NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

MODIFYING THE COMET ASSAY FOR MEASURING GLOBAL DNA METHYLATION IN A VARIETY OF TISSUE CELLS

Johannes Frederik Wentzel Hons. B.Sc

Dissertation submitted in partial fulfilment of the requirements for an Masters degree in Biochemistry.

Division for Biochemistry, School of Physical and Chemical Sciences, North-West University, Potchefstroom Campus, Potchefstroom, 2520, South Africa.

Supervisor: Prof. P.J. Pretorius

Student supervisor: Chrisna Gouws

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I am indebted to many individuals who gave their time, expertise, support and assistance to make this study possible. To each and everyone who contributed in any way, my most heartfelt thanks. I am privileged to express my sincere appreciation and profound thanks to the following:

- The Division Biochemistry, who generously permitted me the use of reagents, apparatus and laboratory facilities during this study.
- My supervisor, Prof. Piet Pretorius - an academic giant, mentor and teacher - for his indispensable guidance, inspiration, moral support, availability and wisdom.
- Crisna Gouws, an outstanding scholar and tutor, for her generous assistance, motivation, encouragement, patience and proof reading.
- My parents, brother and sister, for their unconditional love, trust, support and tolerance.
- My Heavenly Father who blessed me with wonderful opportunities and perseverance to accomplish this study with endurance and great joy.

"A task becomes a duty from the moment you suspect it to be an essential part of that integrity which alone entitles a man to assume responsibility."

Dag Hammarskjöld

OPSOMMING

Dit is baie duidelik dat DNS metilering 'n krities belangrike rol in geenregulering speel en dat die wanregulering van hierdie proses aanleiding tot sekere siektetoestande, insluitend kanker, kan gee. Alhoewel daar tans 'n verskeidenheid analiseringstegnieke vir DNS metilering bestaan, is die meeste van die metodes steeds relatief duur en platformspesifiek. Die komeetanalise (enkelsel jel elektroforese) is 'n koste-effektiewe, sensitiewe en eenvoudige tegniek wat tradisioneel aangewend word vir die analisering en kwantifisering van DNS integriteit van individuele selle. Die doel van hierdie studie was om vas te stel of die komeetanalise aangepas kan word om veranderinge in die DNS metileringsvlakke in individuele selle aan te dui.

Die alkaliese komeetanalise is gestandaardiseer om gebruik te maak van die verskille in die metilerings-sensitiwiteit van die isoskisomeriese restriksie ensieme, *Hpa*II en *Msp*I, ten einde die globale DNS metileringsvlakke van individuele selle te meet. Gekweekte selle wat blootgestel is aan die demetileringsagent 5-azacytidine en suksinielasetoon - 'n geakkumuleerde tirosinemie tipe I metaboliet - is gebruik om te bepaal of dit moontlik sou wees om met die aangepaste komeetanalise verskille in die vlak van globale DNS metilering waar te neem. Die resultate van die metileringssensitiewe komeetanalise is verder deur die gebruikmaking van die gevestigde sitosien inbouings analise bevestig. Die sitosien inbouings analise is ook gebaseer op die selektiewe gebruik van die metileringspesifieke restriksie ensieme *Hpa*II en *Msp*I, wat beide 'n 5' guanien oorhang vorm nadat die DNS gesny is. Dit word gevolg deur 'n enkele inbouing van tritium gemerkte sitosien-trifosfaat. In hierdie studie is tot die gevolgtrekking gekom dat die metilerings-sensitiewe komeetanalise daarin slaag om veranderinge in DNS metilering in gekweekte selle aan te toon.

Die komeetanalise is dus suksesvol aangepas om veranderinge in DNS metilering op 'n globale en area-spesifieke vlak in individuele selle waar te neem. Hierdie aanpassing brei die meerdoeligheid van die komeetanalise verder uit deur die verskeidenheid waarnemings wat in een analise gemaak kan word, te vermeerder. Omdat DNS metilering 'n weefselspesifieke gebeurtenis is, bied hierdie aanpassing van die komeetanalise die geleentheid om die DNS metileringstatus van verskillende weefsels, onder 'n verskeidenheid fisiologiese omstandighede, te bestudeer.

ABSTRACT

It is becoming abundantly clear that DNA methylation plays a crucial role in gene regulation and that aberrant regulation of DNA methylation influences the development of certain diseases such as cancer. Although a wide variety of methylation analysis techniques are available today, they are still relatively expensive and a large number of them is platform specific. The comet assay (single cell gel electrophoresis) is a cost-effective, sensitive and simple technique which is traditionally used for analysing and quantifying DNA integrity in individual cells. The aim of this study was to determine whether the comet assay could be modified to detect changes in the levels of DNA methylation in single cells.

The alkaline comet assay was standardised to use the difference in methylation sensitivity of the isoschizomeric restriction endonucleases *Hpa*II and MspI to measure the global DNA methylation levels of individual cells. Cultured cells exposed to the demethylating agent 5-azacytidine and the accumulating tyrosinemia type I metabolite, succinylacetone, was used to investigate whether it was possible to detect differences in the degree of global DNA methylation with the comet assay. The methylation sensitive comet assay's results were then verified using the well established cytosine extension assay (CEA). The CEA is also based on the selective use of the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I, both of which leave a 5' guanine overhang after DNA cleavage followed by a single nucleotide extension with [^{3}H]dCTP. The study concluded that the methylation sensitive comet assay is indeed able to show clear variations in DNA methylation after treatment of cultured cells.

In conclusion, the comet assay was successfully modified to determine changes in the level of global and regional DNA methylation in single cells. This modification further expands the versatility of the comet assay by increasing the variety of observations that can be made in one experiment. Since DNA methylation was shown to be a tissue-specific event, this modification of the comet assay provides the opportunity to study the DNA methylation status of single cells that are prepared from different tissues under various physiological conditions.

LIST OF ABBREVIATIONS

A:

A:	Adenine
ALS:	Alkali-labile sites

B:

bp:	Base pair

C:

C:	Cytosine
CEA:	Cytosine Extension Assay
CH_3:	Methyl group
CpG island:	A cytosine-guanine rich sequence area

D:

ddH_2O:	Double distilled water
DMSO	Dimethyl sulfoxide
DNMT:	DNA methyltransferases
ds*:	double stranded nucleotide
DSB:	Double strand break

E:

EDTA:	Diaminoethanetetraacetic acid
et al:	And others (Latin)
EtOH:	Ethanol (70%)
EMS:	Ethylmethanesulphonate

G:

x *g*:	Times gravity
G:	Guanine
H:	
HCl:	Hydrochloric acid
HCC:	Hepatocellular carcinoma
HMPA:	High Melting Point Agarose
HT1:	Hereditary Tyrosinemia type 1
L:	
LMPA:	Low melting point agarose
M:	
M:	Molar
MBD:	Methyl Binding Domain proteins
MMC:	Mitomycin C
M.SssI :	Methyltransferase
O:	
OD:	Optical density
P:	
pHPPA:	4-Hydroxyphenylpyruvate
R:	
RE:	Restriction enzyme
rpm:	Revolutions per minute

S:

SA:	Succinylacetone	
SAM:	S-adenosylmethionine	
SCGE:	cell gel electrophoresis SRA:	SET-and-Ring-finger-Associated
SSB:	single strand break	

T:

T:	Thymine

U:

U:	Uracil
UV:	Ultra violet

V:

V:	Volt

W:

WGA:	Whole genome amplification

SYMBOLS

α: Alpha

µl: Micro litre

µM: Micro molar

%: Percentage

LIST OF TABLES, FIGURES AND DIAGRAMS

Tables:

Figures:

Diagrams:

CHAPTER ONE

INTRODUCTION

Maintaining global genome stability is crucial for the development and normal functioning of any organism. In eukaryotic organisms, DNA methylation is vital for maintaining genome stability in the numerous DNA sequences, as well as playing an important role in gene expression (Bird, 2002; Nag and Smerdon, 2008). DNA methylation is part of the epigenetic events taking place in mammalian cells and can be described as the inherited chemical modification of DNA involving the addition of a methyl group to cytosine without changing the original DNA sequence (Waggoner, 2007). Apart from ensuring a stable genome in adults, DNA methylation also plays a crucial role in the ordered differentiation of mammalian cells during embryonic development by activating specific genes and silencing others (Mohn and Schübeler, 2009). DNA methylation, as part of the epigenetic code, ensures diverse cellular differentiation despite a primarily static genome. There exists a complex collaboration between epigenetic mechanisms (including DNA methylation, histone modification and micro RNA's) to orchestrate this sophisticated and cell specific gene regulation (Guil and Esteller, 2009). These epigenetic processes connect the genome (DNA sequences) and transcriptome (proteins) by introducing complex and dynamic networking layers of gene control (Ohgane *et al.*, 2008). Due to this intensive involvement of DNA methylation in the epigenome it comes as no surprise that the abnormal regulation of these mechanisms may cause altered gene expression and ultimately disease.

Since the late 1980s, researchers started noticing abnormal DNA methylation patterns in most cancer types. Today there is ample evidence indicating that disruption of epigenetically regulated expression of genes play a direct, and maybe even a causative, role in the development and manifestation of many diseases (Sawan *et al.*, 2008; Hirst and Marra, 2009). This includes the unusual methylation patterns observed in most cancers, strengthening belief that aberrant DNA methylation plays a direct role in carcinogenesis (Franco *et al.*, 2008; Patra, 2008; Dobrovic and Kristensen, 2009). Taking into account the role DNA methylation may play in carcinogenesis, it may be theoretically possible to reverse methylation patterns by pharmacological means by inducing demethylation of targeted sequences and subsequently reactivating tumour suppressor genes. In summary, it is

becoming widely accepted many diseases (including Alzheimer's disease, depression and many carcinomas) are as much epigenetic disorders as they are genetic diseases (Wilson *et al.*, 2007; Gräff and Mansuy, 2008). Better insight into these epigenetic mechanisms involved in diseases are of cardinal importance if we want to develop more effective medical treatments in the future and ultimately prevent disease.

