes d'incubation, l'activité de l'AChE a été exprimée en quantité de thiocholine produite par unité de temps (μmol/min ou U). Pour calculer l'activité spécifique de l'AChE (μmol/min/mg protéine) on a rapporté l'activité de l'AChE, calculée selon la procédure présentée précédement, à la quantité totale de protéines qui a été dosé séparément par spectrophotométrie.

4.5. WESTERN BLOT

Cette technique a été décrite dans l'article #2 dans la section de Matériels et méthodes. En plus des anticorps mentionnés dans l'article #2, d'autres anticorps ont été utilisés. Il s'agit de l'anticorps polyclonal de chèvre contre la caséine humaine (« goat polyclonal anti-human β-casein ») à une dilution de 1 :1000, de l'anticorps polyclonal de lapin contre la protéine humaine c-myc (« rabbit polyclonal anti-c-myc ») à une dilution de 1 :500, de l'anticorps polyclonal de lapin contre l'histone H3 acétylée (« rabbit polyclonal anti-acetyl-histone H3 ») à une dilution de 1 :5000 et un antisérum de lapin contre l'histone H4 acétylée (« rabbit antiserum anti-acetyl-histone H4 ») à une dilution de 1 :2000.

4.6. RT-PCR

Cette technique a été mentionnée dans l'article #2 dans la section de Matériels et méthodes. Ce qui suit donnera plus de détails sur cette technique, les séquences d'amorces utilisées pour analyser l'expression de l'unité catalytique de la télomérase, la hTERT (human telomerase reverse transcriptase), ainsi que les conditions pour la PCR.

Les cellules MCF-7, MDA-MB-231 et Hs578T ont été ensemencées dans des plaques de 6 puits à une densité allant de 300 000 jusqu'à 1 million de cellules par puits. Le lendemain, elles ont été traitées avec l'histone H2A (50 µg/ml). À la fin du traitement, l'extraction d'ARN a été faite avec une solution de TRIzol. Les cellules ont été traitées avec 1 ml de TRIzol pendant 3 minutes et avec 0.2ml chloroforme/ml de TRIzol pendant 10 minutes. Les tubes ont été ensuite centrifugés à 13 200 RPM pendant 15 minutes à 4°C. Le surnageant a été récupéré et traité avec 0.5 ml isopropanol/ml de TRIsol pendant 10 minutes. Par la suite, les tubes ont été centrifugés à 13 200 RPM pendant 10 minutes à 4°C. Le culot a été lavé avec de l'éthanol 75 %, séché à la température ambiante et puis dissout dans l'eau exempte de RNase.

La concentration de l'ARN isolé a été déterminée par spectrophotométrie (Bio-rad SmartSpec^{TM} 3000). La qualité de l'ARN a été déterminée par spectrophotométrie et par électrophorèse en gel d'agarose 1.4 %.

La transcription inverse ou la rétrotranscription (RT) a été faite en utilisant 3 µg d'ARN comme matrice, 4µl de dNTP 5mM, 5 µl de tampon RT 5X, 2 µl de DTT 0.1M, 1 µl d'amorce aléatoire et 1 µl inhibiteur de RNase (RNaseOUT), dans un volume total de 20 µl. Le mélange a été chauffé 5 minutes à 65° C. Ensuite, 1 µl de

transcriptase inverse a été ajoutée et la réaction de rétrotranscription (RT) s'est poursuivie pendant 2 heures à 42° C.

La réaction en chaîne par polymerase (PCR, qui est l'abréviation anglophone de polymerase chain reaction,) pour quantifier la hTERT a été faite en utilisant 2µl d'ADNc, 2 µl de dNTP (2mM), 2 µl de tampon 10X avec $MgCl_2$, 1µl de l'amorce sens (10 µg/ml) AGAACGTTCCGCAGAGAAAA, 1 µl de l'amorce anti-sens (10 µg/ml) AAGCGTAGGAAGACGTCGAA et 1 µl de Taq ADN polymerase (5U/µl). Les séquences de l'amorce d'actine sont mentionnées dans l'article #2. Les conditions pour la PCR étaient les suivantes : une dénaturation initiale de 1 minute à 94° C suivie de 35 cycles de dénaturation (94° C pour 15 secondes), hybridation (52° C pour 30 secondes), élongation (72° C pour 2 minutes) et une élongation finale à 72° C pendant 7 minutes. Les produits obtenus par PCR ont été analysés par électrophorèse en gel d'agarose 1% avec bromure d'ethidium et visualisés sous lampe UV (Typhoon 8600).

4.7. TEST D'APOPTOSE PAR MARQUAGE AVEC ANNEXIN V-FITC/PI

Les cellules HeLa ont été ensemencées dans des plaques de 6 puits à la densité de 300 000 cellules par puits. Le lendemain, elles ont été traitées avec l'histone H1.2 recombinante (150 µg/ml) pendant 2, 3, 4, 5 et 6 heures. Par la suite, les cellules ont été récoltées et marquées à l'annexine V-FITC et iodure de propidium (PI) en utilisant la trousse « Annexin V-FITC Apoptosis Detection kit 1 » (BD Biosciences) selon les directives du fabricant. Le pourcentage des cellules

apoptotiques a été déterminé par cytometrie en flux (FACS-calibur, Becton-Dickinson).

4.8. EXTRACTION ACIDE DES HISTONES

Les cellules MDA-MB-231 et Hs578T ont été ensemencées dans des plaques de 6 puits à une densité de 200 000 cellules par puits. Le lendemain, elles ont été traitées avec l'histone H2A (50 µg/ml). À la fin du traitement, les cellules ont été récoltées et les histones ont été extraites selon le protocole décrit par la compagnie Abcam. En bref, après l'ajout de 100 µl de tampon TEB (0.5% Triton X, 2mM de PMSF et 0.02% de NaN_3 dans PBS), les cellules ont été conservées sur la glace pendant 10 minutes sous légère agitation. Par la suite, elles ont été centrifugées à 2000 RPM pendant 10 minutes à 4°C. Le culot a été récupéré, lavé dans TEB et traité avec 0.2N HCl pendant toute la nuit. Le lendemain, les échantillons ont été centrifugés à 2000 RPM pendant 10 minutes à 4°C. Le surnageant a été récupéré et la concentration des protéines a été déterminée en utilisant la trousse BCA Protein Assay.

4.9. COLORATION DES GELS SDS-PAGE AU BLEU DE COOMASSIE

Afin de vérifier si des quantités égales de protéines ont été chargées dans chaque puits, nous avons procédé à une coloration au bleu de Coomassie. Après la migration des protéines dans le gel d'acrylamide, une partie du gel a été placée dans une solution de bleu de Coomassie (0.2 g bleu de Coomassie, 7.5 ml d'acide acétique et 40 ml du méthanol dans 100 ml d'eau) pendant 1 heure à la température de la

pièce. Ensuite, le gel a été placé dans une solution de décoloration (75 ml d'acide acétique et 50 ml de méthanol dans 1 L d'eau) et des lavages ont été effectués successivement jusqu'à ce que les bandes apparaissent clairement.

4.10. ÉTUDES IN VIVO

Afin de vérifier l'efficacité de l'histone H2A à inhiber la prolifération des cellules cancéreuses in vivo, nous avons procédé à une étude chez la souris. Le protocole expérimental a été soumis à l'approbation du Comité institutionnel de protection des animaux du CHUM (CIPA) et l'étude a été réalisée en suivant leurs directives.

Bien que la majorité des nos expériences aient été réalisées sur la lignée cellulaire MCF-7, nous avons aussi utilisé la lignée MDA-MB-231 pour les études in vivo. Cette décision a été prise pour deux raisons : premièrement, la lignée MCF-7 est une lignée estrogène - dépendante, alors que la lignée MDA-MB-231 est estrogène – indépendante (ATCC), c'est-à-dire qu'elle ne requiert pas de suppléments d'estrogène pour s'implanter et proliférer lorsqu'elle est injectée chez la souris. Ceci présente un grand avantage, puisque l'administration des suppléments d'estrogène chez les souris est associée à des effets secondaires tels que la formation de lithiases rénales. De plus, puisque la lignée MDA-MB-231 est une lignée invasive (contrairement à la lignée MCF-7 qui est non-invasive) (Nagaraja et al. 2006), elle offre la possibilité d'étudier la formation des métastases et ainsi de tester le pouvoir anti-métastatique de l'histone H2A,

Avant d'injecter les cellules MDA-MB-231 chez les souris, nous avons fait tester cette lignée cellulaire selon les Procédures normalisées de fonctionnement de CIPA concernant l'évaluation du matériel biologique. Il s'agit d'un test réalisé par la compagnie MuRail (États Unis) pour s'assurer que la lignée en question est exempte de tout pathogène murin potentiellement dangereux pour les souris.

Le protocole expérimental était le suivant : les cellules MDA-MB-231 ont été ensemencées dans des plaques de 6 puits à une densité de 300 000 cellules par puits et le lendemain, elles ont été traitées avec l'histone H2A (50 µg/ml). Après 3 jours de traitement, les cellules ont été récoltées et injectées chez des souris immunodéficientes FOX CHASE SCID (severe combined immune deficiency) âgées de 6 semaines provenant de Charles River Laboratories (Wilmington, MA).

Pour l'étude sur la tumorigénicité, les souris ont été divisées en deux groupes de 6 souris. Le premier groupe a été injecté par la voie sous-cutanée (s.c.) avec 1 million de cellules MDA-MB-231 non traitées et le deuxième groupe a été injecté avec 1 million de cellules MDA-MB-231 traitées avec l'histone H2A.

Pour analyser la formation des métastases, une étude pilote avec 3 souris a été suggérée par CIPA. Les 3 souris ont été injectées par la voie intra- veineuse (i.v.) avec 1 million de cellules MDA-MB-231 non traitées.

Les souris ont été suivies deux fois par semaines et nous nous sommes assurés que les points limites décrits dans les Protocoles et procédures de CIPA étaient respectés. Ainsi, pour l'étude de tumorigénicité, les souris ont été pesées et les tumeurs ont été mesurées deux fois par semaine. L'expérience a été cessée après 7 semaines, une fois que la grosseur de la tumeur sous-cutanée ait atteint 17 mm.

La longueur et la largeur des tumeurs ont été mesurées avec un pied à coulisse (la Figure 9 montre l'aspect macroscopique d'une tumeur formée en sous-cutané sur le flanc des souris). Le volume des tumeurs a été calculé selon la formule suivante (Julien et al. 2006) :

$$V = \pi/6 \times l \times w \times [l + w]/2$$

l = longueur

w = largeur

Figure 9 : L'apparence macroscopique d'une tumeur formée chez les souris après l'injection des cellules cancéreuse MDA-MB-231.

Les cellules MDA-MB-231 traitées et non-traitéees ont été injectées dans le flanc des souris. Le volume des tumeurs formées a été déterminé en mesurant la longueur et la largeur de la tumeur.

Pour l'étude pilote des métastases, les souris ont également été pesées 2 fois par semaine et ont été sacrifiées 6 semaines plus tard. La présence des métastases pulmonaires a été vérifiée en faisant une autopsie des animaux sacrifiés.

4.11. TEST POUR LA DÉTECTION ET LE TRAITEMENT DES MYCOPLASMES

Toutes nos lignées cellulaires ont été testées pour la présence de mycoplasmes, parasite souvent présent dans les cultures cellulaires. Le test a été fait avec la trousse MycoTect™ de Gibco, selon les instructions du fabricant.

Les lignées parasitées par les mycoplasmes ont été traitées en utilisant la trousse BM-Cyclin de Roche, selon les instructions du fabricant.

5. RÉSULTATS

Les résultats qui suivent n'ont pas été décrits dans l'article #2.

5.1. HISTONE H1.2 RECOMBINANTE PROVOQUE UNE DIMINUTION DU NOMBRE DE CELLULES MÉTABOLIQUEMENT ACTIVES

L'effet de l'histone H1.2 recombinante a été évalué par le test MTT fait sur plusieurs lignées cellulaires, soit les cellules MCF-7, NXS2 et HeLa. Comme on peut le voir sur la Figure 10, l'histone H1.2 recombinante entraîne une diminution du nombre de cellules métaboliquement actives de façon dose-dépendante.

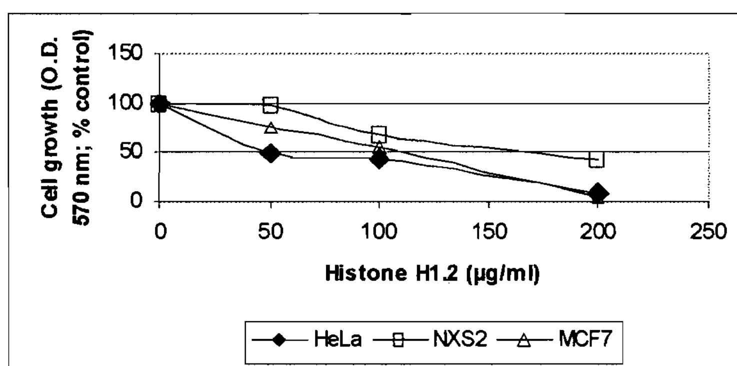


Figure 10 : Effet de l'histone H1.2 recombinante sur les cellules cancéreuses. Les cellules MCF-7, NSX2 et HeLa ont été traitées avec l'histone H1.2 recombinante (50-200 µg/ml) pendant 48H. Le nombre de cellules métaboliquement actives a été évalué par le test MTT. L'expérience a été réalisée au moins 2 fois.

5.2. L'HISTONE H1.2 RECOMBINANTE N'INDUIT PAS DE CHANGEMENTS MORPHOLOGIQUES DANS LES CELLULES MCF-7

Beaucoup d'agents de différenciation induisent des changements dans la morphologie des cellules cancéreuses. Nous avons montré dans l'article #2 que l'histone H2A provoquait des changements morphologiques des cellules MCF-7 (Figure 5A et B). Selon des résultats obtenus précédemment par un autre étudiant de notre laboratoire, il semble que les histones H2B, H3 et H4 soient incapable d'induire des changements morphologiques comme l'histone H2A (résultats non-montrés). Nous avons testé également l'histone H1.2, le principal membre de la famille d'histone H1 (Pohlmeyer et al. 2000) et nous avons constaté que cette histone n'induisait pas de changements morphologiques (Figure 11).