Although our understanding of DNA methylation mechanisms and functions has vastly improved over the past two decades, the entire spectrum of influences of this potent mechanism is not fully grasped yet. Today, a wide variety of techniques are used to examine DNA methylation patterns but most of these methods are still relatively expensive and many are platform specific (Ho and Tang, 2007). The need has arisen to develop an economically viable and uncomplicated technique for DNA methylation analysis. The Comet Assay (single cell gel electrophoresis or SCGE) is a cost-effective, sensitive and simple technique which is traditionally used for analysing and quantifying DNA damage in individual cells (Fairbairn *et al.*, 1995). By modifying this assay to be methylation sensitive, we can routinely measure global DNA methylation in cell cultures and simultaneously determine the integrity of their genetic material. The aim of this study is to expand the comet assay for global DNA methylation measurement in a variety of samples. After optimization, this assay will be compared to the cytosine extension assay to validate its effectiveness and the standardized method will then be used to examine the effect of the accumulating metabolite, succinylacetone, in hereditary Tyrosinemia type I (HT1), since hepatocellular carcinoma (HCC) frequently develops into this disease.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epigenetic Alterations

Conrad Waddington first used the term epigenetics in 1936 to describe "the causal interactions between genes and their products, which bring the phenotype into being" (Waddington, 1939). Today our understanding of epigenetics has greatly improved since the 1940's and although Waddington's definition was very simplistic, it only underwent minor alterations in the past few decades. Currently, epigenetic changes are described as stable, reversible and heritable modifications that do not directly influence the primary DNA sequence (Ohgane *et al.*, 2008). These epigenetic modifications enable cell types sharing the same DNA sequence to have different characteristics under specific conditions. Due to intensive research in this field over the past two decades, it is apparent that epigenetic regulation plays a critically important role in cellular differentiation during early development as well as in adult life (Mohn and Schübeler, 2009). Apart from its role in differentiation, epigenetic inheritance is also essential in other cellular processes such as gene transcription, protection against viral genomes and general gene regulation (Métivier *et al*, 2008; Sawan *et al.*, 2008).

There are three main mechanisms known to initiate and sustain epigenetic modifications - micro RNAs, histone modification and DNA methylation (**Figure 2.1**) (Sawan *et al.*, 2008). These processes are crucial for normal cellular function and influence important processes such as chromatin compaction, DNA folding, transcriptional stability and the activation or silencing of genes (Fuks, 2005; Ho and Tang, 2007).

2.1.1 Micro RNAs

Micro RNAs (miRNAs) can be described as a special type of small non-coding RNA acting in the translation phase which regulates gene expression of mRNA (Mirnezami et al, 2009). RNA Polymerase II is responsible for synthesizing miRNA that can later be expressed by their own transcriptional units found mainly in the introns of associated genes

(Guil and Esteller, 2009). Bartel estimates that at least 30% of mammalian genes are regulated by miRNAs (Bartel, 2004).

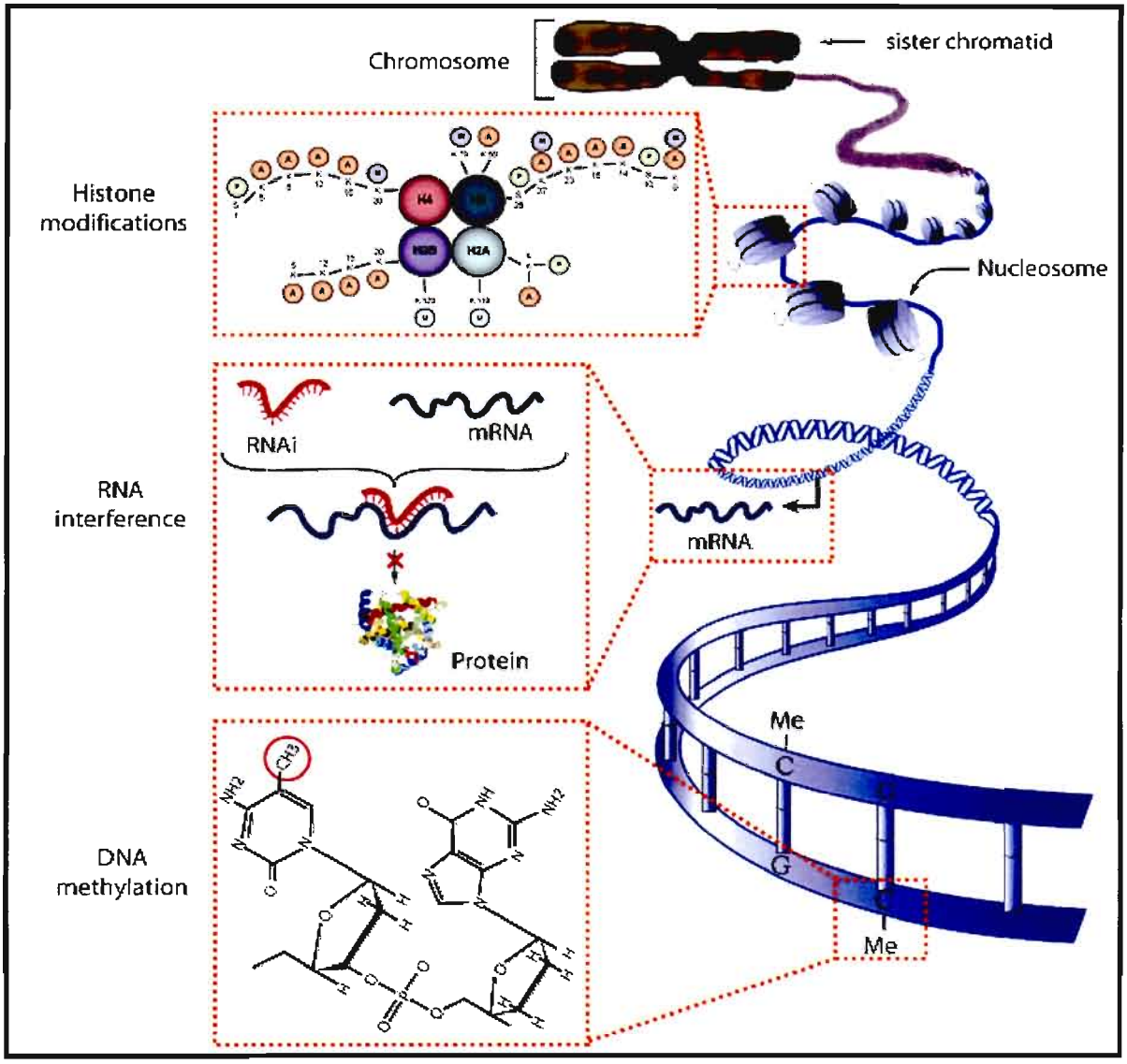

Figure 2.1: The three mechanisms that are known to initiate and sustain epigenetic modifications: Histone modification, small-interfering RNAs and DNA methylation (Sawan et al. 2008)

There is also strong evidence suggesting that mRNAs play crucial roles in apoptosis, differentiation and cellular proliferation, this is especially apparent in disease states

(Ding *et al.*, 2009; Moazed, 2009). Although our understanding of non-coding RNAs have progressed leaps and bounds over the past decade, we are still far from fully comprehending the extent by which this non protein coding transcriptome influences the epigenome.

2.1.2 Histone modification

In chromosomes, DNA is stably packaged around the proteins called histones. There is a number of different histone modifications known and include methylation, acetylation, and phosphorylation (Lennartsson and Ekwall, 2009). The term "histone code" is used to refer to the manner in which histone modifications influence each other to ultimately coordinate the unique genome expression in various cells (Escargueil *et al*, 2008). Histones undergo post-transcriptional modifications which have a profound influence on the structural features of chromatin and due to this direct impact on the physical properties of chromatin it also affects the transcriptional outcome of the genome of a cell, orchestrating activation or repression of specific genes (Nag and Smerdon, 2008; Vaissière *et al*, 2008).

In a nutshell, epigenetic alterations ensure diverse cellular differentiation despite a mainly unchanging genome. It must also be stressed that epigenetic regulation is not the product of a single mechanism (such as DNA methylation, histone modification or miRNA) but rather the sum of all these mechanisms working together and influencing each other to orchestrate this complex and cell specific gene regulation. Epigenetics connects the genome (DNA sequences) and transcriptome (proteins) by introducing an active layer of gene control (Ohgane *et al.*, 2008).

2.2 DNA methylation

DNA undergoes several modifications after every replication cycle of which DNA methylation is one. DNA methylation, as part of the epigenetic code, involves the addition of a methyl group to cytosine without altering the original DNA sequence. DNA methylation takes place on the number 5 carbon of the cytosine pyrimidine ring and has been found in every vertebrate examined to date (Herman and Baylin, 2001; Waggoner, 2007). DNA methylation alters the biophysical characteristics of DNA which may either inhibit the recognition of

certain DNA sequences by some proteins or enable the binding of others (Prokhortchouk and Defossez, 2008). Two common patterns of cytosine methylation at CpG dinucleotides have been described in the eukaryotic genome - genome-wide CpG methylation density, and non-random regional CpG methylation (Cottrell, 2004; Ohgane *et al.*, 2008). Despite the progress science has made to better our understanding of DNA methylation it seems we are only at the first chapter of this intricate book and many unanswered questions remain surrounding the extent in which DNA methylation influences normal as well as malignant cell activity. One thing that can be said with certainty, after more than 50 years of research, is that the main methylation function involves the regulation of gene expression. This function is extended via other actions, e.g. chromatin compaction, x-chromosome inactivation, maintaining genome stability and maintaining cellular identity (Guil and Esteller, 2009). These functions are to a great extent dependent on the physical properties of cytosine methylation.

2.2.1 Physical and chemical properties of mammalian cytosine methylation

It is estimated that between 50 - 60% of all CpG bases are methylated in mammals (Herman, 2001; Klose and Bird, 2006). DNA methylation is essential in mammals and its loss causes growth arrest or apoptosis in cells. This was confirmed by studies where the DNA methyltransferases of rodents were silenced which led to aberrant differentiation and death (Okano *et al.*, 1999; Prokhortchouk and Defossez, 2008). In contrast to prokaryotes, where DNA methylation occurs on both cytosine and adenine bases, DNA methylation in mammals (**Figure 2.2**) involves the addition of a methyl group to the 5' carbon of the cytosine pyrimidine ring (Klose and Bird, 2006). DNA methylation in adult somatic tissues typically occurs in regions called CpG islands while non-CpG methylation (methylation outside of CpG islands) is prevalent in embryonic stem cells (Haines *et al.*, 2001). CpG islands are characterized by high CpG density and tend to be unmethylated under normal conditions (Duffy *et al.*, 2009). According to Gardiner-Garden sequence criteria, a CpG island is defined as a region greater than 200 bp with a G + C content greater than 50% (Gardiner-Garden, 1987). Over 50% of human genes contain CpG islands in their promoter regions and the classical viewpoint is that CpG islands are typically methylation-resistant except for CpG Islands methylated during imprinting and X-chromosome inactivation (Klein and Costa, 1997; Ohgane *et al.*, 2008; Illingworth and Bird, 2009). Fan and his colleges constructed a

computational method (MethCGI) to predict the methylation status of CpG island fragments (segments of CpG islands fragmented into identical lengths) based on DNA sequence features.

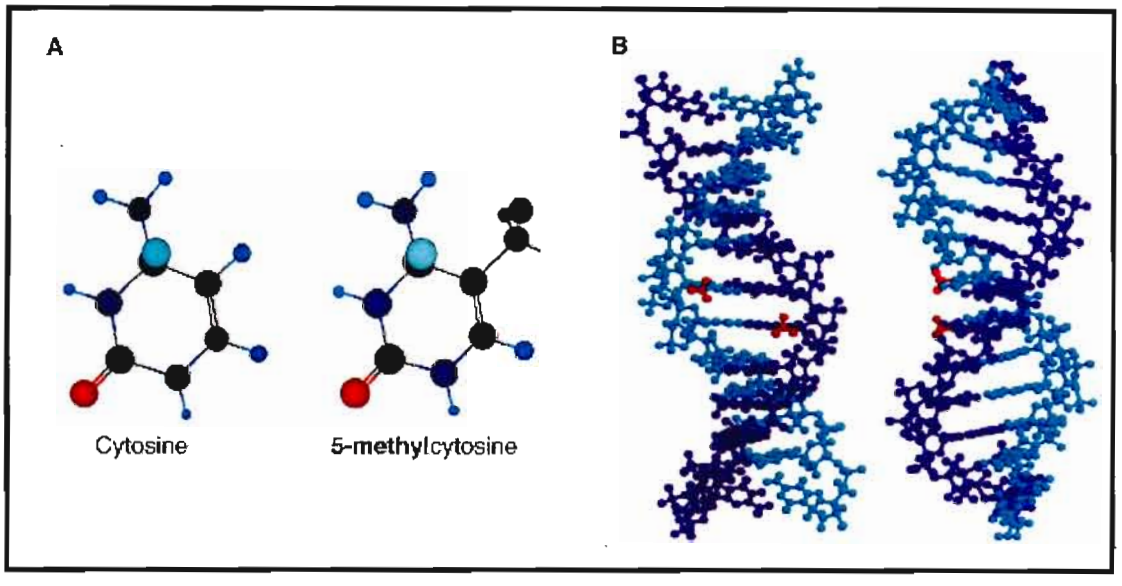

Figure 2.2: ***DNA methylation in mammals.*** *(A) Methylated 5 carbon of a cytosine pyrimidine ring. (B) The methyl groups on modified cytosines are accessible in the major groove. The two strands of the DNA helix are coloured light-blue and purple, and the methyl groups are highlighted in red (Prokhortchouk and Defossez, 2008).*

By using this method they concluded that about 60% of CpG Islands found in X and Y chromosomes are methylation prone and that only ~13% of the CpG Islands located in promoters are methylated (Fan *et al.*, 2008). These results strengthen the current viewpoint that many genes are repressed in sex chromosomes and CpG islands located in promoter regions are seldom methylated. On the other hand, promoters that lack CpG Islands have been known to show some tissue-specific methylation patterns linked to their transcriptional status (Guil and Esteller, 2009). Due to DNA methylation's vital role in gene regulation, it is obvious to conclude that it plays a major role in cellular differentiation.

Cellular differentiation can be described as the complex process in which a less specialized cell type becomes structurally and functionally more specialized cell (Guyton and Hall,

2006b). Epigenetic processes play a crucial role in the ordered differentiation of mammalian cells during embryonic development by activating specific genes and silencing others (Mohn and Schübeler, 2009). *De novo* DNA methylation has been well studied in cell culture model systems and examples of global *de novo* methylation have been thoroughly documented during germ-cell development (Bird, 2002). These epigenetic processes are regulated by DNA methyltransferases (DNMTs) which silence specific gene promoters through methylation (Sweatt, 2009). But despite this, the origins and targeting of this methylation mechanism remains somewhat of a mystery. It is still unclear what determines which regions of the genome are to be methylated and what mechanisms participate. One theory suggests that in early mammalian development all CpGs are indiscriminately methylated. This theory is partially supported by the fact that DNM3A and DNM3B are highly expressed in early embryonic development (Bird, 2002; Illingworth and Bird, 2009). But this is difficult to confirm due to the small amount of biological material available for biochemical study in embryos.

2.2.2 Mechanism of DNA methylation

DNA methylation is carried out by a group of enzymes called DNA methyltransferases (DNMTs) (Progribny *et al*, 2004). These enzymes not only determine the DNA methylation patterns during the early development, but are also responsible for copying these patterns to the new strands formed during DNA replication. Based on their preferred DNA substrates, these enzymes can be divided into two different classes – maintenance enzymes (DNMT1) and *de novo* or pioneering enzymes (DNMT3A + B) (Sweatt, 2009).

The maintenance methyltransferase DNMT1 is responsible for copying existing methylation patterns to newly replicated, hemimethylated DNA (Kanai, 2002; Sweatt, 2009). DNMT1 is also associated with other epigenetic processes such as silencing gene transcription and is known to interact with histone deacetylase 1 and 2 (HDAC1/2) (Kanai *et al*, 2002). While some studies have indicated that DNMT1 has some *de novo* methylation activity *in vitro*, there is no substantial evidence indicating that this is the case *in vivo* (Okano *et al.*, 1999). Due to the relation of DNMT1 with HDAC1 and 2, it directly affects gene transcription and chromatin structure. Although the identification of hemimethylated sequences by DNMTs are still a point of discussion and uncertainty, there is some evidence suggesting that DNMT1 may be recruited by UHRF1 which can bind to partially methylated DNA (Prokhortchouk and

Defossez, 2008). In short, DNMT1 is the key maintenance DNA methyltransferases which is responsible for copying the previously established methylation blueprint after every replication cycle.

The *de novo* methyltransferase DNMT3 typically establishes new DNA methylation patterns in specific regions, mainly in satellite repeats and retrotransposon (transposons via RNA intermediates) sequences (Gopalakrishnan *et al.*, 2008). The *dnmt3* gene family consists of *dnmt3a* and *dnmt3b* which seems to have related functions (Okano *et al*, 1999). Studies done on rodent embryos lacking the *dnmt3a* gene develop normally but died a few weeks after birth (Bird, 2002; Mohn and Schübeler, 2009). On the other hand, mice lacking *dnmt3b* suffered from multiple developmental defects and was aborted (Gopalakrishnan *et al.*, 2008; Mohn and Schübeler, 2009). This is not only a clear indication of the importance of DNMT3 in *de novo* methylation but also illustrates the fundamental role of DNA methylation in early development. To summarize, DNMT3A and B are responsible for methylating previously unmethylated DNA and seem to play an imperative role in development and growth.

A fourth DNA methyltransferase, DNMT2, exists and is structurally similar to prokaryotic and eukaryotic methyltransferases. However, it shows weak or no DNA methyltransferase activity *in vitro* and targeted deletion of the DNMT2 gene in embryonic stem cells causes no detectable effect on global DNA methylation, suggesting that this enzyme has little involvement in establishing DNA methylation patterns (Okano *et al*, 1999; Klose, 2006). The methyltransferase DNMT3L is thought to be a co-factor for DNMT3a and DNMT3b and modulates their catalytic activity (Klose and Bird, 2006; Prokhortchouk and Defossez, 2008). S-adenosylmethionine (SAM) acts as a methyl donor and donates a methyl group to unmethylated cytosines within CpG islands of genomic DNA.

There is ample evidence suggesting that accessory factors are needed to target *de novo* DNA methylation. The need for methylation co-factors was first demonstrated in plants where it was shown that the protein DDM1 is necessary for full methylation of the *Arabidopsis thaliana* genome (Bird, 2002). Evidence also suggests that DNMT3a and DNMT3b may take part in active DNA demethylation (Kangaspeska *et al.*, 2008). Kangaspeska and his co-workers found that DNMT3a and DNMT3b were recruited in the *PS2* promoter before demethylation commenced and that this phenomenon is part of a methylation/demethylation cycle. There have been three possible *de novo* methylation targeting mechanisms suggested:

firstly, DNA methyltransferases (DNMT3 in particular) might directly recognize specific DNA sequences; or DNMT3a and DNMT3b may be recruited by transcriptional repressors or other factors through protein-protein interactions; or lastly, RNAi mediated systems may guide *de novo* methylation to a specific DNA domain (Sawan *et al.*, 2008). It was furthermore shown that in order for *de novo* methylation to be initiated, the protein, Myc, must first associate with DNMT3a. As soon as Myc recruited DNMT3a to a *p21cip1* promoter region, *de novo* methylation of the promoter was initiated (Brenner *et al.*, 2005). These findings support the theory that DNA methyltransferases needs some kind of co-factor to initiate *de novo* methylation. It is clear that not all DNMTs have equal access to all the regions of the genome. Kondo and his co-workers demonstrated this when they showed that mice and humans with DNMT3B mutations are unable to methylate repetitive DNA sequences and CpG islands on the inactive X chromosome. This shows that DNMT3B may be specialized to methylate regions of silent chromatin (Kondo *et al*, 2000).

There are specific proteins that recognise methylated DNA areas causing gene expression inhibition by repressing chromatin structure. There are three protein families that can target DNA methylation: Methyl Binding Domain proteins (MBD), Zink-finger proteins and SET-and-Ring-finger-Associated (SRA) domain proteins (***table 2.1***).

Currently, there is still some uncertainty whether these proteins have individual or similar recognition sequences. These proteins may also differ in location, DNA-binding affinities and expression time (Prokhortchouk and Defossez, 2008). Métivier and his co-workers recently showed that the *pS2/TFF1* gene promoter is subjected to a DNA methylation/demethylation transcriptional cycle (Métivier, 2008). Conventionally DNA methylation is seen primarily as a silencing mechanism with its main role being gene expression regulation. This new evidence may indicate that DNA methylation is not a standalone mechanism but a crucial part of transcription and more in depth studies are necessary to confirm this cycle.

To summarize, DNMT's are at the core of the DNA methylation machinery and is not only responsible for establishing new methylation patterns in DNA but also to maintain these patterns after replication. It may even be an inseparable part of transcription. Further studying DNMTs full extent of influence will undoubtedly better our understanding of DNA methylation and will be indispensable for future medical endeavours to ultimately prevent disease.

Table 2.1: Summary of methyltransferase enzymes and methyl recognition proteins that play a role in DNA methylation[#]

Binding Domain Proteins	*Methylated/unmethylated DNA binding*
MBD	**Recognises methylated DNA**
MecP	
MBD1	
MBP2	
MBP3	
Zinc finger	**Recognises methylated as well as unmethylated DNA**
Kaiso	
ZBTB4	
ZBTB38	
SRA	**Binds methylated DNA**
UHRF1	
UHRF2	

[#] *Adapted from Prokhortchouk and Defossez, 2008*

2.3 Epigenetics in disease

Taken into account the crucial role epigenetics play in many cellular processes, it is logical that abnormal regulation of these mechanisms may lead to misinterpreted gene expression and eventually disease. This relationship between diseases and the epigenome prompted scientists to start comprehensive research into the role epigenetics play in disease states. Today there is mounting evidence of irregular epigenetic regulation in many diseases (see **table 2.2** for some epigenetically linked diseases).