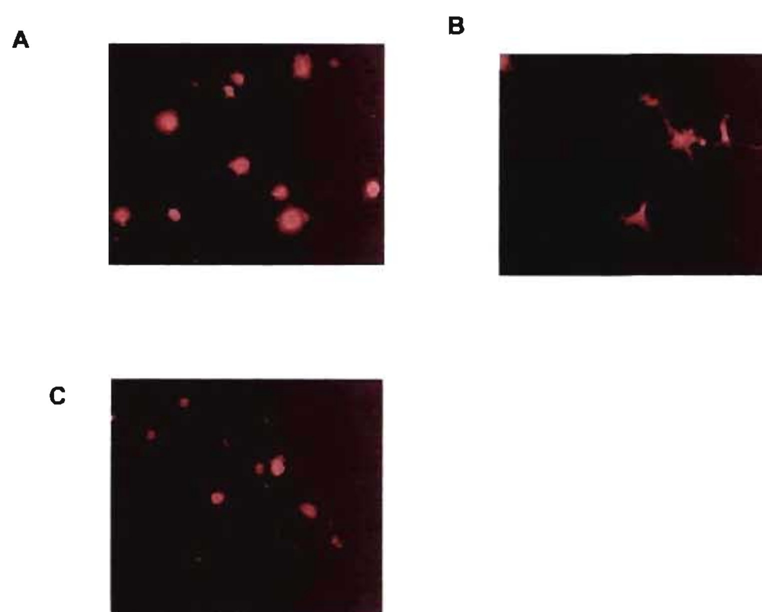


Figure 11 : Changements morphologiques des cellules MCF-7 suite au traitement avec l'histone H1.2 recombinante et H2A.

Des cellules MCF-7 (A) ont été traitées avec 50 µg/ml d'histone H2A (B) et 150 µg/ml d'histone H1.2 recombinante (C) pendant 24H. Les cellules ont été colorées au

violet de crystal et photographiées à un grossissement de 200X. L'expérience a été réalisée au moins 2 fois.

5.3. HISTONE H1.2 RECOMBINANTE INDUIT L'APOPTOSE DANS LES CELLULES HELA

Plusieurs médicaments employés dans la thérapie contre le cancer ont comme mode d'action l'induction de l'apoptose. Nous avons évalué si l'histone H1.2 recombinante était capable d'induire l'apoptose dans les cellules cancéreuses. Après un traitement avec 150 µg/ml d'histone H1.2 recombinante, nous avons constaté une augmentation rapide du pourcentage de cellules apoptotiques (Figure 12).

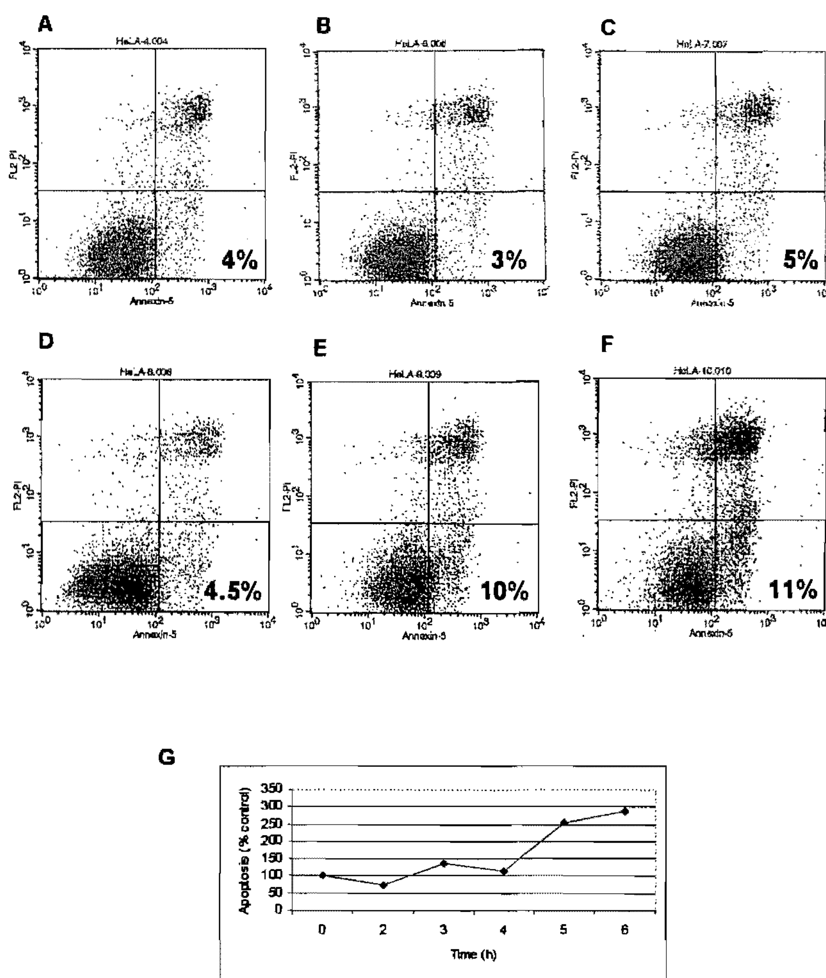


Figure 12 : Induction de l'apoptose dans les cellules Hela après le traitement avec l'histone H1.2 recombinante.

Des cellules Hela ont été traitées avec l'histone H1.2 recombinante (150 μg/ml) pendant 2, 3, 4, 5 et 6H (B,C,D,E,F, respectivement). Le pourcentage de cellules apoptotiques (cellules positives pour l'annexine V, mais négative pour le PI) a été évalué après le marquage à l'annexineV-FITC/PI (G). Les profils cytométriques sont montrés dans les Figures A-F. L'expérience a été répétée en changeant les temps d'exposition à l'histone H1.2. Une augmentation du pourcentage des cellules apoptotiques a été également constatée (résultats non-montrés)

5.4. L'IMPACT DE LA PRÉSENCE DES MYCOPLASMES DANS LES CELLULES SUR LES EFFETS DE L'HISTONE H2A.

La contamination par des mycoplasmes est fréquente dans des cultures cellulaires. Comme celle-ci peut affecter les résultats expérimentaux et entraîner d'importantes pertes de temps et de matériel, nous avons testé toutes nos lignées cellulaires pour la présence de mycoplasmes. Dans les cas où la contamination était présente, un traitement avec une combinaison d'antibiotiques a été effectué. Ainsi, nous avons pu constater que la présence d'une contamination par des mycoplasmes altère les effets de l'histone H2A. Tel que la Figure 13 le montre, les cellules HepG2 contaminées avec des mycoplasmes sont résistantes aux effets de l'histone H2A, tandis que les mêmes cellules deviennent sensibles lorsqu'elles sont décontaminées.

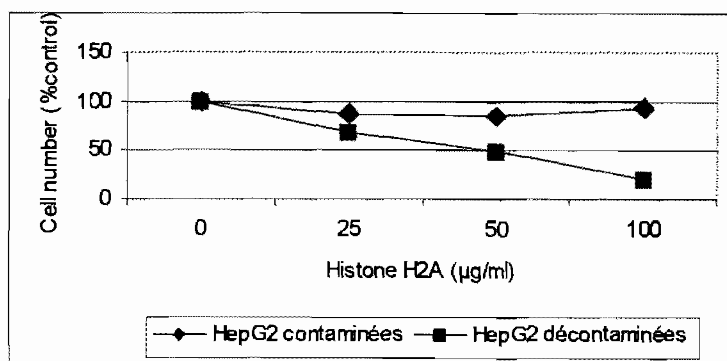


Figure 13 : L'impact de la contamination par les mycoplasmes sur les effets de l'histone H2A.

Des cellules HepG2 contaminées par des mycoplasmes et décontaminées ont été traitées avec l'histone H2A (25-100µg/ml) pendant 72H. Le nombre de cellules viables a été déterminé par l'exclusion du bleu de Trypan.

5.5. HISTONE H2A INDUIT DES CHANGEMENTS MORPHOLOGIQUES ET INHIBE LA PROLIFÉRATION DES CELLULES PRIMAIRES D'ADÉNOCARCINOME MAMMAIRE HUMAIN

Nous avons démontré que l'histone H2A inhibe la prolifération et induit des changements morphologiques dans des lignées cellulaires cancéreuses. Nous avons testé cet effet sur des cellules primaires humaines provenant d'un adénocarcinome mammaire. Nous avons constaté que l'histone H2A induit des changements morphologiques dans ces cellules. Une diminution du nombre de cellules viables a été également observée (Figure 14).

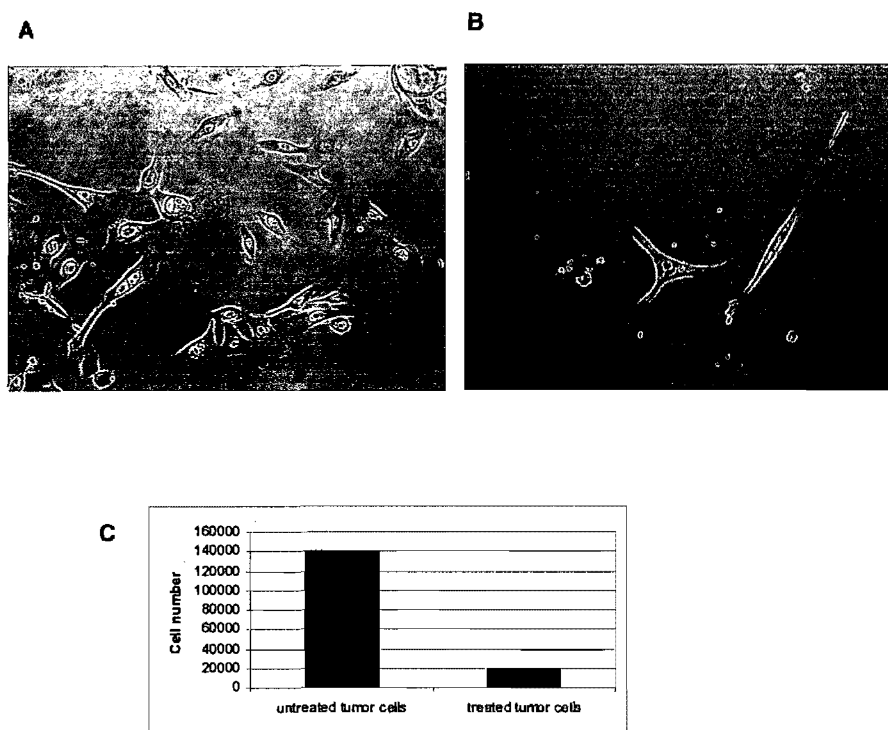


Figure 14 : Effet de l'histone H2A sur les cellules primaires d'adénocarcinome mammaire humain.

Des cellules provenant d'une patiente atteinte d'un adénocarcinome mammaire ont été traitées avec 50 µg/ml d'histone H2A (B). Trois jours plus tard, des photos ont été prises à un grossissement de 200X (A,B). Les cellules viables ont été comptées après une coloration au bleu de Trypan (C). L'expérience a été réalisée une fois.

5.6. HISTONE H2A INHIBE LA PROLIFÉRATION CELLULAIRE (MÉTHODE D'INCORPORATION DE THYMIDINE TRITIÉE)

L'augmentation de la prolifération cellulaire est une caractéristique des cellules cancéreuses. Un des objectifs de la thérapie anti-cancéreuse est de freiner cette prolifération incontrôlée. Ainsi, la mesure de viabilité et de la prolifération cellulaire est un instrument important dans la recherche sur le cancer. Nous avons

montré dans l'article #2 que le traitement avec l'histone H2A provoque une diminution du nombre de cellules viables (par coloration avec le bleu de Trypan, Figure 1A et Figure 2) et le nombre de cellules métaboliquement actives (par le test MTT, Figure 1B et C).

Nous démontrons ici que l'histone H2A inhibe la prolifération des cellules MCF-7 et MDA-MB-231. L'effet de l'histone H2A dépend de la dose administrée et du temps d'exposition (Figure 15).

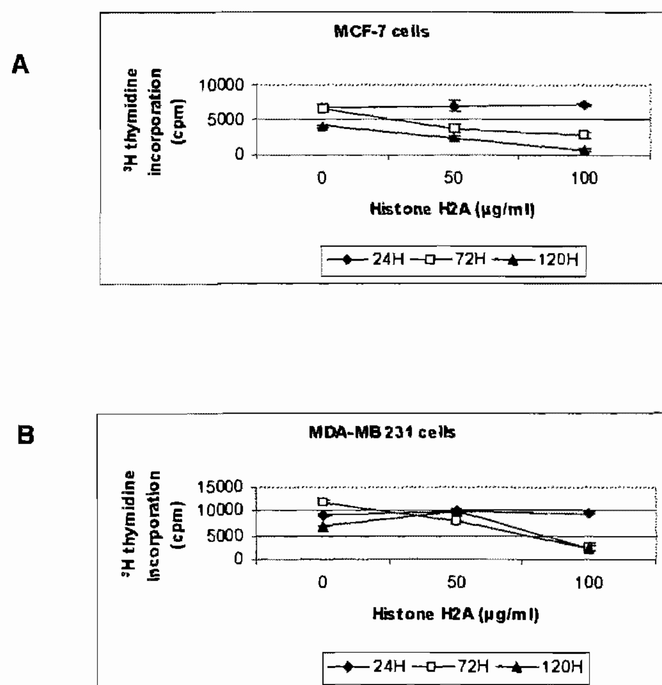


Figure 15 : Inhibition de la prolifération des cellules MCF-7 et MDA-MB-231 par l'histone H2A.

Les cellules MCF-7 (A) et MDA-MB-231 (B) ont été traitées avec différentes concentrations d'histone H2A pendant 24, 72 et 120 heures et l'inhibition de la prolifération a été évaluée par le test d'incorporation de la thymidine tritiée. L'effet

de l'histone H2A était significatif à 72 et 120 heurs (**$p<0.01$ établi par le test de Student). Cette expérience a été réalisée au moins 2 fois.

5.7. HISTONE H2A INDUIT DES CHANGEMENTS MORPHOLOGIQUES DANS LES CELLULES MDA-MB-231

Les changements dans la morphologie des cellules sont souvent associés à la différenciation cellulaire. Tel que nous avons montré dans l'article #2, le traitement des cellules MCF-7 avec l'histone H2A est associé à des changements morphologiques (Figure 5A et B). Nous avons constaté que l'histone H2A induit également des changements morphologiques dans les cellules MDA-MB-231 (Figure 16).