Table 2.2: Diseases associated with aberrant DNA methylation

Pathology Class	Disease Name	Gene(s) Involved
Cognitive [1]	*Fragile X Syndrome (FXS)*	***FMR1* and *FRM2***
	Alzheimer's disease (AD)	***APP***
	Huntington's disease (HD)	***htt***
Psychiatric disorders [1]	*Schizophrenia*	***Reln***
	Drug addiction	***c-Fos, Cdk5, FosB, Bdnf (cocaine addiction)***
	Depression	***Bdnf***
	Predisposition to stress	***GR***
Carcinomas[2]	*Prostate cancer*	***Cytochrome P450 1B1***
	Invasive breast cancer	***P-Cadherin***
	Lung cancer	***p53***
	Hepatocellular cancer	***MAGE 1; MAGE-A1; MAGE A3***

[1] (Gräff and Mansuy, 2008) [2] (Wilson et al, 2007)

The brain contains very high levels of cytosine methylation and in recent years many studies have been done to determine the role of DNA methylation in cognitive brain functions such as learning and memory (Spencer, 2000; Kalda *et al.*, 2007; Keverne and Curley, 2008). It has also been indicated that there is a decline in the brain's DNA methylation status with aging, which may point to a possible link between DNA methylation and higher cognitive processes like memory (Liu *et al.*, 2009). Aberrant DNA methylation patterns have also been detected in many neurological disorders including Rett syndrome and Alzheimer's disease (Gräff and

Mansuy, 2008; Liu *et al.*, 2009). Although the exact functions of DNA methylation in higher brain function is still elusive, it is clear that these epigenetic mechanisms are orchestrating diverse changes in the central nervous system. These changes can then be expressed at molecular, cellular and even behavioural levels (Sweatt, 2009). The influence of epigenetic mechanisms on higher brain function is at the moment a poorly studied and underappreciated field. It is of paramount importance that more in-depth research must be done in this exciting field involving epigenetic regulation of cognition.

It is now clear that genome-wide DNA methylation profiles are the epigenetic memory that is indispensable for cells and tissues to maintain their unique features in ever changing conditions. It is also apparent that properly established and maintained DNA methylation patterns are essential for normal functioning of the adult organism and that aberrant epigenetic regulation may have dire consequences, one of them being cancer.

2.3.1 DNA methylation in cancer

Cancer is a multistage process which causes specific changes in the transcriptional activity of genes (Franco *et al*, 2008). Since the late 1980s researchers started observing abnormal DNA methylation patterns in basically all cancer types. In cancer cells, methylated CpGs loses their DNA methylation status and unmethylated promoter regions become densely methylated. This puzzling phenomenon prompted scientists to believe that both the loss and gain of DNA methylation are linked to cancer (Ehrlich, 2002; Szyf, 2003; Gopalakrishnan *et al.*, 2008; Hirst and Marra, 2009). As mentioned earlier the epigenome is critical in expanding and regulating the genome which in its turn influences the phenotype (***see paragraph 2.2.1***). The epigenome is subsequently influenced by internal cues as well as large range of environmental factors (Lambert and Herceg, 2008). If this processes are not correctly regulated it can lead to changes in DNA methylation and histone modification patterns resulting in the disruption of important cellular processes including gene expression, DNA repair and tumour suppression which may lead to cancer.

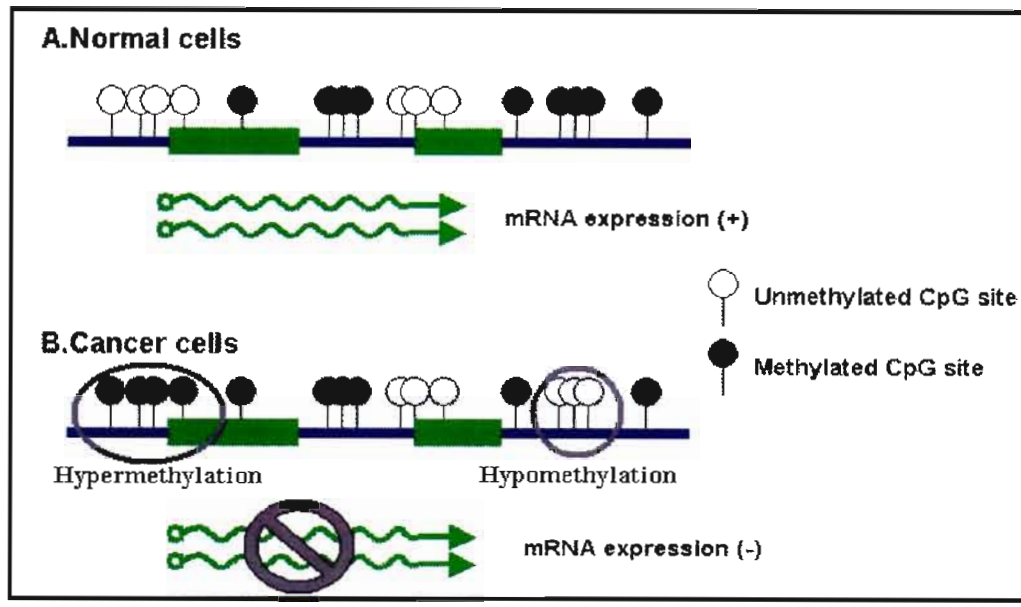

Figure 2.3: Different methylation patterns in normal (A) and malignant (B) cells. Cancer cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation. (Adapted from

Mutations in genes encoding DNA methyltransferases lead to changes in gene expression and altered DNA methylation patterns (Brenner *et al.*, 2005). These changes are associated with cancer and congenital diseases due to defects in imprinting. As described before, CpG islands are usually located in non-tissue specific promoter regions of genes and are normally unmethylated. Tumour cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation (**Figure 2.3**) (Szyf, 2003).

2.3.2 DNA Hypomethylation

DNA Hypomethylation can be described as the demethylation of the typically methylated CpG islands of genes. This may lead to the weakening, or even lifting, of the transcriptional repression in silenced gene promoters which in turn may contribute to the unwanted and abnormal expression of genes that is seen in many cancers (Wilson *et al.*, 2007). This abnormal demethylation can also cause chromosomal rearrangement and translocations which might negatively influence genome stability (Ehrlich, 2002). The three main mechanisms proposed to contribute to cancer development due to hypomethylation:

(1) increase in genomic instability, (2) reactivation of transposable elements and (3) loss of imprinting (Hirst and Marra, 2009).

2.3.3 DNA Hypermethylation

Hypermethylation of DNA mainly occurs in the promoter regions of genes leading to their transcriptional silencing (Moss and Wallrath, 2007). This is apparent from the inactivation of the tumour suppressor genes, adhesion molecules and repair enzymes which contributes to cancer development (Gopalakrishnan *et al.*, 2008). CpG island hypermethylation seems to increase in metastasis suppressor genes while increasing histone alterations (Lujambio, 2007).

Metastasis is the spreading of a disease, usually trough the lymphatic system and blood vessels, to another non-adjacent part of the body. Of recently, scientists are starting to brand metastasis not only as a genetic disease but as an epigenetic disorder as well. According to Lujambio "*... metastasis can be explained by epigenetic mechanisms that regulate both metastasis-associated genes and mi-RNAs*" (Lujambio and Esteller, 2007). Metastasis plays a prominent role in the spread of cancer and the development of secondary tumours.

A large number of recent review papers put forth very convincing evidence that epigenetic changes (including DNA methylation) play a direct causative role in cancer initiation and development (Gopalakrishnan *et al.*, 2008; Sawan *et al.*, 2008; Hirst and Marra, 2009). If this is true, it means that it is possible to reverse methylation patterns by pharmacological agents that will initiate demethylation and reactivate tumour suppressor genes. Epigenetic alterations are present in many malignancies and in contrast to genetic alterations, epigenetic changes occur gradually and are in theory reversible - making them attractive targets for the development of therapeutic and preventive drugs. In principle these drugs can reverse DNA methylation while reactivating silenced genes in cancer cells (Lambert and Herceg, 2008).

Methylated genes are also attractive targets for new cancer biomarkers. Protein biomarkers in blood are not suitable for early cancer detection. On the other hand aberrant DNA methylation of gene promoters are known to exist in the early stages of cancer and are

potentially a better biomarker (Duffy *et al.*, 2009). Advantages of methylation biomarkers include:

- ***Serum concentrations of methylated genes display a high specificity for malignancy*** (Methylated genes have high specificity for cancer and is present in body fluid, making it an attractive target for biomarkers)
- ***Certain genes exhibit relative tissue specificity in their methylation pattern*** (CpG island profiles differ from tissue to tissue)
- ***Gene methylation is associated with defined DNA regions*** (malignant gene promoters are usually found in confined genomic regions which means only one set of primers is necessary).

In conclusion, it is becoming widely accepted that epigenetic alterations are universally present in human malignancies and that cancer is as much a disease of abnormal epigenetics as it is a genetic disease and by better understanding these epigenetic mechanisms (including aberrant methylation) we can develop more effective cancer treatments and may even one day be able to prevent it.

2.4 Comet Assay (Single Cell Gel Electrophoresis assay)

The Comet Assay (single cell gel electrophoresis or SCGE) is a cost-effective, sensitive and simple technique which is traditionally used for analyzing and quantifying DNA damage in individual cells. This method was first used by Östling and Johanson in 1984 to study radiation-induced DNA damage in individual mammalian cells and has since been regularly used in biomonitoring and mechanistic studies in a large range of *in vitro* and *in vivo* systems (Östling and Johanson, 1984; Lovell and Omori, 2008). By modifying this assay to be DNA methylation sensitive, we envisage to routinely measure global DNA methylation in cell cultures and simultaneously determine the integrity of their genetic material.

A variety of DNA lesions can be detected using the alkaline version of the single cell gel electrophoresis (comet) assay, including DNA double (DSB) and single strand breaks (SSB), as well as alkali-labile sites (ALS) (Collins and Gaivão, 2004). Modifications to the comet

assay have allowed the use of lesion specific endonucleases to detect specific base modifications as DNA single strand breaks (Epe *et al.*, 1993; Collins and Gaivão, 2004). For example, Fpg acts both as an AP-lyase and an *N*-glycosylase allowing it to release modified purines from double stranded DNA (Collins *et al.*, 1993; Tice *et al.*, 2000). It was shown that Fpg strongly enhances the detection of mitomycin C (MMC) and ethylmethanesulphonate (EMS) induced DNA modifications (Andersson and B.E. Hellman, 2005). Damaged pyrimidines are removed in a similar manner from double stranded DNA by the endonuclease *Endo III* (Speit *et al.*, 2004).

This simplistic, inexpensive, sensitive and easily modifiable assay will present us with an alternative and viable way to simultaneously determine methylation status and genome integrity of tissue and/or cultured cells. Through better understanding the role of DNA methylation and the manner in which it influences normal as well as diseased states, we are not only unravelling the mysteries of life but paving the way to more effective screening procedures and treatments.

2.5 Aims and approach of this study

Aims:

The main aim of this study was to modify the alkaline comet assay to measure global DNA methylation in cultured cells.

Approach:

1. Establishing the comet assay in our laboratory by applying proven published methods.
2. Modifying the comet assay to be DNA methylation sensitive by using the isoschizomeric restriction enzymes *Hpa*II and *Msp*I.
3. Comet assay efficiency will be validated by performing the cytosine extension assay which utilizes the same restriction enzymes as the methylation sensitive comet assay.
4. Finally, the methylation sensitive comet assay will be applied to cultured cells treated with the demethylation agent 5-azacytidine and the accumulating Tyrosinemia type I metabolite, succinylacetone (SA).

(As summarized in ***diagram 2.1***)

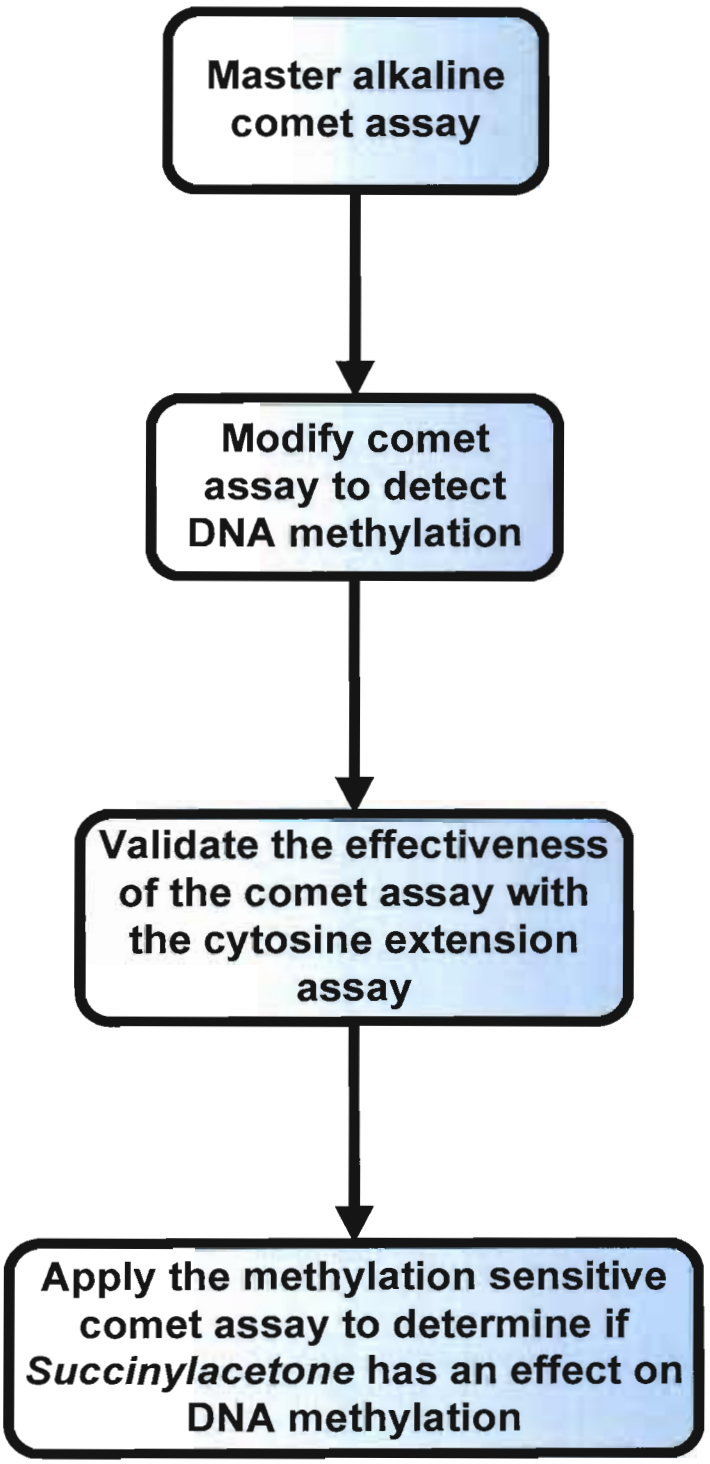

Diagram 2.1: *A flow diagram illustrating the proposed study*

CHAPTER THREE

THE METHYLATION SENSITIVE COMET ASSAY

A series of experiments were performed in order to modify the alkaline comet assay for global DNA methylation measurement and the outline of the proposed methylation sensitive comet assay is given in **diagram 3.1**.

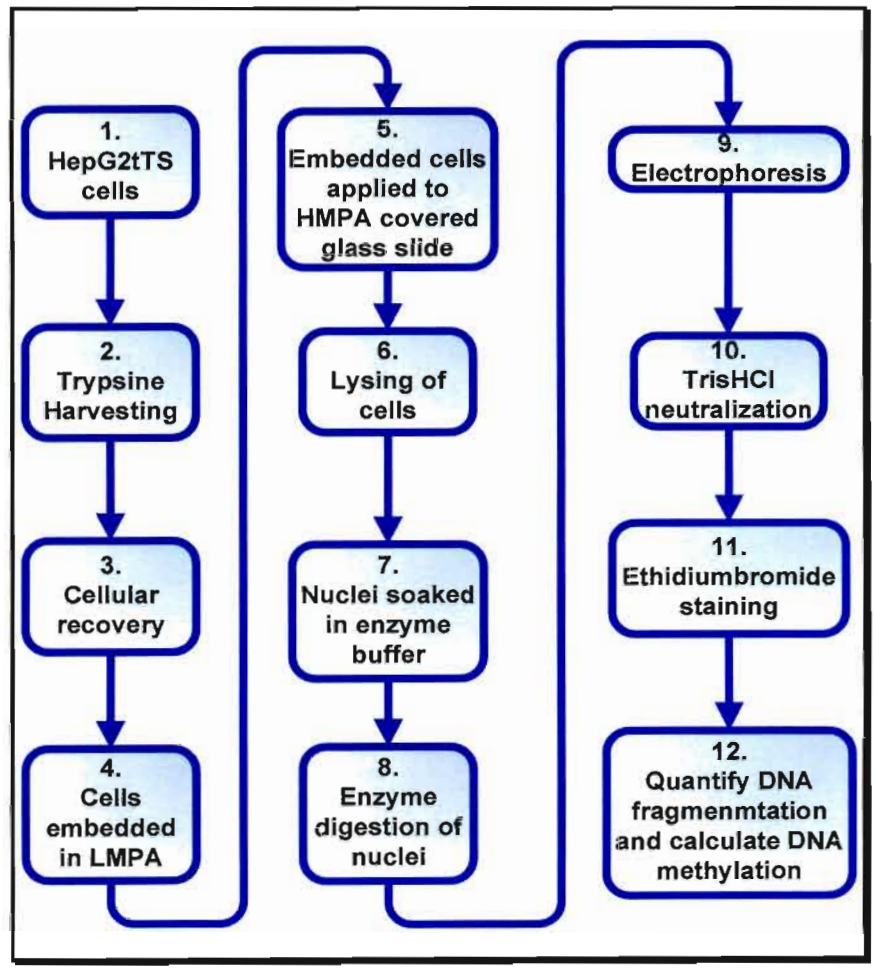

Diagram 3.1 **A diagram showing the outline of the proposed methylation sensitive comet assay**

3.1 Cell line, culture conditions and harvesting

Two different mammalian cell types, isolated leukocytes and cultured HepG2tTS, were used in the standardisation of the methylation sensitive comet assay. This specific HepG2 cell line is regularly used in our laboratory and was therefore available for this study. Distinct cell lines require specific cellular harvesting techniques, for instance, HepG2tTS is an adherent cell line and must be separated from their growth chambers, adjacent cells and other material in suspension, while leucocytes have to be separated from other cells and substances found in blood.

3.1.1 Leukocytes

Leukocytes are isolated from blood and are the mobile component of the immune system. The average adult human being has approximately 7000 leukocytes per micro liter whilst about 62% of these consist of polymorphonuclear neutrophils (Guyton and Hall, 2006a). To isolate leukocytes, 1.5ml blood is placed on top of 1.5ml Histopaque™ and centrifuged at 4 000 g for 15min. The buffy coat is removed and mixed with 250µl PBS followed by centrifugation at 1 100 *g* for 3min. The supernatant is discarded and the pellet washed again with PBS. This step can be repeated two to three times to ensure high leukocyte purity. After the wash steps are completed, the supernatant is discarded and the pellet is resuspended in 500µl DMEM nutrient medium. The cells are then counted and the required number of cells (~25 000 cells per 50 µl) is then used in the comet assay. Leukocytes were always isolated within an hour from collecting blood.

3.1.2 HepG2 cells

The HepG2tTS cell line is derived from the hepatocellular carcinoma cell line HepG2 which adheres to their neighbouring cells and the bottom of the growth flask. HepG2 cells were transfected with the G418 resistant tTS-Neo vector (Clontec). HepG2tTS were cultured in HyClone medium (Separations) containing 10% FBS, G418, penstrep (WhiteSci) and non-essential amino acids (Lonza). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. This particular cell line was chosen due to its availability in our laboratory and ease of handling and culturing. Since the comet assay focuses on single cells, it is of fundamental

importance that the HepG2tTS cells are separated from each other during the harvesting step. To this end it was found that trypsin harvesting yielded the best results.

3.2 HepG2 cellular repair

Trypsin is a serine proteinase which hydrolyses proteins. During the harvesting process, HepG2tTS cells are exposed to trypsin which negatively influences the cell's cellular integrity that may lead to DNA fragmentation. This makes a repair phase essential before the cells can be used in the comet assay. To determine a suitable recovery time, cells were incubated in an orbital shaker (to prevent cells from adhering to the container) in DMEM nutrient medium for 1, 2, 3 and 4 hours at 37˚C directly after trypsin harvesting (***Figure 3.1***).