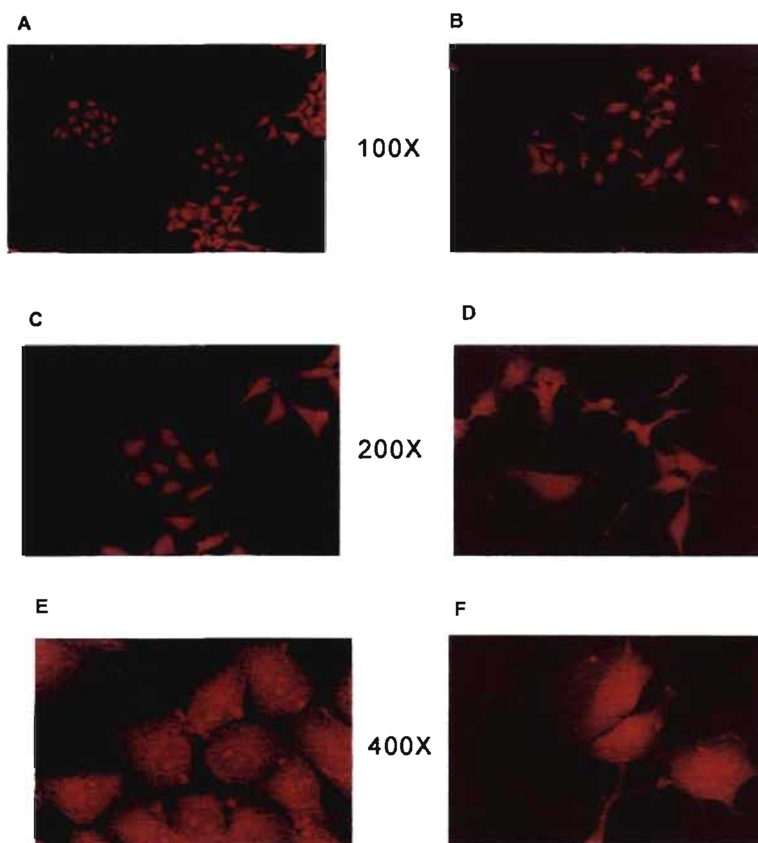


Figure 16 : Changements morphologiques des cellules MDA-MB-231 suite au traitement avec l'histone H2A.

Des cellules MDA-MB-231 (A,C,E) ont été traitées avec 50 µg/ml d'histone H2A pendant 72H (B,D,F). Les cellules ont été colorées au violet de crystal et photographiées à un grossissement de 100X (A,B), 200X (C,D) et 400X (E,F). L'expérience a été réalisée au moins 2 fois.

5.8. HISTONE H2A INDUIT L'EXPRESSION DE LA B -CASÉINE

L'augmentation de l'expression de la β-caséine est un marqueur de différenciation des cellules mammaires. Nous avons analysé par western blot l'effet de l'histone H2A sur l'expression de la β-caséine dans deux lignées de cancer du sein, soit les cellules MCF-7 et les cellules Hs578T (Figure 17). Nous avons constaté que le traitement avec l'histone H2A augmente l'expression de la β-caséine et que l'effet augmente avec le temps d'exposition.

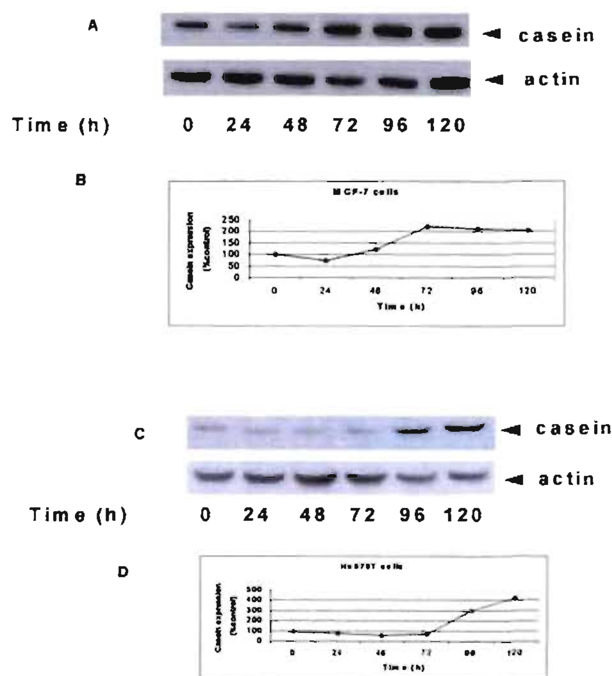


Figure 17 : Effet de l'histone H2A sur l'expression de la β-caséine.

Les cellules MCF-7 (A,B) et les cellules Hs578T (C,D) ont été traitées avec 50 μg/ml d'histone H2A pendant 1 à 5 jours et l'expression de la β-caséine a été déterminée par western blot (A,C). L'intensité des bandes a été évaluée par densitométrie à l'aide du logiciel « Image J » (B,D). L'expérience a été réalisée au moins 2 fois pour les cellules MCF-7 et une fois pour les cellules Hs578T.

5.9. HISTONE H2A INDUIT L'ACCUMULATION DES LIPIDES

L'accumulation des lipides, un autre marqueur de différenciation des cellules mammaires, peut être mise en évidence par la coloration au rouge de Nil. La fluorescence obtenue après le marquage à l'aide de ce colorant est différente pour les lipides neutres et pour les lipides polaires. Ainsi, le rouge de Nil permet de différencier les gouttelettes lipidiques, qui contiennent les lipides neutres, des lipides polaires qui constituent les membranes cellulaires. Il est donc possible de mesurer la fluorescence cellulaire due spécifiquement aux lipides neutres. Le pourcentage de cellules marquées au rouge de Nil augmente après le traitement avec l'histone H2A (Figure 18). L'accumulation des lipides augmente avec la concentration de l'histone H2A utilisée. L'effet maximal est observé après 120H de traitement pour les cellules MCF-7 (Figure 18G), alors que pour les cellules Hs578T on observe un plus grand effet après 96H (Figure 18N).

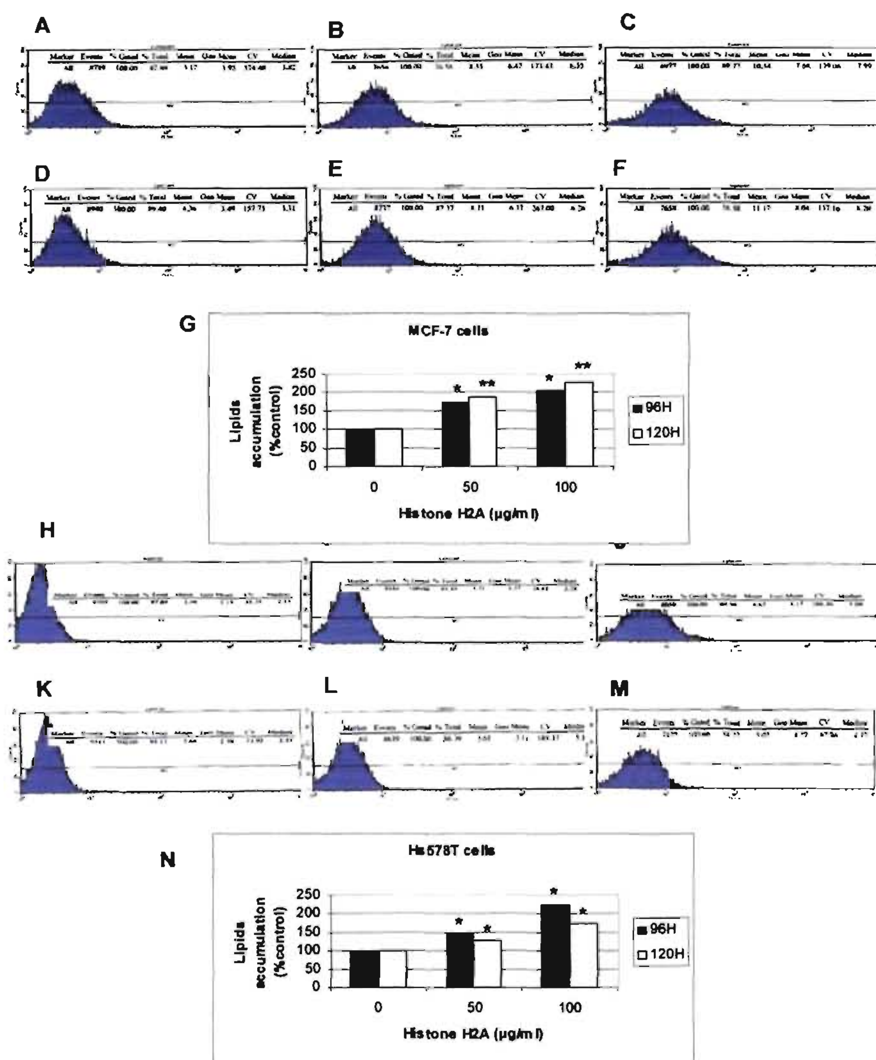


Figure 18 : Accumulation des lipides dans les cellules MCF-7 et Hs578T suite au traitement avec l'histone H2A.

Des cellules MCF-7 (A-G) et Hs578T (H-N) ont été traitées avec histone H2A (50 et 100 µg/ml) pendant 96 et 120H. Les cellules ont été marquées au rouge de Nil et l'intensité de la fluorescence a été mesurée à l'aide de la cytométrie en flux (FACS). Des profils cytométriques représentatifs sont montrés pour les cellules MCF-7 (A-F) et Hs578T (H-M). L'effet de l'histone H2A est significatif (**$p<0.01$, *$p<0.05$ établi par le test de Student). L'expérience a été réalisée 2 fois.

5.10. HISTONE H2A AUGMENTE L'ACTIVITÉ DE L'ACÉTYLCHOLIESTÉRASE (ACHE) DANS LES CELLULES SH-SY5Y

Les cellules SH-SY5Y sont des cellules de neuroblastome humain qui expriment des marqueurs spécifiques de différenciation tels que des changements dans l'expression des neurotransmetteurs. Pour évaluer si l'histone H2A était capable d'induire la différenciation des cellules de neuroblastome, nous avons mesuré l'activité de l'AchE, une enzyme responsable de la dégradation de l'acétylcholine. Après une incubation de 72H, on constate une augmentation de l'activité de l'AchE dans les cellules SH-SY5Y traitées avec l'histone H2A (Figure 19).

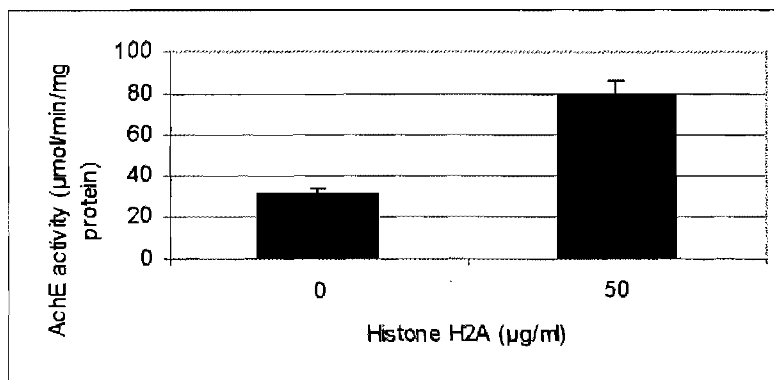


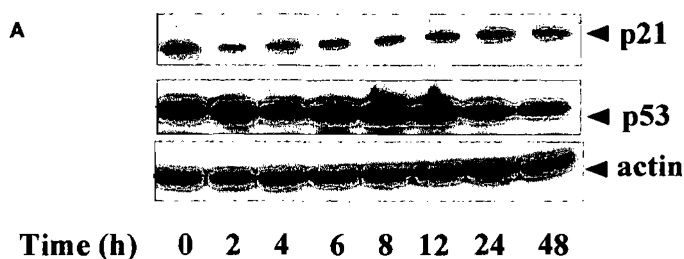
Figure 19 : Changements dans l'activité de l'AchE dans des cellules de neuroblastome suite au traitement avec l'histone H2A.

Les cellules SH-SY5Y ont été traitées avec l'histone H2A pendant 72H. L'activité de l'AchE a été déterminée par le test Ellman. L'effet de l'histone H2A sur l'activité de l'AchE des cellules SH-SY5Y était significatif ($**p<0.01$ établi par le test de Student). Les expériences ont été faites 2 fois.

5.11. EXPRESSION DE P21 ET P53 DANS LES CELLULES MDA-MB-231 APRÈS LE TRAITEMENT AVEC L'HISTONE H2A

Les protéines p21 et p53 sont des protéines impliquées dans le contrôle de la prolifération des cellules cancéreuses et souvent ciblées par des agents anti-cancéreux. Nous avons montré dans article #2 que le traitement avec l'histone H2A augmente l'expression de p21, mais ne change pas l'expression de p53 dans les cellules MCF-7 (Figure 6). Nous avons analysé ici les changements dans l'expression de p21 et p53 dans les cellules MDA-MB-231 après le traitement avec 50 µg/ml d'histone H2A pendant 2 à 48H. Nous avons constaté une augmentation de l'expression de p53 et une diminution de l'expression de p21 (Figure 20). Les changements ont lieu dans les premières 24H, tel qu'observé pour les cellules MCF-7.

A



B

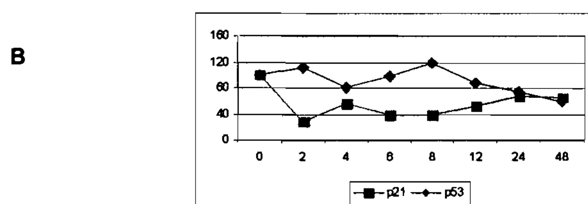
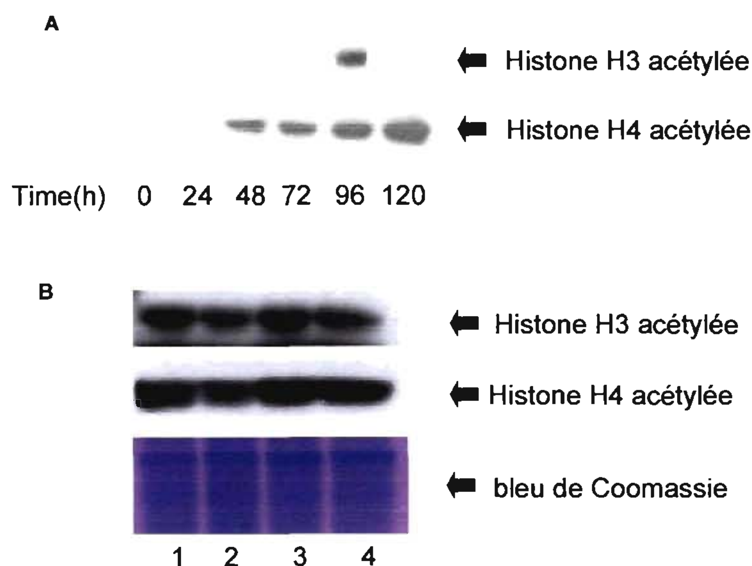


Figure 20 : L'expression de p21 et p53 dans les cellules MDA-MB-231 après le traitement avec l'histone H2A.

Des cellules MDA-MB-231 ont été traitées avec 50 µg/ml d'histone H2A pendant les temps indiqués. L'expression de p21 et p53 a été déterminée par western blot (A). L'intensité des bandes a été évaluée par densitométrie à l'aide du logiciel « Image J » (B). L'expérience a été réalisée une fois.

5.12. EFFET DE L'HISTONE H2A SUR LE NIVEAU D'ACÉTYLATION DES HISTONES H3 ET H4

Nous avons proposé un modèle pour expliquer les effets de l'histone H2A (voir le mécanisme de compétition proposé dans l'article #1). Pour vérifier cette hypothèse, nous avons analysé si le traitement avec l'histone H2A menait à une augmentation du niveau d'acétylation des histones H3 et H4, tel que rapporté pour les HDACi. Le premier essai que nous avons réalisé, montrait une augmentation significative d'histone H4 acétylée après le traitement avec 50 µg/ml d'histone H2A (Figure 21A). Malheureusement, nous n'avons pas pu répéter ce résultat (Figure 21B).



1. cellules MDA-MB231 non-traitées
2. cellules MDA-MB231 traitées avec 50µg/ml pendant 72H
3. cellules Hs578T non-traitées
4. cellules Hs578T traitées avec 50µg/ml pendant 72H

Figure 21 : Acétylation des histones H3 et H4 après le traitement avec l'histone H2A.

Dans la Figure A, des cellules Hs578T ont été traitées avec 50 µg/ml d'histone H2A pendant 1 à 5 jours. Dans la Figure B, des cellules MDA-MB-231 et Hs578T ont été traitées avec l'histone H2A (50 µg/ml) pendant 72H. Le niveau d'acétylation des histones H3 et H4 a été mesuré par western blot. La coloration avec le bleu de Coomassie a été faite pour s'assurer que les puits ont été chargés avec la même quantité de protéines totales. L'expérience a été réalisée 2 fois.

5.13. HISTONE H2A DIMINUE LA TAILLE DES TUMEURS IN VIVO SANS AFFECTER LE POIDS DES SOURIS

Nous avons démontré précédement que l'histone H2A inhibait la prolifération cellulaire in vitro. Nous voulions savoir si l'effet de l'histone H2A était similaire dans un modèle in vivo. Des cellules MDA-MB-231 non-traitées et traitées avec l'histone H2A ont été injectées en sous cutané (s.c.) chez des souris SCID. Les souris ont été divisées en 2 groupes, soit le groupe contrôle (C : C1-C6) et le groupe traité (T : T1-T6). Nous avons constaté que la taille des tumeurs formées à partir des cellules non-traitées était plus grande que la taille des tumeurs dérivées des cellules traitées avec l'histone H2A (Figure 22A-C). Cependant, la différence n'était pas statistiquement significative. En effet, nous avons constaté récemment que le dernier lot d'histone H2A reçu ne fonctionnait pas de la même façon dans certaines expériences que les lots antérieurs (résultats non- montrés). Les expériences in vivo ont été réalisées avec ce lot d'histone et l'absence d'effets significatifs pourrait être donc secondaire à l'utilisation de ce dernier lot. Des expériences sont présentement en cours dans le laboratoire pour expliquer le problème de perte d'efficacité de l'histone H2A.

Le poids des souris n'a pas été affecté par le traitement (Figure 22D,E). Durant l'expérience, une souris du groupe C a due être sacrifiée (voir la flèche dans la Figure 22A et D) car la taille de la tumeur nuisait à sa démarche (un des points limites de l'expérimentation chez les animaux).

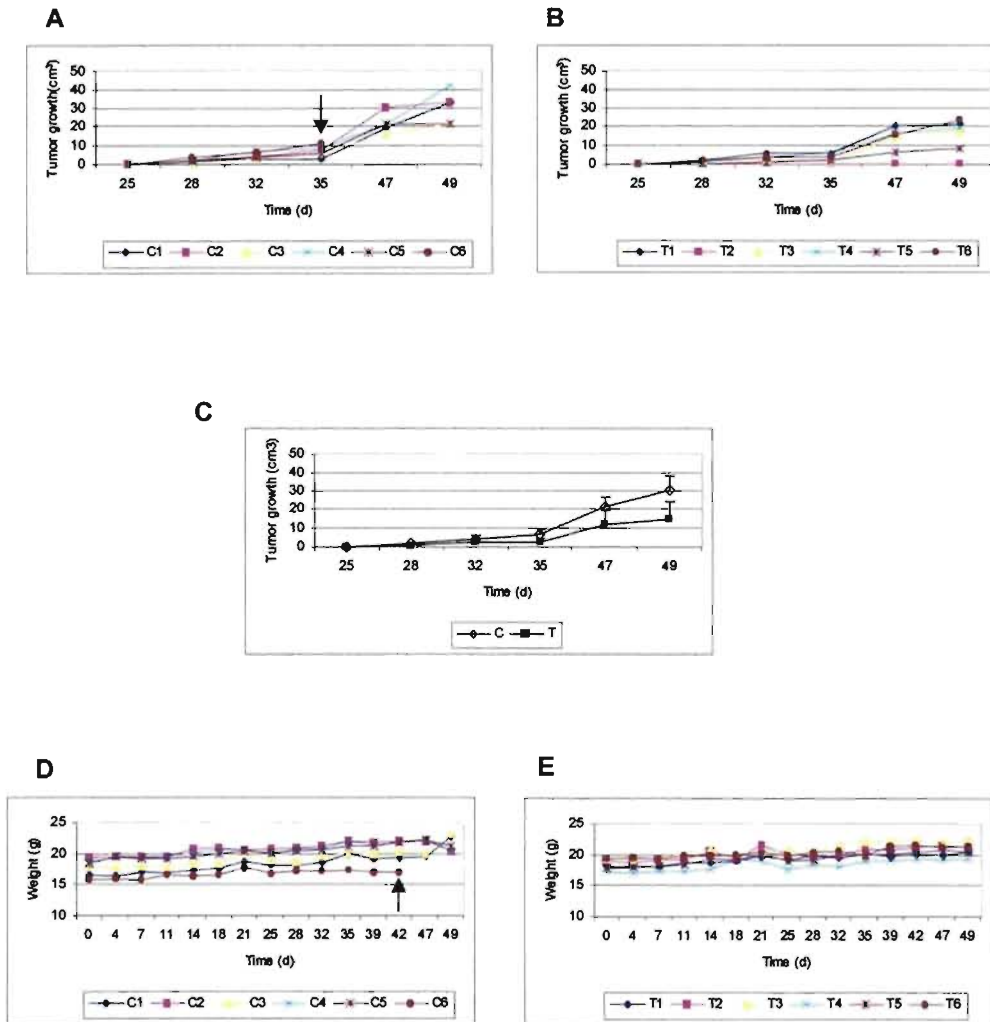


Figure 22 : Effet de l'histone H2A in vivo.

Des souris SCID ont été injectées en s.c. avec des cellules MDA-MB-231 non-traitées et traitées avec 50 µg/ml d'histone H2A. La taille des tumeurs a été mesurée avec un pied à coulisse. Les Figures A et B montrent l'évolution de la taille des tumeurs pour chaque souris du groupe contrôle (C1-C6) et du groupe traité (T1-T6). La Figure C montre les moyennes des tailles des tumeurs des 2 groupes de souris : contrôle (C) et traité (T). L'évolution des poids de souris durant l'expérience est

indiquée dans les Figures D (groupe contrôle) et E (groupe traité). L'expérience a été réalisée une fois.

Pour l'étude pilote des métastases, les souris ont été sacrifiées 6 semaines après l'injection intra-veineuse (i.v.). Tel que prévu (Guo et al. 2006), la présence des métastases au niveau des poumons a été identifiée (Figure 23).

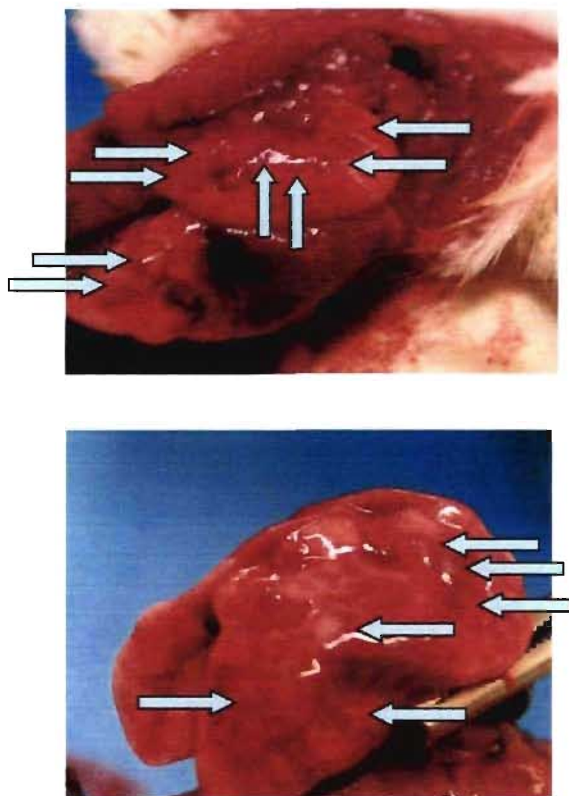


Figure 23 : Métastases au niveau des poumons.

Les souris injectées avec des cellules MDA-MB-231 par voie i.v. ont développé des métastases pulmonaires après 42 jours. Les flèches montrent des foyers de métastases.

5.14. HISTONE H2A INHIBE L'EXPRESSION DE LA PROTÉINE C-MYC

La protéine c-myc est souvent surexprimée dans les cancers et associée aux tumeurs agressives et indifférenciées. Ainsi, cette protéine est devenue une cible pour la thérapie anti-cancéreuse (Vita and Henriksson 2006). Nous avons constaté qu'après le traitement avec l'histone H2A, l'expression de c-myc diminue (Figure 24).

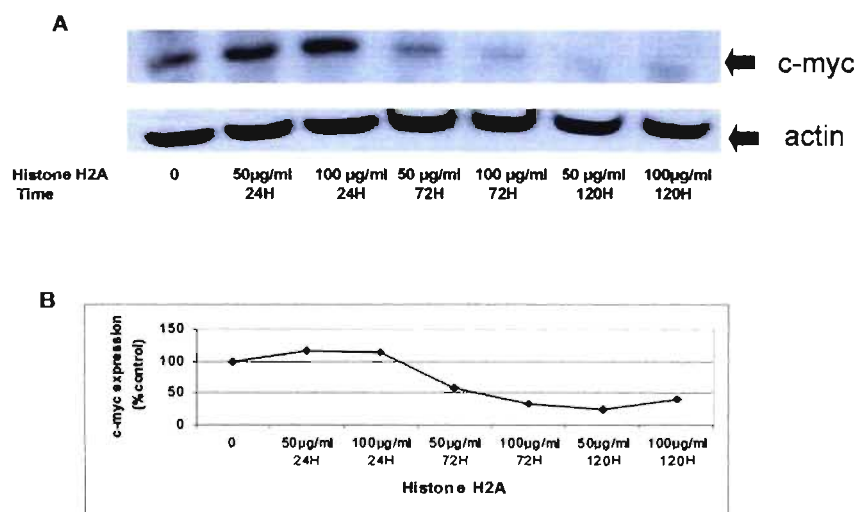


Figure 24 : L'expression de c-myc après le traitement avec l'histone H2A.

Des cellules Hs578T ont été traitées avec 50 et 100 µg/ml d'histone H2A pendant 24, 72 et 120H. L'expression de la protéine c-myc a été évaluée par western blot (A). L'intensité des bandes a été déterminée par densitométrie à l'aide du logiciel « Image J » (B). L'expérience a été réalisée une fois. Elle a été répétée pour la lignée MCF-7. L'intensité des bandes était très faible, mais les résultats étaient semblables (résultats non-montrés). L'expérience a été réalisée une fois.

5.15. HISTONE H2A INHIBE L'EXPRESSION DE LA TÉLOMÉRASE

La télomérase est présente dans beaucoup de cancers. Elle permet aux cellules de se diviser indéfiniment. Nous avons analysé l'effet de l'histone H2A sur l'expression de hTERT, l'unité catalytique de la télomérase. Nous avons observé une diminution de l'expression de hTERT suite au traitement avec l'histone H2A (Figure 25).

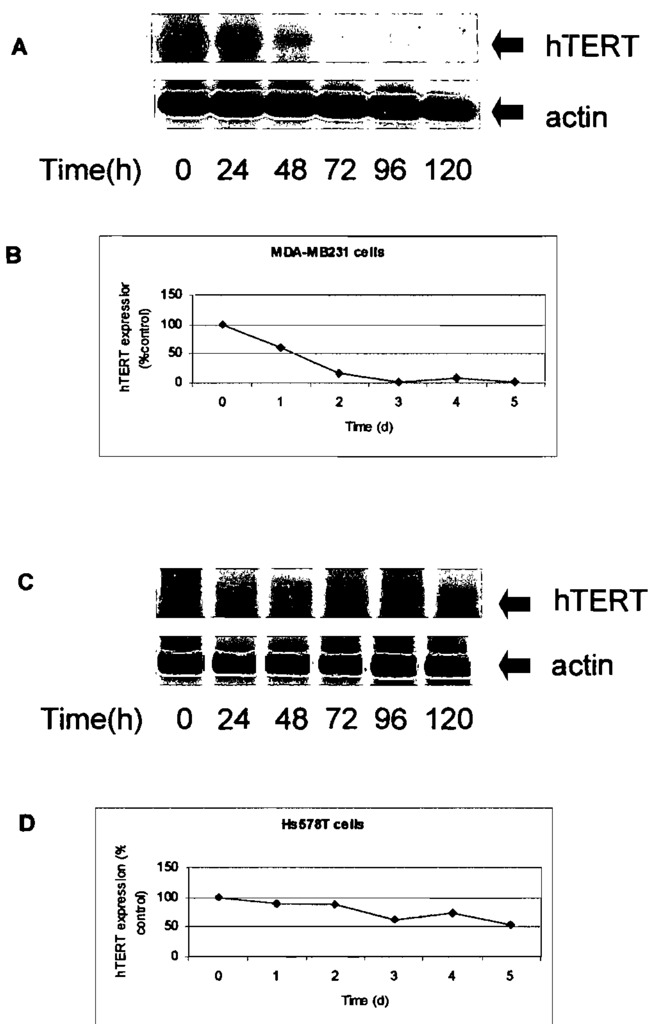


Figure 25 : Inhibition de l'expression de la hTERT par l'histone H2A.

Des cellules MDA-MB-231 (A,B) et Hs578T (C,D) ont été traitées avec 50µg/ml d'histone H2A pendant les temps indiqués. L'expression de hTERT a été déterminée

par RT-PCR (A,C). L'intensité des bandes a été évaluée par densitométrie à l'aide du logiciel « ImageQuant 5.2 » (B,D). L'expérience a été réalisée une fois.

6. DISCUSSION

6.1. MISE EN CONTEXTE

Les stratégies anti-tumorales ont pour objectif principal l'arrêt de la prolifération cellulaire. Plusieurs médicaments sont actuellement utilisés pour la thérapie anticancéreuse. Cependant, plusieurs de ces substances sont très toxiques et parfois peu efficaces. En conséquence, de nombreuses compagnies et groupes de recherches sont activement à la recherche de nouveaux médicaments moins nocifs et plus efficaces.