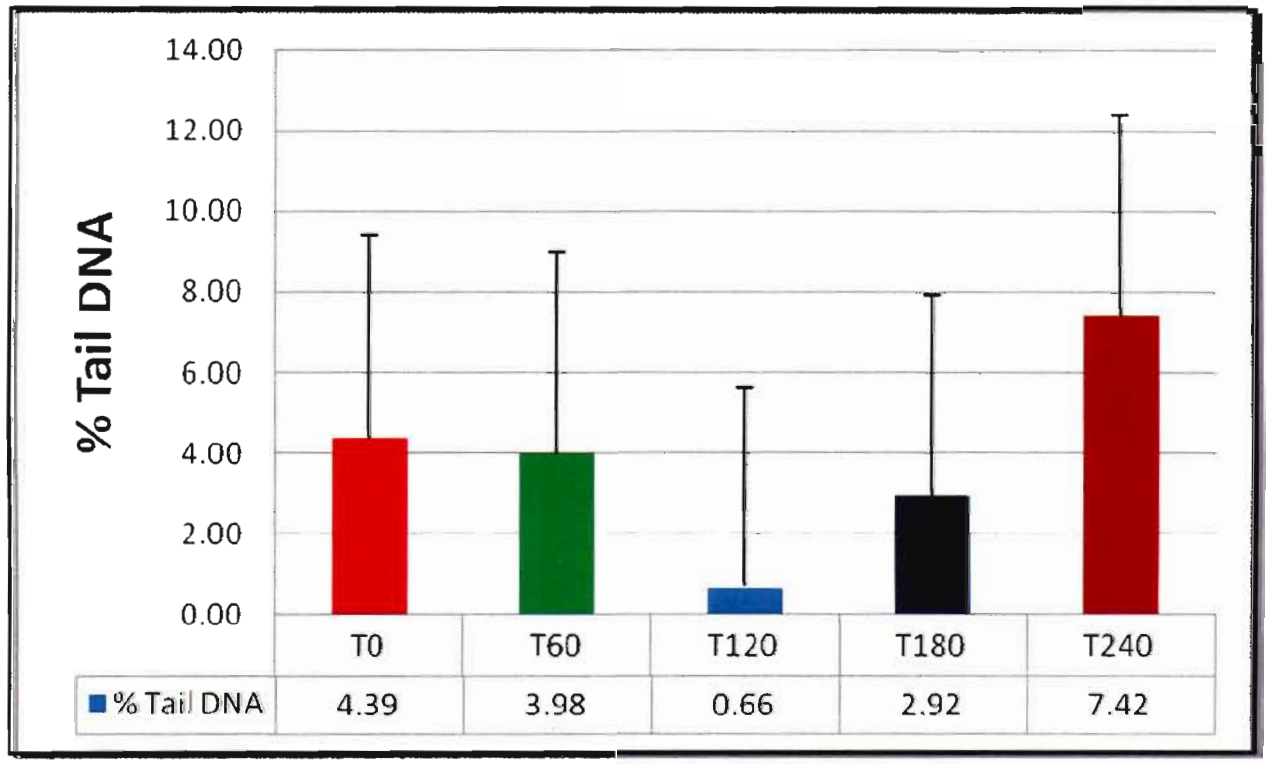

Figure 3.1 **The DNA integrity in HepG2tTS cells following trypsin harvesting.** The cells were incubated for various times (0, 60, 120, 180 and 240 minutes) before being investigated with the comet assay.

The results indicate a linear decrease in tail intensity of the comets (DNA damage) within the fist two hours of incubation, followed by a rapid increase in DNA fragmentation in hours three and four. The most significant recovery in DNA integrity can be seen after two hours of incubation (**diagram 3.2**). A possible explanation for the rapid DNA degradation following two hours of incubation may be due to the fact that the cells cannot adhere to the container as a result of the container that being shaken.

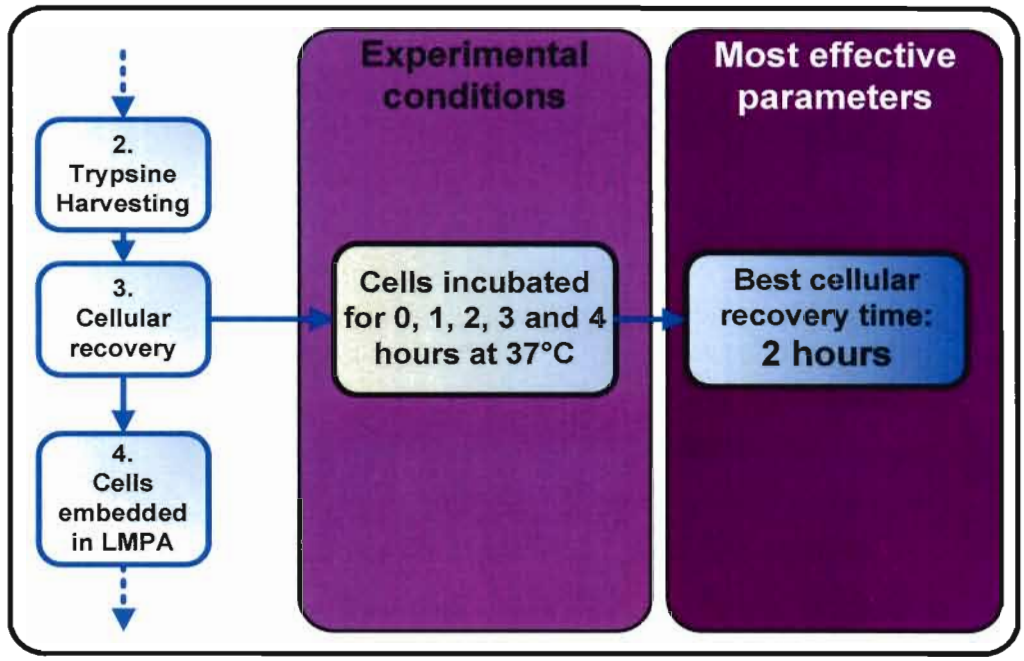

Diagram 3.2 **Summary of the experiments preformed to determine the most effective recovery time after trypsin harvesting**

3.3 Comet assay

The basic principle of the comet assay involves the electrophoresis of single nuclei in order to determine its genetic integrity. To summarize the method: harvested cells are encapsulated in a low melting point agarose gel and applied to a glass slide covered in high melting point agarose. The agarose gel acts to keep the cells separate from each other and to serve as staging point for all the treatments that follows. Encapsulation is followed by chemical (chemical detergent) or physical (mechanical or sonification) lysis. The supercoiled

DNA attached to the nuclear matrix unwinds due to the high pH (~12.3) of the alkaline lysing solution which makes sites available for digestion with methylation sensitive restriction enzymes eg. *Hpa*II. After lysing the cells, the nucleoids are treated with *Msp*I and *Hpa*II respectively on separate slides.

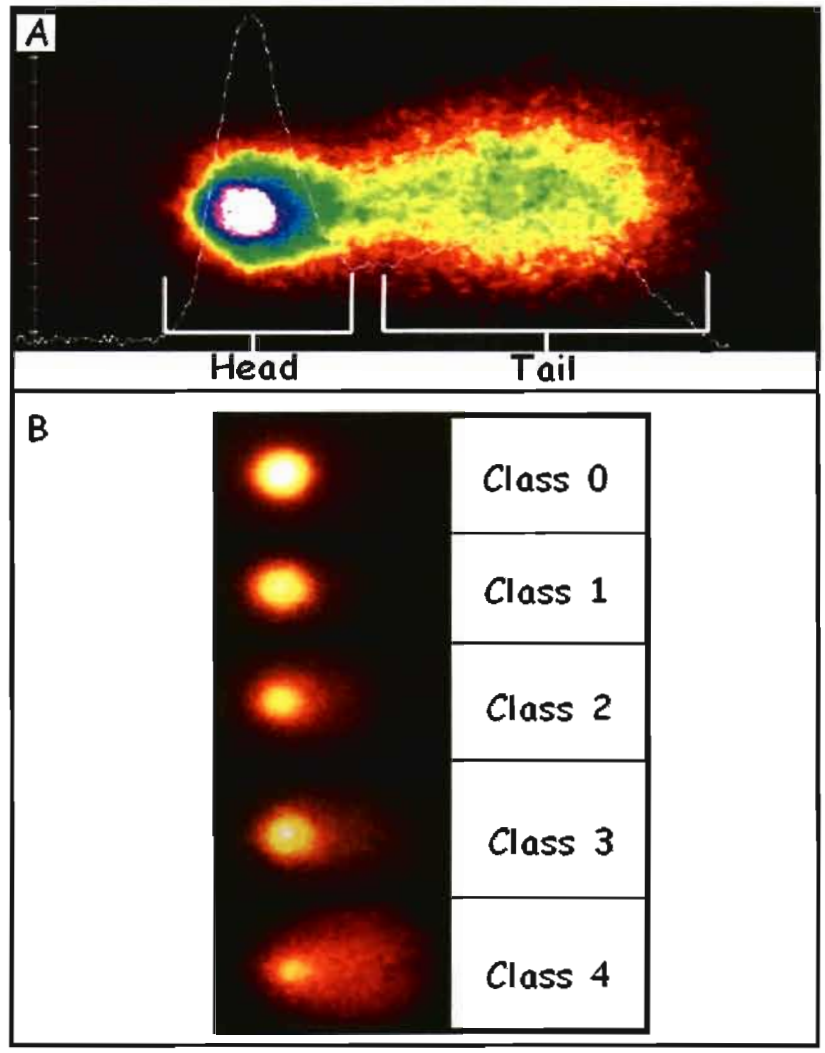

Figure 3.2 **The DNA fragments migrate from the nucleoid forming a comet like form.** (A) The general shape of a comet clearly indicating its head (nucleoid) and tail (migrated DNA fragments) after electrophoresis and staining. (B) Illustration of different degrees of DNA damage which can be divided into five classes, class 0: <6%; class 1: 6.1-17%; class 2: 17.1-35%; class 3: 35.1-60% and class 4: >60%.

Electrophoresis is carried out causing the damaged and/or digested fragments of the DNA to migrate towards the anode - forming the so called tail of the comet. After electrophoresis, Tris-HCl is used to neutralize the alkaline electrophoresis buffer followed by staining with ethidium bromide. Imaging software is then used to measure the fluorescence and to determine the extent of DNA damage and methylation percentage (Collins *et al.*, 1997; Rojas *et al.*, 1999). The tail migration may be determined by either measuring the tail length, tail moment or percentage DNA in the tail (Lovell, 2008). Comets are then divided in different classes according to their DNA integrity (***Figure 3.2***).

3.3.1 Encapsulation of cells

The cells are encapsulated in a low melting point agarose gel and applied to a glass slide covered with a thin layer of high melting point agarose. This enables the nucleoids prepared from the HepG2 cells to undergo electrophoreses in order to quantify the extent of DNA damage and/or fragmentation.

The High Melting Point Agarose (HMPA [1%]) serves as a foundation for the agarose containing the encapsulated cells, making electrophoresis more effective and assuring the low melting point agarose (LMPA) does not dry out. To prepare 1% HMPA, 1g of the HMPA agarose powder is dissolved in 100ml EDTA by heating the solution until completely dissolved. Between 350µl and 500µl of this solution is applied eventually to a frosted glass plate as a smooth layer and left to solidify at room temperature.