Tel que déjà mentionné dans l'introduction, en plus de leurs fonctions dans la structure de la chromatine, les histones exercent aussi d'autres rôles importants dans la thérapie contre le cancer. Ceci est le cas pour l'histone H1, H1.2, H2A et H2A.X, dont l'implication dans la progression et dans la thérapie contre le cancer a été décrite dans l'introduction (voir article # 1).

Les premières expériences réalisées par un stagiaire de notre laboratoire ont montré que parmi les 5 histones, les histones H1 et H2A purifiées à partir de thymus de veau (résultats non-montrés, obtenus précédemment par un autre étudiant de notre laboratoire et Figure 1) étaient capables d'inhiber la prolifération des cellules cancéreuses. Nous avons également observé que l'histone H1.2 recombinante, qui est le composant majeur de la famille d'histone H1 (Pohlmeyer et al. 2000) provoque une diminution du nombre de cellules métaboliquement actives (Figure 10). Dans le cadre de la thérapie anticancéreuse, plusieurs stratégies sont mises en oeuvre pour freiner la prolifération cellulaire: l'induction de l'apoptose, de la différenciation et de la sénescence. Nos premières observations recueillies après le traitement des cellules

MCF-7 avec l'histone H2A ont montré que cette histone induisait des changements morphologiques (Figure 5A et B). Puisque les changements morphologiques sont souvent associés à la sénescence et/ou la différenciation, nous avons décidé de vérifier si l'histone H2A induisait la sénescence et la différenciation dans les cellules cancéreuses. De plus, d'autres résultats obtenus dans notre laboratoire ont révélé que l'induction des changements morphologiques est un effet spécifique du traitement à l'histone H2A. Les autres histones du cœur du nucléosome tels que H2B, H3 et H4 purifiées à partir de thymus de veau (résultats non-montrés, obtenus précédemment par un autre étudiant de notre laboratoire) et l'histone H1.2 recombinante (Figure 11) n'induisaient pas de tels changements. Des études récentes ont montré que la translocation de l'histone H1.2 du noyau vers le cytoplasme serait impliquée dans l'induction de l'apoptose (Konishi et al. 2003). Nous avons aussi montré que l'histone H1.2 recombinante induit l'apoptose dans les cellules cancéreuses (Figure 12).

Comme il est mentionné dans l'article #1, des fonctions insoupçonnées des protéines endogènes, tels que les histones, commencent à être mieux caractérisées. Nos résultats préliminaires suggèrent que les histones H2A et H1.2, lorsqu'administrées de façon exogène, peuvent avoir des applications dans la thérapie anti-cancéreuse.

Cette étude propose l'analyse des effets anti-prolifératifs de l'histone H2A purifiée à partir de thymus de veau et l'élucidation de son mécanisme d'action.

6.2. EFFETS ANTI-PROLIFÉRATIFS

Les résultats issus de nos travaux démontrent clairement que l'histone H2A diminue la prolifération des cellules de cancer de sein MCF-7 (Figure 1). Nous avons également démontré que plusieurs lignées cellulaires étaient sensibles aux effets de l'histone H2A : les cellules humaines de carcinome cervical HeLa, les cellules humaine du carcinome hépatocellulaire HepG2, les cellules IM-9 qui appartiennent à une lignée de lymphocytes dérivés du sang d'un patient atteint d'un myélome multiple, les cellules de rein d'un embryon humain HEK293, les cellules MCF-10A dérivées d'une lignée mammaire humaine qui tire son origine d'une lésion fibrokystique non-tumorale et les fibroblastes humains BJ.

Puisque la présence de mycoplasmes, qui sont des contaminants fréquents des cultures cellulaires, influence les effets de l'histone H2A (Figure 13), toutes nos lignées ont été testées pour la présence de mycoplasmes et traitées lorsqu'une contamination était identifiée.

En plus de ses effets sur les lignées cellulaires, nos résultats préliminaires suggèrent que l'histone H2A inhibe la prolifération des cellules tumorales primaires (Figure 14).

Nous avons également constaté que l'effet de l'histone H2A sur les cellules de cancer du sein ne dépend pas de l'expression du récepteur d'estrogène (ER) puisque l'inhibition de la prolifération est observée tant dans les cellules hormono-dépendante MCF-7 (ER+) que dans les cellules hormonoindépendante MDA-MB-231 (ER-) (Figure 15).

Nous avons ensuite voulu savoir si les effets anti-prolifératifs de l'histone H2A étaient spécifiques à celle-ci ou généralisés à tous les autres membres de la

famille des histones retrouvées dans le cœur du nucléosome. Nous avons ainsi constaté que les histones H2B, H3 et H4 n'exercent aucun effet anti-prolifératif (Figure 3A). L'histone H2A.X, un membre de la famille de l'histone H2A, qui contient 142 acides aminés, soit 13 acides aminés de plus par rapport à l'histone H2A est dépourvu d'effet sur la prolifération cellulaire (Figure 3B).

Une autre caractéristique des cellules tumorales est l'altération des signaux d'adhésion cellulaire qui se traduit par la perte du mécanisme de contrôle de la prolifération par inhibition de contact et par la croissance dépendante de l'ancrage à la matrice extracellulaire («anchorage independency») (Kufe et al. 2003). Cette propriété être mis en évidence *in vitro* en cultivant les cellules dans un milieu semi-solide d'agarose. Nos résultats démontrent une diminution de la taille et du nombre de colonies en présence de l'histone H2A (Figure 4A et B). Ceci suggère que l'histone H2A diminue un des attributs des cellules malignes MCF-7 en interférant avec les mécanismes régulateurs qui permettent la croissance en absence d'ancrage à un support solide.

L'arrêt de la prolifération induit par l'histone H2A se traduit par un blocage dans le cycle cellulaire. Nous avons analysé la distribution des cellules MCF-7 au travers des différentes phases du cycle cellulaire et nous avons constaté que l'histone H2A induit une réduction du nombre de cellules en phase G1 et une accumulation des cellules en phase G2/M. Selon la littérature, une accumulation des cellules en phase G2/M signifie l'arrêt de cycle cellulaire à cette phase. Donc, l'histone H2A induit un arrêt du cycle cellulaire en phase G2/M (Figure 3C). L'arrêt du cycle cellulaire en phase G2/M a été identifié pour divers médicaments anticancéreux. Par

exemple, Strait et al. (Strait et al. 2002) ont démontré que le trichostatin A (TSA), un HDACi, inhibe la prolifération des cellules du cancer des ovaires. Cette inhibition est associée avec une diminution du pourcentage de cellules en phase S et une accumulation de cellules en phase G2/M, donc un arrêt du cycle cellulaire en phase G2/M, tel qu'observé pour l'histone H2A. Un autre exemple est le taxol, un agent chimiothérapeutique qui induit également l'arrêt du cycle cellulaire en phase G2/M (Allman et al. 2003).

6.3. MÉCANISME D'ACTION

6.3.1. SÉNESCENCE ET DIFFÉRENCIATION

Tel que nous l'avons déjà mentionné, le traitement des cellules avec l'histone H2A transforme la morphologie de ces cellules. Les cellules deviennent étoilées et développent beaucoup de prolongements filamenteux (Figure 5A et B et Figure 16A-F). Cet aspect phénotypique évoque une cellule en voie de sénescence ou de différenciation. C'est pourquoi nous avons décidé de vérifier si l'histone H2A induisait la sénescence et/ou la différenciation des cellules cancéreuses. En perdant leur caractère différencié et leur capacité d'entrer en sénescence, les cellules cancéreuses ont acquis la capacité de proliférer indéfiniment. Ainsi, l'induction de la différenciation et la réactivation de la sénescence sont des pistes prometteuses dans la lutte contre les cancers.

La sénescence est un état d'arrêt irréversible du cycle cellulaire causé par le raccourcissement des télomères (sénescence réplicative) ou par d'autres signaux

endogènes ou exogènes (Hayflick 1965;Shay and Roninson 2004;Dimri et al. 1995;Marcotte and Wang 2002). D'ailleurs, Sharpless et al. (Sharpless and DePinho 2004) ont défini la sénescence comme une forme spécialisée de différenciation terminale.

Une étape importante dans le processus de carcinogenèse est de vaincre la sénescence cellulaire et ainsi, d'acquérir un potentiel réplicatif illimité. Partant, l'induction de la sénescence cellulaire par l'entremise de différents agents pourrait être appliquée à la thérapie contre le cancer. De fait, il a été démontré que des agents chimiothérapeutiques, tels que l'étoposide (te Poele et al. 2002), des HDACi (Munro et al. 2004) et DNMTi (Kulaeva et al. 2003) sont capable d'induire la sénescence.

La sénescence se caractérise par des changements phénotypiques, comme une morphologie aplatie, une augmentation du cytoplasme, une hausse caractéristique de l'activité de la β-galactosidase à pH 6, connue sous le nom de « senescence associated β-galactosidase » (SA-β-galactosidase ou SA-β-gal), et l'accumulation de lipofuscine dans le cytoplasme (Hayflick 1965;Shay and Roninson 2004;Dimri et al. 1995;Marcotte and Wang 2002).

La β-galactosidase est une enzyme lysosomale essentielle pour la cellule car elle est impliquée dans l'hydrolyse des glucides. La déficience de cette enzyme est responsable du syndrome de Morquio (Mucopolysaccharidosis type IVB; β-galactosidase deficiency; MPS IV) (Santamaria et al. 2006). Dimri et al. (Dimri et al. 1995) ont mis au point une technique pour le marquage des cellules sénescentes (voir matériels et méthodes de l'article #2). La β-galactosidase atteint son maximum d'activité à pH 4 et s'exprime dans la majorité des cellules. Lors d'un marquage

pour la β- galactosidase réalisé à pH 4, les cellules sénescentes et non-sénescentes ne peuvent être différenciées. Cependant, lorsque le test est réalisé à pH 6, seules les cellules sénescentes sont marquées, probablement en raison de l'activité lysosomale élevée.

Dans cette étude nous démontrons que l'histone exogène H2A induit la sénescence cellulaire. Nous avons constaté que le traitement avec l'histone H2A augmente l'expression de la SA-β-gal dans les cellules MCF-7 (Figure 5C et D). Cependant, nous n'avons pas observé de sénescence dans les cellules MDA-MB-231 (données non montrées). Des caractéristiques génétiques distinctes de ces cellules pourraient peut-être rendre compte de cette observation. En effet, des lignées cellulaires distinctes peuvent répondre différemment au traitement avec le même agent anti-proliferatif. Par exemple, le TSA inhibe la prolifération des cellules humaines du cancer des ovaires A2780 en induisant des changements morphologiques et la différenciation de ces cellules. Cependant, le même produit induit l'apoptose dans d'autres lignées de cancer d'ovaires (Strait et al. 2002). Aussi bien, Li et al. (Li et al. 2005) ont démontré que si l'acide valproïque induit l'arrêt de la prolifération et la sénescence des cellules de médulloblastome, les cellules de tumeurs primitives neuroectodermiques supratentorielles (sPNET) y sont entièrement résistantes. Les auteurs ont émis l'hypothèse que des mécanismes compensatoires étaient à l'origine de cette résistance. De la même manière, on peut concevoir que des mécanismes intra-cellulaires spécifiques puissent rendre les cellules MDA-MB-231 résistantes à la sénescence induite par l'histone H2A.

L'induction de la différenciation s'ajoute aux autres stratégies utilisées dans la lutte anti-tumorale. Un des agents de différenciation le mieux caractérisé est l'acide rétinoïque. Ce dernier est utilisé dans le traitement de la leucémie promyelocytaire aiguë (APL) (Grignani et al. 1998). L'induction de la différenciation s'observe non seulement dans les tumeurs hématopoïétiques mais aussi dans les tumeurs solides. Par exemple, Munster et al. (Munster et al. 2001) ont montré que l'acide hydroxamique de suberoylanilide (SAHA), un HDACi, stoppe la prolifération et induit la différenciation des cellules de cancer du sein.

Nous avons démontré que l'histone H2A produisait les mêmes effets sur les cellules de cancer du sein, indépendamment de leur état de différenciation et de leur expression des récepteurs oestrogéniques (ER). L'accumulation de gouttelettes lipidiques dans les cellules du cancer du sein consécutive à l'induction de la différenciation a été rapportée dans plusieurs études (Li et al. 2001;Adan et al. 2003). L'augmentation de l'expression de la β-caséine a été également associée à un phénotype différencié des cellules du cancer de sein (Nobuhisa et al. 2005;Logarajah et al. 2003).

Dans cette étude, nous avons démontré que l'histone H2A provoque une augmentation de l'expression de la β-caséine (Figure 17) et induit l'accumulation de gouttelettes lipidiques (Figure 18) aussi bien dans les cellules bien différentiées MCF-7 et qui expriment ER que dans les cellules peu différentiées Hs578T qui ne l'expriment pas.

Nous avons ensuite vérifié si l'histone H2A induisait la différenciation d'autres types cellulaires, en l'occurrence la lignée de neuroblastome humain SH-

SY5Y. Les cellules SH-SY5Y sont des cellules issues d'une tumeur maligne des crêtes neurales où les cellules conservent un phénotype indifférencié. On sait que le neuroblastome peut se différencier spontanément en tumeurs bénignes, non invasives. Ainsi, l'induction de la différenciation des cellules neuroblastiques par des agents exogènes peut être envisagée comme traitement du neuroblastome (Hill and Robertson 1997). Comme dans le cas des cellules de cancer du sein, l'histone H2A est capable d'induire la différenciation des cellules neuroblastiques SH-SY5Y. En effet, le traitement des cellules SH-SY5Y provoque une augmentation de l'activité de l'AchE, une enzyme impliquée dans l'hydrolyse de l'acétylcholine (Figure 19). L'induction de la différenciation, évaluée par l'augmentation de l'activité cholinestérasique a déjà été rapportée suite au traitement avec différents agents exogènes, comme l'acide rétinoïque (Hill and Robertson 1997) et le tribromophénol (Rios et al. 2003).

La différenciation et la sénescence partagent des caractéristiques communes, telles que les changements morphologiques et l'arrêt irréversible de la croissance (Leszczyniecka et al. 2002). Des événements moléculaires et épigénétiques communs ont été identifiés dans ces deux processus, par exemple des changements dans l'expression de p21 (Munro et al. 2004;Gui et al. 2004) ou de la méthylation de la lysine 9 de l'histone H3 du promoteur de plusieurs gènes (Narita et al. 2003;Ait-Si-Ali et al. 2004).

Plusieurs agents sont capables d'induire à la fois la différenciation et la sénescence. Par exemple, Wainwright et al.(Wainwright et al. 2001) ont démontré

que l'acide rétinoïque stimule la différenciation et la sénescence dans deux clones de la lignée cellulaire humaine de neuroblastome SK-N-SH, soit les cellules SK-N-SH-N et SK-N-SH-F, respectivement. Les auteurs ont suggéré que des inhibiteurs de kinases dépendantes des cyclines (cdk) de la famille Cip/Kip (ex. p21) et de la famille Ink4 (ex. p16) soient impliqués dans cette réponse différenciée. Ainsi, p21 favoriserait la différenciation neuronale et p16 favoriserait la sénescence. Il y a aussi d'autres exemples d'agents susceptibles d'induire la différenciation et la sénescence: les rayons X (Fournier et al. 2007), l'acide valproïque (Akare et al. 2006) et l'acide ursodeoxycholic (Li et al. 2005). Nos résultats indiquent que l'histone H2A provoque l'induction coordonée de la différenciation et de la sénescence.

6.3.2. LA VOIE DE SIGNALISATION P53/P21

L'arrêt du cycle cellulaire, l'induction de la différenciation et de la sénescence sont souvent associés à des changements dans l'expression des inhibiteurs de cdk tels que p21 (Butler et al. 2001;Beausejour et al. 2003;Weinberg and Denning 2002), p16 et p27 (Weinberg and Denning 2002). De plus, il a été suggéré que la contribution de ces inhibiteurs se succède. Ainsi, p21 subirait une augmentation rapide et transitoire au début du processus de différenciation alors que p27 et p16 augmenteraient plus tard et seraient responsables du maintien de l'état de différenciation (Weinberg and Denning 2002).

Quoique la plupart des études démontrent une augmentation de l'expression de p21 au cours de la différenciation cellulaire, Di Cunto et al. (Di Cunto et al. 1998) ont démontré une diminution de p21 lors de la différenciation des keratinocytes. Les

auteurs suggèrent que l'augmentation de p21 concerne l'induction et les premières étapes de la différenciation, tandis que la diminution subséquente de p21 serait essentielle aux étapes tardives de la différenciation.

Nous avons démontré que l'histone H2A provoque l'augmentation transitoire de l'expression de la protéine p21 dans les cellules MCF-7 (Figure 6). Une augmentation transitoire de l'expression de p21 a également été rapportée dans des fibroblastes dont la sénescence a été induite par un traitement avec 2 agents chimiothérapeutiques : la bléomycine et l'actinomycine (Robles and Adami 1998).

p21 est une protéine instable (Sheaff et al. 2000) essentielle à la régulation du cycle cellulaire. Ainsi, n'est-il pas surprenant que plusieurs facteurs en modulent les niveaux d'expression. Le mécanisme le mieux connu dans la régulation de la cinétique d'expression de p21 est l'induction par p53. Mais il y en a plusieurs autres : dégradation par le protéasome, phosphorylation et stabilité de l'ARNm (Weinberg and Denning 2002).

La protéine p53 est un facteur de transcription qui régit le cycle cellulaire en vertu de l'activation de plusieurs gènes tels que p21, 14-3-3 sigma, Gadd45 et Bax (el Deiry et al. 1993;Hermeking et al. 1997;Kastan et al. 1992;Miyashita et al. 1994). On savait que la protéine p53 agissait à plusieurs niveaux : cycle cellulaire, contrôle de l'apoptose et suppression des tumeurs. Or, de nouvelles fonctions ont été décrites, telles que le contrôle de la différenciation et le développement. Par exemple, Porrello et al. (Porrello et al. 2000) ont montré que p53 est impliqué dans la régulation de la différenciation des myoblastes. Beaucoup de cellules cancéreuses présentent des

anomalies de la différenciation et celles-ci peuvent être liées à une mutation dans p53. Ainsi, la réactivation de p53 pourrait induire la différenciation dans ces cellules (Stiewe 2007).

Nous avons constaté qu'après le traitement des cellules MCF-7 avec l'histone H2A il n'y avait pas de changements significatifs dans l'expression de p53 pendant les premières 24 heures (Figure 6). Cependant, l'arrêt de la prolifération cellulaire ne dépend pas exclusivement de l'induction de p53. Par exemple, le traitement des cellules de cancer du sein avec le 6-anilino-5,8-quinolinequinone (LY83583), un inhibiteur de guanylate cyclase, induit l'arrêt de la prolifération et la sénescence cellulaire d'une manière dépendante de p21, mais indépendante de p53 (Lodygin et al. 2002).

Donc, nos résultats suggèrent que l'histone H2A inhibe la prolifération des cellules MCF-7 par un processus dépendant de p21 et indépendant de p53. Des résultats analogues ont été déjà rapportés par Atadja et al., qui ont démontré une augmentation de l'expression de p21 en l'absence de p53 dans des fibroblastes en voie de sénescence (Atadja et al. 1995).

Nous avons ensuite voulu déterminer si ce mécanisme d'action était spécifique aux cellules MCF-7 ou s'il pouvait être aussi appliqué à d'autres lignées cellulaires. Ainsi, nous avons observé une diminution de l'expression de p21 et une augmentation de l'expression de p53 dans les cellules MDA-MB-231 traitées avec l'histone H2A (Figure 20). Li et al. (Li et al. 2001) ont démontré que l'oncostatin M induit l'arrêt de la prolifération des cellules MCF-7 et MDA-MB-231 par un mécanisme spécifique à chaque lignée. Ainsi, l'augmentation de l'expression de p21

et p27 n'a été observée que dans les cellules MCF-7 et non dans les cellules MDA-MB-231. Ceci semble aussi être le cas de l'histone H2A. Alors que chez les cellules MCF-7 traitées à l'histone H2A l'expression de p21 augmente, elle diminue au contraire dans les cellules MDA-MB-231 soumise au même traitement. Cependant, une augmentation transitoire de l'expression de p53 a été observée dans les cellules MDA-MB-231 traitées à l'histone H2A.

En conclusion, nos résultats suggèrent que le mécanisme d'action de l'histone H2A implique à la fois p21 et p53 mais que le type cellulaire puisse en moduler l'expression différentielle. Cette versatilité de l'histone H2A pourrait avoir un avantage thérapeutique face à la grande hétérogénéité des cellules tumorales.

6.3.3. MÉCANISME DE COMPÉTITION

Tel que déjà mentionné, et sauf indication contraire, la préparation d'histone H2A utilisée dans cette étude a été extraite à partir du thymus de veau, préparation contenant à la fois la forme acétylée et la forme non-acétylée (Figure 8A).

En traitant les cellules MCF-7 avec l'histone humaine H2A recombinante exprimée chez Escherichia coli (E. coli) nous avons observé que celle-ci n'avait aucun effet sur la prolifération cellulaire (Figure 3C). Les séquences primaires de ces protéines sont pourtant très similaires. Mais, l'histone recombinante exprimée chez E. coli est non-acetylée, puisque chez les bacteries, les modifications post-traductionnelles des protéines n'existent pas (Sandman et al. 1998). Les histones subissent des modifications post-traductionnelles seulement dans les cellules eucaryotes où ces modifications altèrent leurs interactions avec l'ADN et d'autres

protéines nucléaires (Strahl and Allis 2000). De telles modifications incluent l'acétylation, l'ubiquitination, la méthylation, la phosphorylation, l'ADP-ribosylation, la sumoylation et la citrullination. Il est donc raisonnable d'envisager que les modifications post-traductionnelles sont impliquées dans les effets anti-prolifératifs de l'histone H2A. À cet égard, il est intéressant de signaler que Huyen et al. (Huyen et al. 2004) ont montré que l'histone H3 de thymus de veau interagit avec 53BP1, une protéine de contrôle dans la voie de signalisation activée par le dommage au double brin de l'ADN, alors que l'histone H3 recombinante (exprimée chez les bactéries) n'interagit pas dans les mêmes conditions. Ceci souligne l'importance des modifications post-traductionnelles dans les fonctions des histones. Nos résultats suggèrent que les modifications post-traductionnelles de l'histone H2A sont essentielles pour ses propriétés anticancéreuses.

Les modifications post-traductionnelles des histones sont parmi les événements épigénétiques liés à la progression du cancer les plus étudiés. La modulation des modifications post-traductionnelles des histones, en particulier de l'acétylation, a ouvert une nouvelle voie thérapeutique qu'on appelle la thérapie épigénétique. Par ailleurs, les médicaments épigénétiques s'imposent de plus en plus en tant que substances prometteuses dans la thérapie contre le cancer. Le mécanisme d'action de ces composés n'est pas entièrement élucidé, mais une combinaison d'apoptose, de différenciation et de sénescence pourrait expliquer leurs effets (Issa 2007).

Les HDACi font partie de la famille des médicaments épigénétiques. L'inhibition des HDACs a pour conséquence l'accumulation des histones acétylées,

affectant ainsi la transcription des gènes impliqués dans la prolifération cellulaire (Munster et al. 2001;Butler et al. 2001;Butler et al. 2000;Glaser et al. 2004).

Il est intéressant de noter les effets communs des HDACi et de l'histone H2A. Parmi ces effets, mentionnons l'arrêt du cycle cellulaire, les changements morphologiques, l'induction de la différenciation cellulaire et de la sénescence et l'augmentation de l'expression de p21 (Munster et al. 2001;Munro et al. 2004;Butler et al. 2001;Gui et al. 2004).

En considérant ces similitudes, nous avons proposé un modèle pour expliquer le mécanisme d'action de l'histone H2A (voir article # 1). Balicki et al. (Balicki et al. 2002) ont démontré que l'histone H2A exogène pénètre dans le cytoplasme et dans le noyau. De plus, Hariton-Gazal et al. (Hariton-Gazal et al. 2003) ont démontré que les histones du cœur du nucléosome sont capables de transloquer directement à travers les membranes cellulaires. Ainsi, nous pouvons supposer que suite au traitement des cellules avec l'histone H2A, celle-ci entrera dans le cytoplasme et puis dans le noyau grâce à son signal de localisation nucléaire. Dans le noyau, la fraction acétylée des histones exogènes pourrait servir de substrat pour les HDAC et ainsi, exercer un effet compétitif à l'égard des histones acétylées endogènes. Cette compétition aurait pour conséquence d'augmenter le taux d'acétylation global des histones endogènes tel qu'observé après le traitement avec les HDACi.

Afin de vérifier le modèle proposé, nous avons analysé les niveaux d'histones H3 et H4 acétylées après le traitement avec l'histone H2A. Bien que l'acétylation des

histones soit une modification post-traductionnelle commune à toutes les histones du cœur du nucléosome, l'acétylation des histones H3 et H4 corrèle fortement avec une chromatine ouverte et favorable à la transcription (Yoo and Jones 2006). Ainsi, Richon et al. (Richon et al. 2000) ont démontré que le traitement de cellules cancéreuses avec le SAHA s'accompagne d'une accumulation des histones H3 et H4 acétylées, activant la transcription des gènes impliqués dans l'arrêt du cycle cellulaire tel que p21. La corrélation entre l'augmentation de l'expression de p21 et l'augmentation du niveau d'acétylation des histones H3 et H4 a été également démontrée par d'autres études (Wu et al. 2001;Butler et al. 2001). Notre premier essai indiquait clairement une augmentation du niveau d'acétylation de l'histone H4. Cependant, des expériences subséquentes réalisées dans les mêmes conditions à l'exception d'un nouveau lot d'histone n'ont pas donné les mêmes résultats (Figure 21). Malgré que le dernier numéro de lot d'histone H2A que nous avons reçu n'ait pas le même niveau d'activité que les lots précédents, nous avons certains résultats préliminaires avec des concentrations plus élevées de ce lot qui sont semblables aux résulats obtenus avec les lots précédents. Donc, les expériences pourront continuer avec ces concentrations plus élevées d'histone H2A.

Pour la suite de cette étude, deux approches seront envisagées pour éviter les écueils liés à la variabilité de l'activité de l'histone H2A : la recherche d'un nouveau fournisseur d'histone H2A de thymus de veau et l'expression et la purification d'histone H2A recombinante dans des cellules eucaryotes dans notre laboratoire.

7. CONCLUSIONS ET PERSPECTIVES

Les études précédentes liant les histones exogènes à la thérapie du cancer ne concernaient que l'histone H1. Ainsi, il a été démontré que l'histone H1 exogène inhibait la prolifération in vitro des cellules du cancer du sein (Vani et al. 2006) et des cellules leucémiques (Class et al. 1996). Vani et al. ont également démontré que l'histone H1 inhibait la croissance tumorale in vivo (Vani et al. 2003). Nos résultats indiquent que l'histone H2A inhibe la prolifération cellulaire in vitro. De plus, nos études préliminaires suggèrent que l'histone H2A pourrait inhiber la croissance tumorale in vivo, dans un modèle de souris (Figure 22). En plus de confirmer ces résultats, des études ultérieures sont prévues pour analyser le pouvoir anti-métastatique de l'histone H2A.

Dans le but de mieux caractériser le mécanisme d'action d'histone H2A d'autres études seront réalisées, afin d'analyser les effets de l'histone H2A sur l'expression de c-myc et de la télomérase.

Nos résultats préliminaires suggèrent que l'histone H2A inhibe l'expression de c-myc, un facteur de transcription souvent surexprimé dans les cancers (Figure 24). C-myc est associé à plusieurs fonctions cellulaires, comme la croissance, la différenciation et l'apoptose et ainsi, joue-t-il un rôle important dans la progression des cancers. Il n'est pas surprenant que plusieurs études s'intéressent au développement de nouveaux médicaments anti-cancéreux ayant comme cible thérapeutique c-myc (Liao and Dickson 2000;Vita and Henriksson 2006).

Nous avons pu constater dans une étude préliminaire que l'histone H2A inhibait l'expression de hTERT, l'unité catalytique de la télomérase (Figure 25). La télomérase est l'enzyme responsable du maintien des télomeres, qui sont des unités

répétitives de nucléotides qui protègent les extrémités des chromosomes. Dans les cellules normales somatiques qui n'expriment pas la télomérase, les télomères se raccourcissent à chaque division cellulaire, jusqu'au moment où la cellule entre en sénescence. Dans les cellules cancéreuses la télomérase permet à la division cellulaire de se poursuivre indéfiniment. Il a été démontré que la télomérase est exprimée dans 90% des cancers (Berletch et al. 2007).

En conclusion, nous avons démontré dans cette étude que l'histone H2A exogène inhibe la croissance des cellules cancéreuses par un mécanisme qui implique l'induction de la différenciation et de la sénescence cellulaire. Le mécanisme moléculaire sous-jacent implique l'induction des protéines p21 et p53 dépendamment de la lignée cellulaire analysée. Enfin, nous proposons un modèle illustrant le mécanisme d'action probable de l'histone H2A. Ce modèle tient compte des similitudes observées entre les effets de l'histone H2A et ceux de HDACi. Ce modèle permet d'expliquer l'absence d'effet anti-prolifératif de l'histone H2A recombinante non-acétylée. Pour compétitioner avec les histones endogènes pour les HDAC, il est essentiel que le substrat offert à ces enzymes soit acétylé. Ainsi, lorsque les cellules sont traitées avec l'histone recombinante non-acétylée, l'arrivée de cette histone dans le noyau est sans effet sur les HDAC.