The 0.5% Low Melting Point Agarose (LMPA) is prepared by dissolving 0.5g LMPA agarose powder in 100ml EDTA. This mixture is kept at 40°C until the cells are ready to be encapsulated. It should be noted that LMPA mixtures hotter than 50°C can cause unwanted DNA damage. When working on heavily damaged cells or cells with small genomes, a higher concentration of LMPA can be used to restrict DNA fragment migration to allow proper measurement of tail intensity.

In order to determine the amount of DNA damage and/or of enzymatic digestion using electrophoresis, the cells must be encapsulated in agarose gel and their nucleoids exposed.

To encapsulate the cells, 50µl of the cell solution is thoroughly mixed to 100µl of the LMPA (40˚C). Between 100µl and 150µl of this solution is then evenly applied on top of the HMPA covered plate and left to solidify at room temperature. By applying as little as possible of the cell-LMPA solution on top of the HMPA covered plate, the individual cells are kept on the same planer dimension, making comet analysis easier and less time consuming.

As soon as the LMPA has settled, the slides are placed in lysing solution for a minimum period of 1 hour. Care must be taken to ensure that the LMPA does not dry out, leading to lysing and electrophoresis complications.

To ensure reproducible and reliable results a considerable effort were spent on the following technical aspects:

- The even distribution of cells on the slides. This was achieved by thoroughly pipetting the harvested cells to separate them from each other. It is also important to carefully mix the LMPA and cell solution before applying it to the HMPA covered plate. A plain microscope slide is then used to evenly spread a small amount (100 µl -150 µl) of LMPA-cell mixture on the HMPA covered slide. A lot of practice and effort was put into mastering the spreading technique to ensure results can be accurately and reliably reproduced.

- An experiment was preformed to determine if cells in different LMPA planer levels had an influence on enzyme digestion. This study was executed by applying one layer of LMPA-cell mixture and letting it set before applying a second layer on top of the first. Results indicated that there was no substantial difference between the enzyme digestions of deeply embedded cells in comparison with the surface layer of cells (***Figure 3.3***).

- An investigation was also done to determine the distribution of nucleoids and extent of enzyme digestion on the frosted slides. This was accomplished by dividing the glass counting plates into grids consisting of 3 mm^2 squares using a permanent marker (***Figure 3.4A*).** Five slides covered in agarose containing HepG2tTS cells were prepared and every cell nucleus was painstakingly counted in correspondence to its grid.

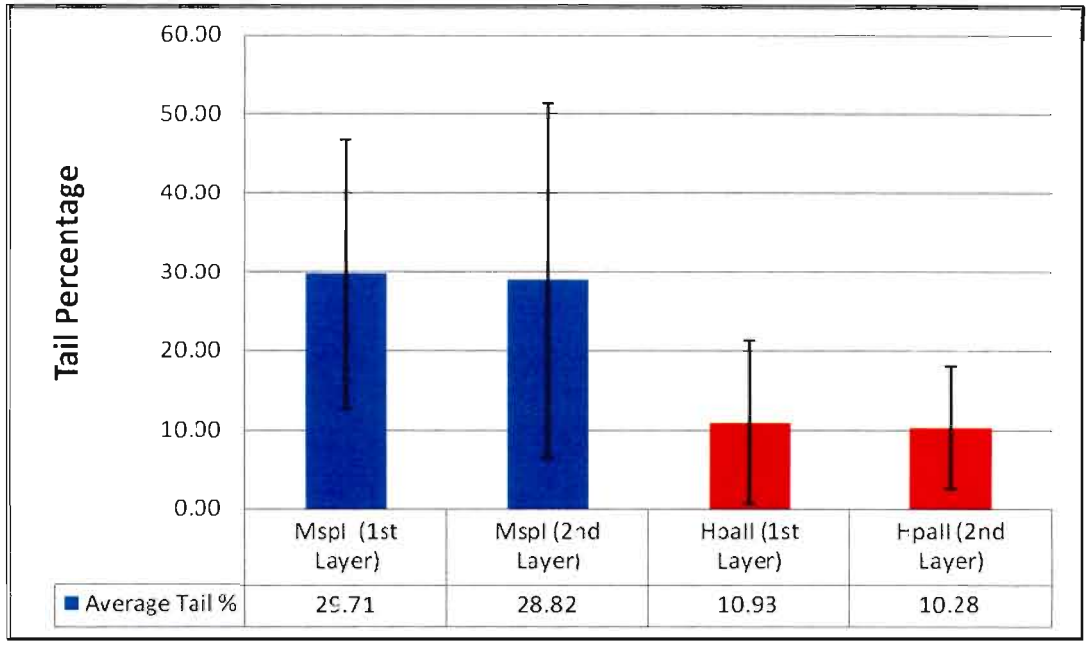

Figure 3.3 Restriction endonuclease treatment of cells in different agarose planer levels. The results indicate that there is no significant difference between deeply embedded (1st layer) cells in comparison with the surface (2nd layer) coat of cells.

Out of the five plates counted there was an average of 250 nucleoids per window. The results of these five plates were pooled to be presented as one diagram (***Figure 3.4B***). Light blue blocks represent areas where less than 5 cells where present per 3 mm^2, green blocks represents between 5 and 7 cells and dark blue more that 7 cells per 3 mm^2. It is clear from the Figure that the nucleoids are not evenly spread across the windows and that the bulk of the cells seem to be concentrated at the centre. This may be due to the agarose applying technique used. The cell solution is applied to the middle of the window before smoothing it out using a pipette tip. This may cause the majority of cells to be concentrated at the centre of the counting windows. Finally, *Msp*I was used to test if restriction digestion is uniform across the slide. Again, the average of the results of the five slides is represented (***Figure 3.4C***).

The blue squares indicate the areas where restriction endonuclease treatment was comparable between different slides, whereas red squares indicate conflicting enzyme digestion. After application of the enzyme, a glass covering slide is used to prevent the

gel from drying out and may result in the digestion pattern observed. Due to the long incubation periods of approximately 60 minutes and, even though the cover slips shields the entire window, the enzyme solution may begin to evaporate at the edges of the plate leading to shorter enzyme digestion in the "red zones". Another interesting observation was that the tail intensity of comets in the second window of the glass counting slides was less intense compared to the first window tail intensity. It seems like electrophoresis is not as effective on the nucleoids of the second window which is further from the cathode and can hence not be used as a control for the first window.

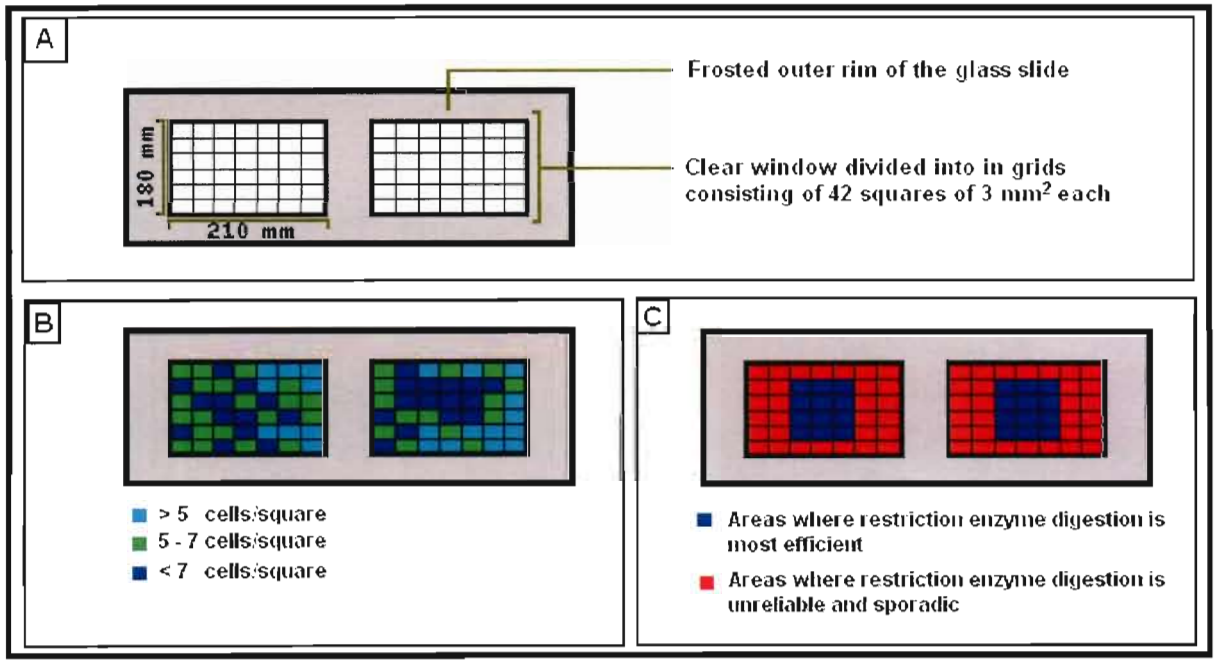

Figure 3.4 **Diagram indicting the distribution of nucleoids and extent of enzyme digestion on frosted slides.** (A): A Figure showing the grid layout used to determine the distribution of nucleoids and effectiveness of enzyme digestion. (B) An indication of the distribution of nucleoids across the counting plate. (C) A diagram showing the most efficient enzyme digestion sites (see text for details).

- All these aspects were taken into account in subsequent experiments to ensure the most reliable results.

3.3.2 *Lysis of cells*

In order for the comet assay to illustrate the integrity of a cell's genetic material, the nucleoides must first be exposed. The encapsulated cells are submerged in lysis solution consisting of 5 mol/l sodium chloride (NaCl), 0.4 mol/l ethylenediaminetetraacetic acid (EDTA), 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100 at 4˚C. This solution is formulated to disrupt cellular material in the shortest amount of time, while leaving the nucleus unharmed. The high NaCl concentration draws water out of the cells by osmotic action. After experimenting with different salt concentrations it seems that the ideal concentration is between 2.5 mol/l and 5mol/l for lysing HepG2tTS cells without negatively influencing the genomic integrity. EDTA is commonly used to forage metal ions in order to deactivate metal dependant enzymes that may cause DNA damage. DMSO is an excellent polar aprotic solvent and is especially effective solvent in reactions involving salts (Bordwell, 1988). It acts as a hydroxyl radical scavenger formed when iron is released from blood while also acting to inhibit the formantion of secondary DNA structures (Chakrabarti, 2001). Studies concluded that an 8-15% dilution of DMSO yields the best lysis results. Finally, the non-ionic detergent Triton X-100 is used to solubilize cell protein and membranes. For a summary of the experiments done to determine the most sufficient lysis conditions see **diagram 3.3**.