Bien que ce modèle demande confirmation, il pourrait bien ouvrir de nouvelles perspectives thérapeutiques. Ainsi, afin d'augmenter le niveau d'acétylation des histones endogènes, plutôt que d'inhiber les HDAC nous pourrions leur offrir des substrats acétylés capable de compétitioner avec les histones endogènes et susceptibles d'en hausser les niveaux d'acétylation.

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9. ARTICLE #3 : SP ANALYSIS MAY BE USED TO IDENTIFY CANCER STEM CELL POPULATIONS

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SP analysis may be used to identify cancer stem cell populations

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9.2. ABSTRACT

Side populations (SP), as defined by Hoechst exclusion in flow cytometry, have been described a few years ago. While they represent only a small fraction of the whole cell population, but their properties confer an important place in several investigations. SP cells express high levels of various members of ABC-transporters family, such as MDR1 and BCRP, which are responsible for drug resistance. Targeting SP could improve cancer therapy by blocking these transporters. In addition, SP appear to be enriched in stem cells, cells that play a pivotal role in normal development and cancer biology. Thus, they could provide a useful tool and a readily accessible source for stem cell studies in both the normal and cancerous settings. However, these cells are poorly defined and pose challenges in their identification and isolation, particularly since they are few in number. Thus, a better characterization of SP will advance our understanding of stem cells and will provide us an accessible target for drug resistance in cancer therapy.

Keywords: cancer, ATP-binding cassette transporters, multidrug resistance, stem cells, side populations, Hoechst 33342, dye efflux

Abbreviations: ABC (ATP-binding cassette) transporters, BCRP (breast cancer resistance protein), CFE (colony-forming efficiency), ER (estrogen receptor), KO (knockout), MDR1 (multidrug resistance transporter 1), P-gp (p-glycoprotein), SP (side population).

9.3. NORMAL AND CANCER STEM CELLS

Despite enormous progress made in the cancer research field, a multitude of unresolved issues remain outstanding regarding the molecular basis of carcinogenesis. For example, what makes some tumors difficult to eradicate, why are some tumors more resistant to therapy than others, and why are some tumors highly aggressive whereas others are not? The answers to these and other questions are currently incomplete. To resolve these pivotal issues, the scientific community has recently turned its attention to adult stem cells, which could become targets for malignant transformation because they have long lifespans, and are thus able to accumulate mutations leading to cellular transformation [1, 2]. In addition, it has been speculated that stem cells underlie tumorigenicity, as they have the capacity to divide indefinitely [3].

Two major characteristics define stem cells. The first is their ability to self–renew, i.e. give rise to identical cells, which are also capable of self-renewal. Second, stem cells are multipotent, and as such differentiate into specific lineages depending on the environmental signals received. Thus, these two characteristics of stem cells confer the unique property of asymmetric division.

Stem cells are classified into two principal types: embryonic and adult stem cells [4]. Embryonic stem cells are totipotent, as they are capable of differentiation into every known daughter cell type. They are also the subject of very intense moral debates because of their implication in human cloning. Although there are numerous potential therapeutic applications of embryonic stem cells, e.g. tissue culture *in vitro,* the use of these cells remains controversial for ethical reasons. Thus, it has been proposed that adult stem cells may serve as an acceptable replacement for embryonic

stem cells in research approaches aiming to create *in vitro* heart muscle cells, insulin-secreting cells and neurons as therapeutic options for myocardial infarct, diabetes, and Parkinson's disease respectively [4].

Stem cells are quiescent or slowly cycling cells maintained in an undifferentiated state until their participation is required in normal functioning of the organism. Thus, under various environmental signals, i.e. massive cell destruction, they will divide and give rise to transiently-amplified cells that will undergo multiple divisions before proceeding to terminal differentiation [4]. Tissue repair following an injury (i.e. muscle repair) and mammary gland development during pregnancy are some examples of the involvement of adult stem cells in normal physiology [4, 5]. Normal stem cells have been described in many normal tissues such as breast [6, 7], brain [8] and liver [9].

While there have been significant advances regarding adult stem cells in the context of normal physiology, recent studies have revealed that they are important players in cancer biology. The significance of adult stem cells in hematological malignancy is well known. However, the presence and roles of these stem cells in solid tumors are poorly understood. Their implication in retinoblastoma [10], melanoma [11] and other malignancies are currently being investigated. Although the origin of cancer stem cells has not yet been elucidated, the malignant transformation of a normal stem cell, or a progenitor cell that has acquired self-renewal ability, or both, has been proposed [3, 12]

Molecular signatures of the stem cells are currently being identified. Thus, the mutations accumulated in these cells could inappropriately activate Bmi-1,

Notch, Wnt/β-catenin or Sonic hedgehog pathways that are implicated in the regulation of self-renewal of normal stem cells and could potentially lead to cancer [13-16]. Common phenotypic markers have also been described in both normal and cancer stem cells. Shackleton et al. [17] have reported the phenotype $Lin^{-}CD29^{hi}$(β1-integrin) $CD24^{+}$ in normal mouse mammary stem cells. Although the two major characteristics of stem cells (self-renewal and differentiation in multiple lineages) are largely accepted as stem cell attributes, the molecular markers that define stem cells remain controversial.

9.4. ABC TRANSPORTERS

The ABC transporters represent a family of proteins with the capacity to bind ATP as an energy source to transport endogenous or exogenous molecules across the cellular membrane. In most cases, this transport is unidirectional, the products being pumped out of the cell or into an intracellular compartment (endoplasmic reticulum, mitochondria, peroxisome) [18]. Although several subfamilies of genes coding for these transporters have been identified (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG), the best characterized are: ABCB1 (MDR1, P-gp) in humans and Mdr1a/1b in mice, and ABCG2 (or BCRP, MXR) in humans and Bcrp1 in mice, members of the ABCB and ABCG sub-families, respectively [19]. The ABC transporters are localized in well-defined tissues. For example, ABCB1 has been identified in liver, kidney, intestine, endothelial cells, pancreas, placenta, and hematopoietic cells [20] and ABCG2 in placenta, brain, kidney, intestine, liver,

testis, and ovary [21]. Although the exact physiological role of these transporters is not fully understood, certain functions are well supported by experimental results.

First, cellular protection against exogenous products able to penetrate the cell membrane barrier by active exclusion is the best characterized function. In normal physiology, this function is illustrated by the presence of these transporters in the blood-brain barrier, resulting in the blockade of penetration of the central nervous system by harmful substances [22]. Additional evidence for this protective function is demonstrated by the presence of ABC transporters in the blood-testis barrier [23].

Second, there is mounting speculation that ABC transporters repress the maturation and differentiation of stem cells. For example, ABCG2/BCRP is overexpressed in hematopoietic stem cells [24] and this overexpression of ABCG2 inhibits hematopoietic development [25]. However, since Bcrp1 knockout (KO) mice display normal hematopoiesis instead of accelerated hematopoietic maturation, it is clear that multiple factors determine the maturation and differentiation of stem cells [26]. This block in differentiation was also reported in cell lines that overexpress ABC transporters. For example, Mdr1 overexpression in the C2C12 myogenic cell line blocks differentiation and myotube formation [27].

Third, protection from hypoxia appears to be another function of ABC transporters. For example, it has been postulated that BCRP expression protects hematopoietic stem cells from hypoxic environments by preventing porphyrin accumulation that causes mitochondrial death [28].

The role of ABC transporters in the normal function of dendritic cells has also been suggested. Thus, MDR1/ABCB1 [29] and MRP1/ABCC1 [30] have been

associated with mobilization of dendritic cells to lymph nodes via lymphatic vessels during the inflammatory response. However, while the mechanism underlying this function is not entirely elucidated, leukotriene (LT) transport and chemotactic cytokine receptor (CCR) expression have been proposed to be involved in this process [31].

9.5. SIDE POPULATIONS (SP)

SP are a particular cell populations enriched in primitive and undifferentiated cells [32, 33]. Most authors consider that SP is an enriched source of stem cells as well as an alternative source that is particularly useful in situation where stem cells molecular markers are unknown [34]. However, some authors remain unconvinced about the existence of stem cells in SP. For example, Triel et al. [35] demonstrated that cells found within SP did not express stem cells markers.

The isolation of SP is based on the technique described by Goodell et al. [36] in 1996. While using Hoechst 33342 vital dye staining to study the cell cycle distribution of whole bone marrow cells, they discovered that the display of Hoechst fluorescence simultaneously at two emission wavelengths (red 675 nm and blue 450 nm) localizes a distinct, small, non-stained cell population (0.1% of all cells) that express stem cells markers (Sca1+lin$^{neg/low}$). SP are a distinct, small cell populations composed of unstained cells in the left lower quadrant of a Flow Activated Cell Sorter (FACS) profile. Goodell et al. [36] have demonstrated that the exclusion of Hoechst 33342 by SP cells is an active process involving multidrug resistance transporter 1 (MDR1), a member of ATP-binding cassette (ABC) transporter

transmembrane proteins. To support this hypothesis they applied the MDR1 inhibitor verapamil, to show that Hoechst 33342 exclusion by SP cells decreases when the MDR1 pump is blocked. However, verapamil shows no specificity toward MDR1 transporter. Furthermore, the MDR1 antibody does not exclusively identify SP cells, as MDR1 is expressed by 65% of bone marrow cells [36] and SP represent 0.1% of such cells [36]. Thus, MDR1 cannot be taken as a single marker to identify and isolate SP cells and additional transporters should be analyzed. For example, Zhou et al. [25] have demonstrated the implication of breast cancer resistance protein (BCRP) in the SP phenotype.

SP have been identified in many normal tissues, including mammary glands [6, 37, 38], lung [39, 40], muscle [41], heart [42], liver [43, 44], brain [45, 46], and skin [32, 47] in both human and animal models.

In addition to the application of SP in normal cells, they have been identified in various tumors and cancer cell lines, e.g. neuroblastoma [48], melanoma [11] and the C6 glioma cell line [49]. SP have been utilized to isolate, define, and study malignant cells, where their ability to expel nuclear dyes correlates with multidrug resistance, i.e. the expulsion of cytotoxic drugs [48]. Two transporters, i.e. ABCB1 (MDR1) and ABCG2 (BCRP), described in the SP context, exhibit specific exclusion of chemotherapeutic agents. For example, ABCB1 expels vinblastin [50] and paclitaxel [51] while ABCG2 expels imatinib mesylate [52], topotecan [53], and methotrexate [54].

While there is some disagreement regarding the phenotypes that best characterize stem cell, the association between these cells and SP is well-

documented. However, there are some exceptions. For example, Morita et al, [55] have demonstrated that hematopoietic stem cells are present in the non-SP compartment. Mammary repopulating cells capable of reconstituting a cleared mammary fat pad have also been found independent of the SP phenotype [7, 17]. Thus, since SP are composed of stem and non-stem cells, and some stem cells do not reside within SP, these compartments do not completely overlap.

9.5.1. SP IN CELL LINES

Cancer cell lines represent an heterogeneous population, as demonstrated by the finding that the injection of approximately one million human breast cancer cell line MCF-7 in SCID mice is required to give rise to a tumor, but 100,000 cells with a specific phenotype, i.e. CD44+/CD24- [56], are sufficient to induce tumor formation. These observations led to the speculation that cell lines are composed of cells that are heterogeneous in terms of their tumorigenicity.

The Hoechst exclusion assay reveals that, as described in normal tissues, SP can be found in cell lines, and ABC transporters are active, e.g. ABCG2 and ABCA3 in human neuroblastoma cell lines [48], ABCG2 and ABCB1 in human gastrointestinal cell lines [57], and Mrd1a in the mouse muscle cell line C2C12 [27]. Indeed, SP and non-SP populations have been identified in the C6 rat glioma cell line [49], in SK-N-SH, IMR-32, and JF human neuroblastoma cell lines [48], the C2C12 mouse myogenic cell line [27, 33], human retinoblastoma WERI-Rb27 [10], and various human gastrointestinal cancer cell lines [57]. However, no SP have as yet been described in cell lines derived from Wilms' tumor, rhabdomyosarcoma or

osteosarcoma (i.e. SK-NEP-1, A 204 and SaO2 respectively) [48] and the liver cancer cell line HepG2 [57].

The characterization of cells within SP demonstrates that they are immature, poorly differentiated and highly tumorigenic. Gene expression profiles of SP show that these cells are less differentiated than non-SP cells [33]. They express high levels of stem cell markers (i.e. hematopoietic and neural stem cell marker CD133) and low levels of differentiation markers (i.e. mature hepatocyte marker ALBU in the cell line HuH7) [57]. Whether SP are capable of asymmetric division, a characteristic of stem cells, while non-SP cannot, remains controversial. While some researchers have reported that only SP are able to generate SP and non-SP, whereas non-SP are not able to do so [48, 49, 57], others have shown that both SP and non-SP are able to generate either SP and non-SP [27]. Thus, SP are most likely heterogeneous populations, and only a fraction of these populations are able to undergo asymmetric division. Indeed, the SP tail has been divided in different regions according to their dye efflux abilities. It has been demonstrated that cells in the tip of SP with highest Hoechst efflux activity show the highest progenitor activity as compared to the distal portion [58-60]. Thus, the level of Hoechst exclusion may be a direct indicator of cellular differentiation, and presumably have higher transporter levels. Since the SP phenotype has been correlated with ABCG2 expression and ABCG2 expression change during progenitors commitment [61], the sub-populations found in SP reflect various degrees of cellular differentiation. Thus, isolation of cells with the highest dye efflux activity within SP could lead to a highly enriched stem cell population. Moreover, SP and non-SP differ in terms of their tumorigenicity *in vivo* and potential

for multilineage differentiation, SP being more malignant and more likely to differentiate than non-SP [49].

Interestingly, Benchaouir et al. [27] have reported that SP cells are smaller than non-SP cells. This observation correlates well with the findings of De Paiva et al. [62] who propose that small cell size is a stem cells characteristic. Moreover, SP cells are present in a quiescent state, and possess an adhesive endoplasmic reticulum with a few ribosomes suggestive of low metabolic activity, elements common to stem cells [62]. Taken together, these results demonstrate that SP isolated from cell lines preserve a stem cell population.

Cell lines are a useful tool to analyze molecular cell markers and cellular behavior under controlled experimental conditions. Moreover, certain parallels exist between cancer cell lines and malignant tumors. For example, cells derived from cancer cell lines are heterogeneous in their capacity to form colonies in soft agar assays, since the number of colonies is significantly lower in number than the number of cells initially plated in this assay. The ability to form colonies in soft agar is associated with malignant transformation. During this multistep process, cells become anchorage-independent, and related testing in semi-solid media for this characteristic change is widely used to evaluate the malignant potential of cells *in vitro* [63, 64]. Thus, cells from cell lines can be divided into those that will generate colonies in soft agar, and those that do not. This heterogeneity may also exist in tissues. Thus, SP derived from cell lines could serve as an experimental model to study the tumorigenic population in malignant samples. Given that this tumorigenic

population has been associated with cancer stem cells, investigating SP will further enhance our understanding of stem cells.

9.5.2. SP IN NORMAL TISSUES

Since the SP phenotype is correlated with ABC transporter activity, several studies have been conducted to establish a specific association between the increased expressions of a given transporter in specific tissues. The ABCG2/Bcrp1 transporter is most often related to the SP phenotype. Thus, high expression of this transporter has been reported in mouse skeletal muscle [41], bone marrow [26] and skin [47], and human skin [35] and cornea [62, 65, 66]. Absent or low levels of SP expression have been observed in Bcrp1 KO mice in the lungs [39], skeletal muscle and bone marrow [26]. However, both ABCG2/Bcrp1 and MDR1/Mdr1 transporter expression have been reported in the mouse mammary gland [37] and human pancreatic islets of Langerhans [67]. Taken together, these results demonstrate that the upregulation of these transporters is tissue-dependent.

Gene expression profiles of the SP in microarray assays using an in-house platform [32] or Affymetrix 430 2.0 array chips [37] have shown that most genes are up-regulated, e.g. transcription factor ID2, translation initiation factor EIF5 and growth factor NRG1[32]. The upregulation of transcription and translational genes [68] has also been reported, while genes involved in the regulation of the immune response and cell adhesion were downregulated [68]. Downregulation of cell adhesion genes suggests that SP contain a particular cell fraction whose survival is not exclusively or entirely dependent on cell-cell interactions, which regulate stem

cell behaviour [12]. Although the interactions between stem cells and their environment, the so called "stem cell niche", are very important in self–renewal and differentiation [69, 70], it has been demonstrated that mammary stem cells are capable of *in vitro* survival under conditions that do not allow the adherence to a substratum, whereas most mammary epithelial cells die under the same conditions [6]. In addition, cell cycle repressive genes have been reported to be upregulated in SP [37]. This suggests that SP cells will arrest at a particular phase of the cell cycle. Indeed, G0/G1 cell cycle arrest has been reported within the SP [37], which supports the relationship between SP and stem cells, which are both believed to be slow-cycling cells [71] that reside in a quiescent state [72].

Universal stem cells markers have been proposed to characterize the SP. For example, stem cell antigen (Sca1+) is present in heart, bone marrow, mammary gland and pituitary cells respectively [42, 73-75], and proteins identified in the Notch-1 and Wnt self-renewal pathways [76, 77] have also been described in SP. These observations suggest that within SP, there are cell populations capable of self-renewal, i.e. stem cells.

Tissue-dependent stem cells markers have been described. For example, CD34+ has been identified in hematopoietic SP [78], keratin 14 (K14) and keratin 19 (K19) in skin SP [47]. This indicates that SP derived from specific tissues express a given pattern of tissue-dependent markers. However, certain exceptions exist in the literature. For example, nestin is a neural stem cell marker, has been reported not only in nervous system tissues [79] but also in pancreatic side populations [67]. Nonetheless, whether or not SP express a universal or a specific stem cell marker,

there is a consensus on the presence of an immature population within SP. While immature cell markers are expressed at high levels within SP, they express low levels of differentiation markers, i.e. epithelial membrane antigen (EMA) in mouse mammary epithelial cells [38].

There is heightened controversy regarding SP markers in tissue samples compared with cell lines, with multiple examples of contradictory results. A pertinent example is provided by ER expression within mammary gland SP. ER has been reported to be absent in SP [80], present at high levels in SP [81], and expressed in SP and non-SP at similar levels [38].

A few factors have been identified to explain these contradictory results; these include the effects of tissue dissociation in the preparation of single cell suspensions, cell counting, Hoechst concentration, and SP gate selection [82]. Age is another factor that could affect these results, since it has been demonstrated that the size of SP decreases with aging [47]. This observation remains controversial as Triel et al. [35] have concluded from their studies that SP size is age-independent. Although, the SP remains an enriched source of stem cells throughout the lifetime of a given organism, a thorough characterization of this cell population ill elucidate the origins, markers, and functions of stem cells.

9.5.3. SP IN TUMORS

Many authors suggest that stem cells play a pivotal role in carcinogenesis [5, 83, 84]. Stem cells can give rise to either another stem cell, or a more differentiated progenitor, a constant number of stem cells being maintained. This strict regulation

of stem cell numbers is another characteristic of stem cells [83], but if a stem cell gives rise to two daughter stem cells, an increase in stem cell numbers will result if at the same time there is no differentiation of one of the two daughter cells to more mature cells. Thus, if a stem cell can give rise to another stem cell in addition to a more differentiated cell, a constant number of stem cells will be preserved. However, if a stem cell gives rise to two stem cells, then in the next division these two stem cells will give rise to 4 stem cells and this process could continue, leading to an increase in cell numbers, which is a characteristic of neoplastic transformation (Figure 1). This phenomenon is also observed in normal stem cell expansion durimg embryogenesis or extreme tissue injury.

Classical theories of carcinogenesis are stochastic and attribute tumor growth to the accumulation of serial genetic events involving genes, which regulate cellular growth and apoptosis. The cancer stem cell model postulates the existence of cancer stem cells, which sustain tumor growth. For the time being, the origin of these cells remains incompletely understood, but it has been speculated that a cancer stem cell could even arise from a normal stem cell or from a more differentiated cell which has acquired self-renewal ability [5].

It has been demonstrated that tumors are composed of heterogeneous cells, and that only a small cell population of each tumor bears the capacity to reconstitute a tumor when injected in mice. For example, Al-Hajj et al. [85] have demonstrated that 200,000 to 800,000 CD44+ or CD24- human breast cells are capable of tumor formation in NOD/SCID mice, whereas the same number of CD44- or CD24+ cells are not. They have also shown that 100 CD44+/CD24- cells are sufficient to give rise

to a tumor in NOD/SCID mice [85]. According to the stem cell theory of carcinogenesis, this small population is enriched in cancer stem cells. While proliferation is a characteristic of all tumor cells, to better understand the nature of a particular tumor, it is critical to define the number and longevity of cancer stem cells within that tumor. Given that stem cells can virtually be maintained indefinitely, thanks to their self-renewal mechanisms, while non-stem cells cannot, these attributes could explain the different behaviors *in vitro* and *in vivo* of various cell populations isolated from tumors [56, 85]. Thus, since cancer stem cells may be responsible for the aggressive behavior of certain tumors as well as their sustained growth, strategies that target these cells will have significant clinical implications [83, 86]. Cancer chemotherapy targets dividing cells. Given that cancer stem cells are mostly quiescent cells, they will probably survive therapy, reconstitute the tumor, and subsequently become responsible for resistance to cancer therapy [72, 86].

Two general approaches could be used to identify and characterize cancer stem cells. First, molecular markers have been proposed, i.e. CD44+/CD24- for breast cancer stem cells [56, 85], CD44+/alpha2beta1^{hi}/CD133+ for prostate cancer stem cells [87], and CD34+/CD38- for leukemia stem cells [88]. Second, SP isolated from tumors have proven to be an attractive alternative to study cancer stem cells, since they are enriched in cancer stem cells, as SP isolated from normal tissues are enriched in normal stem cell (Table 1). For example, Grichnik et al. [11] have isolated SP from human melanoma samples and have shown that SP cells have properties common with stem cells, which include the finding that they are small, possess the capacity to give rise to larger cells, and have the greatest ability to

expand in culture. The authors made their assumption based on *in vitro* assay and they speculate that SP behavior ressembles stem cells behavior under similar conditions. Several *in vitro* assay designed to study stem cells have been proposed, for example mammospheres assay has been design to study mammary stem cells *in vitro* [6]. However, *in vivo* assays remain the most accepted assays used to demonstrate the characteristics of stem cells. Stems cells are distinguished from proliferating cells by true self-renewal, as determined by in vivo assays.

SP enriched in cells that possess stem cells characteristics have been identified in additional malignancies, including retinoblastoma [10], bone marrow from patients with acute myeloid leukemia (AML) [89], and neuroblastoma [48]. A correlation between these two approaches was subsequently proposed. In support of this correlation, Miletti-Gonzalez et al. [90] have demonstrated that CD44 expression correlates with MDR1/P-gp expression in carcinoma cell lines. While MDR1 is one of the ABC transporters responsible for the SP phenotype, a relationship between the SP and CD44 could be speculated.

Since the SP is defined by ABC transporter activity, the expression of these transporters has been analyzed in various malignancies. For example, patients with acute lymphoblastic leukemia (ALL) express high levels of MRP1 to MRP6 (ABCC members) [91], gastrointestinal stromal tumors express high expression levels of ABCB1 [92], and neuroblastoma cells express high levels of ABCG2 and ABCA3 [48].

Taken together, these results indicate that cancer stem cells are pivotal in tumor progression. The implication of stem cells in carcinogenesis was suggested

several years ago, but poor characterization has limited their clinical application. Although a few markers have been proposed to define the cancer stem cells phenotype, none is universally accepted. Further studies are necessary in order to elucidate their full implication in malignant transformation.

9.6. CONCLUSION AND FUTURE GOALS

In the last few years, stem cells have become the subject of intense study. First, they have been analyzed in the context of normal physiology, and preliminary results demonstrate their therapeutic potential in pathological conditions, e.g. Parkinson's disease [4]. The major obstacle in stem cell research is the difficulty in their isolation because of the lack of universally-accepted markers of these cells. Use of the SP, a cell population enriched in stem cells, overcomes some of the barriers in stem cell research, as it entails no specific cellular markers. However there are some limitations to this approach. First, given that SP do not reflect the entire stem cell cohort, additional approaches are required to enrich this source of stem cells for their full characterization. Concomitant study of molecular markers of stem cells is mandatory to complement and complete their characterization. For example, the Sca-1+Lin-Thy-1-c-kit+ phenotype of hematopoietic stem cells is largely accepted [93-95]. However, while CD34+ hematopoietic stem cells are well-documented, recent data suggest that the CD34- phenotype could also be associated with these cells [96]. Thus, isolation of stem cells should not repose only on cell surface markers, as these markers remain controversial. The second limitation of the SP as a stem cells source is the toxicity of Hoechst 33342[77], the fluorescent dye utilized for their isolation.

As Hoescht dyes are toxic to many cells, it is difficult to compare the biological properties of SP and non-SP cells. For example, using the SP phenotype of mobilized human peripheral blood (mPB), Fischer et al. [97] have demonstrated that Hoechst staining depleted mPB of short term repopulating cells with myeloid potential. Thus, additional studies are necessary to optimize this technique and to define the conditions associated with minimum toxicity. Montanaro et al. [82] have shown that the Hoechst concentration should be adjusted to each new tissue in order to limit its toxicity. Despite these limitations, the SP is well-documented as an enriched source of stem cells, and they remain a valid and promising tool for identification, isolation and characterization of stem cells, particularly when this approach is combined with other cell markers.

Recently, stem cells have been associated with the development and propagation of solid tumors [83, 85]. Preliminary results have demonstrated that this new theory of carcinogenesis, which proposes that the transformation of a normal stem cell into a cancer stem cell is the key element in malignant events [3]. While treatments, which selectively target cancer stem cells, hold significant promise, their discovery and clinical applications are currently limited, since well-defined stem cell markers have yet to be characterized. Thus, alternative approaches such as the SP assay should be considered. Moreover, ABC transporters responsible for SP phenotype are implicated in drugs resistance and lower responses to cancer therapy. Thus targeting the SP should offer an alternative option to approach drug resistance. Since ABC transporters are expressed in both normal and cancerous cells, they cannot be used to selectively target cancer cells unless specific strategies are applied.

As one of the most studied mechanisms of therapeutic resistance involves an increase in ABC transporter expression, the combination of ABC transporter inhibitors with anti-cancer drugs has been shown to improve the clinical outcome of conventional cytotoxic cancer therapy. For example, the association of verapamil (a calcium channel blocker and an inhibitor of P-gp activity) [36] with chemotherapy increases the survival of patients with antracycline-resistant metastatic breast carcinoma [98].

Thus, studies of the SP cover two areas of interest in cell biology research. First, is their implication in stem cells biology, and, second in cancer biology, particularly regarding carcinogenesis as well as drug resistance in the context of cancer therapy.

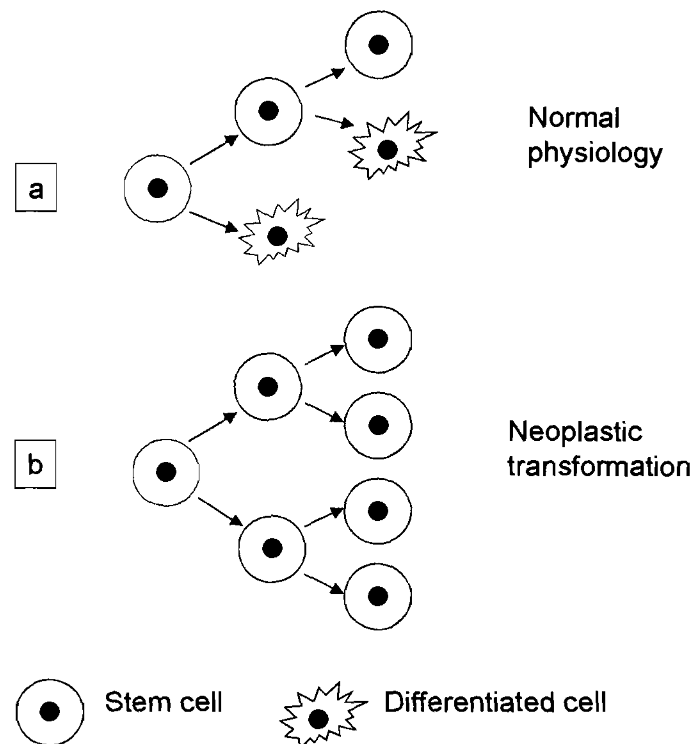

Figure 1 (article #3): Importance of keeping a constant number of stem cells in normal physiology versus malignant transformation.

In normal physiology, in the absense of expansion during embryogenesis and extreme tissues injuries, the asymmetrical division of adult stem cells maintains a constant number (a). In malignant transformation, the mechanisms that control the normal division of stem cells are lost and thus an increase in stem cell numbers is observed (b). This is also the case of normal stem cell expansion during embryogenesis or extreme tissue injury.

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