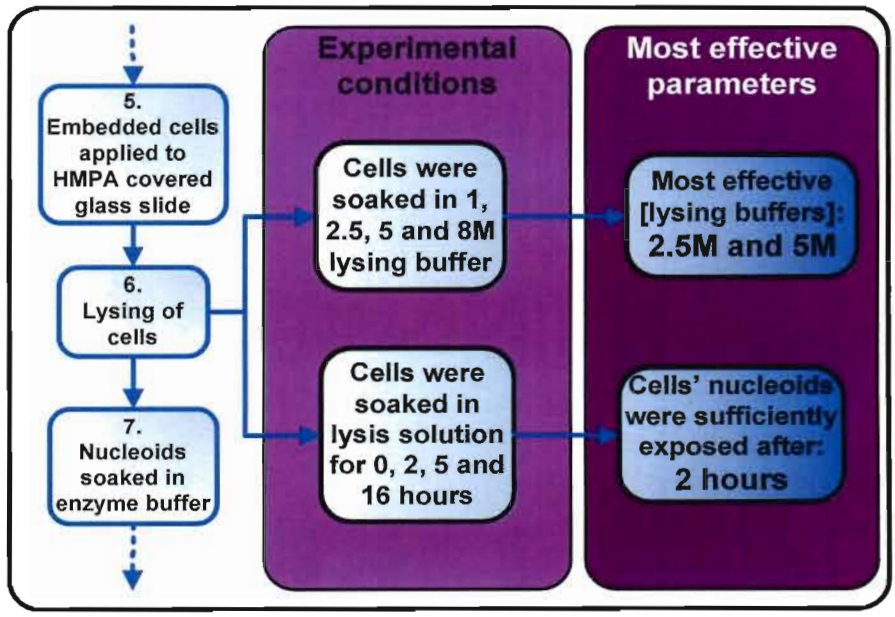

Diagram: 3.3 **Summary of the experiments done to determine the most efficient lysis conditions.**

3.3.3 *Restriction enzyme treatment of exposed nucleoids*

After lysis, the nucleoids are exposed and can be treated with restriction enzymes *Hpa*II and *Msp*I. The isoschizomeric restriction enzymes recognise the same tetranucleotide sequence (5'-CCGG-3') but display differential sensitivity to DNA methylation. *Hpa*II is inactive when any of the two cytosines is methylated, but cuts the hemi-methylated 5'-CCGG-3' at a lower rate compared to the unmethylated sequences. On the other hand, *Msp*I cuts 5'-C^{m}CGG-3', but not 5'-^{m}CCGG-3' (Biolabs, 2005-2006). Thus, when applied to the comet assay, one would expect that a higher level of DNA methylation of the CpG sequence in this site would result in a larger difference in the amount of DNA in the comet tails of *Hpa*II digested versus *Msp* I digested DNA.

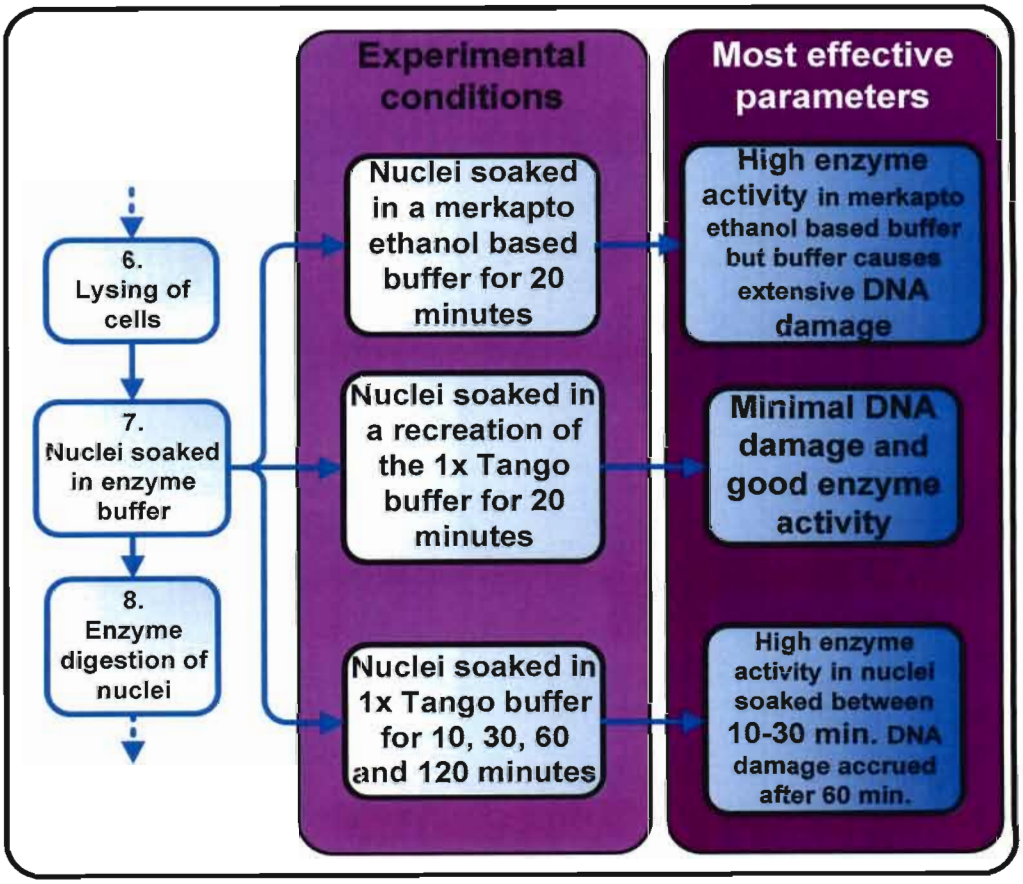

Diagram: 3.4 **Summary of the experiments preformed to determine the most efficient restriction enzyme buffer.**

In order to create favourable restriction conditions, the slides containing the nucleoids were soaked in enzyme reaction buffer for at least 10 minutes. The enzyme reaction buffer consist of $5x10^{-3}$ mol/l TrisHCl, $5x10^{-3}$ mol/l NaCl, $5x10^{-4}$ mol/l merkapto-ethanol and $1x10^{-3}$ mol/l EDTA which is a close recreation of the Tango buffer usually used with these enzymes. It should be noted that preliminary tests indicated that prolonged exposure of nuclei (>60 minutes) to this reaction buffer may have negative effects on the DNA integrity (***diagram 3.4***). After the slides have been soaked in reaction buffer, the enzyme mixture is directly applied to the slides. Each enzyme mixture is composed of 1.5 unit of *MspI/HpaII*, 10 µl of Tango buffer and filled to 100 µl with H_2O. 100 µl of this enzyme mix is then carefully applied to each slide and covered with a cover slip. The slides are then placed in a damp plastic container laced with towel paper that was preheated to 37˚C. After 5 minutes of incubation the slides were covered with towel paper soaked in reaction buffer to keep the slides from drying out while incubating for another 55 minutes. For the experiments done to determine the most effective enzyme incubation conditions see ***diagram 3.5***.

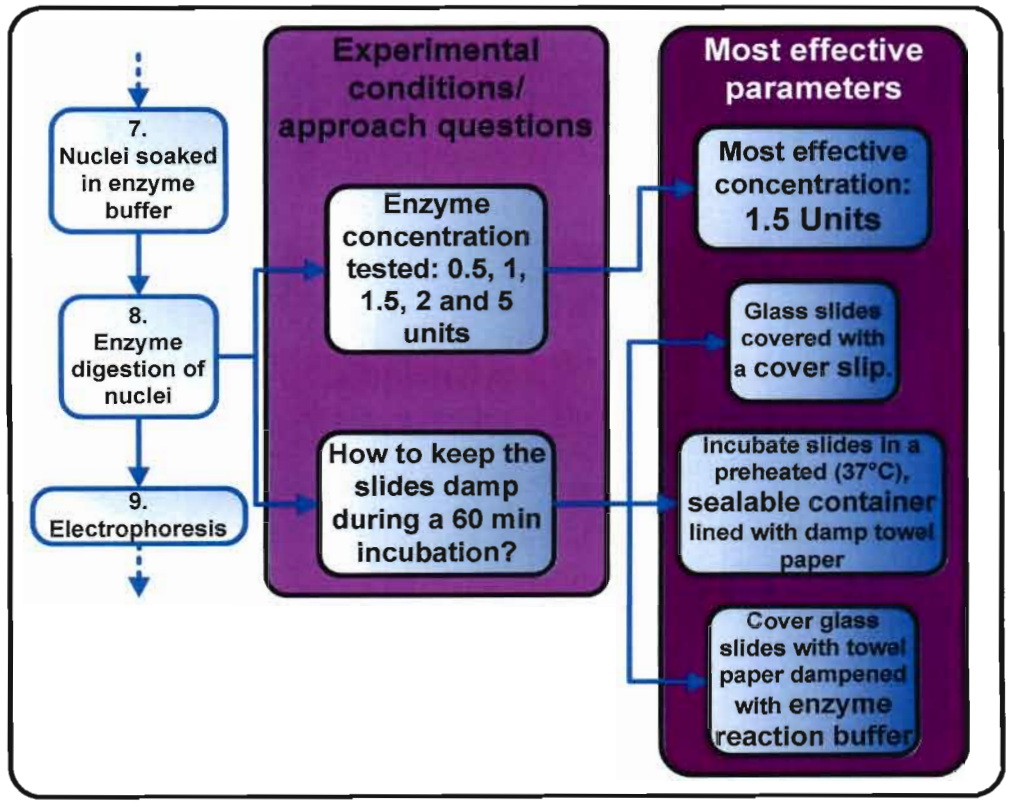

Diagram: 3.5 **Summary of the experiments preformed to determine the most effective enzyme incubation conditions.**

In some cases, experimental results showed little or no DNA digestion. This may be the result of tightly packed nuclei where the majority of the enzymes recognition sites are unavailable, thus leading to poor DNA digestion. This can be overcome by treating the cells directly with a 5% proteinase K solution just after lysis.

3.3.4 Proteinase K treatment

Nucleoids prepared from cells in the later phases of the growth curve tend to be tightly packed on the slides making enzyme digestion only partially effective. This problem can be overcome by soaking the slides in electrophoresis buffer for 30 minutes just before enzyme treatment. This alkaline solution assists with unwinding the nucleus but can cause DNA damage leading to unwanted fragmentation. An alternative is to treat the cells with proteinase K (1.0 -1.5 mM) solution which is a subtilisin-type protease. This broad spectrum serine protease is commonly used to inactivate nucleases when isolating or purifying DNA (QIAGEN, 2005).

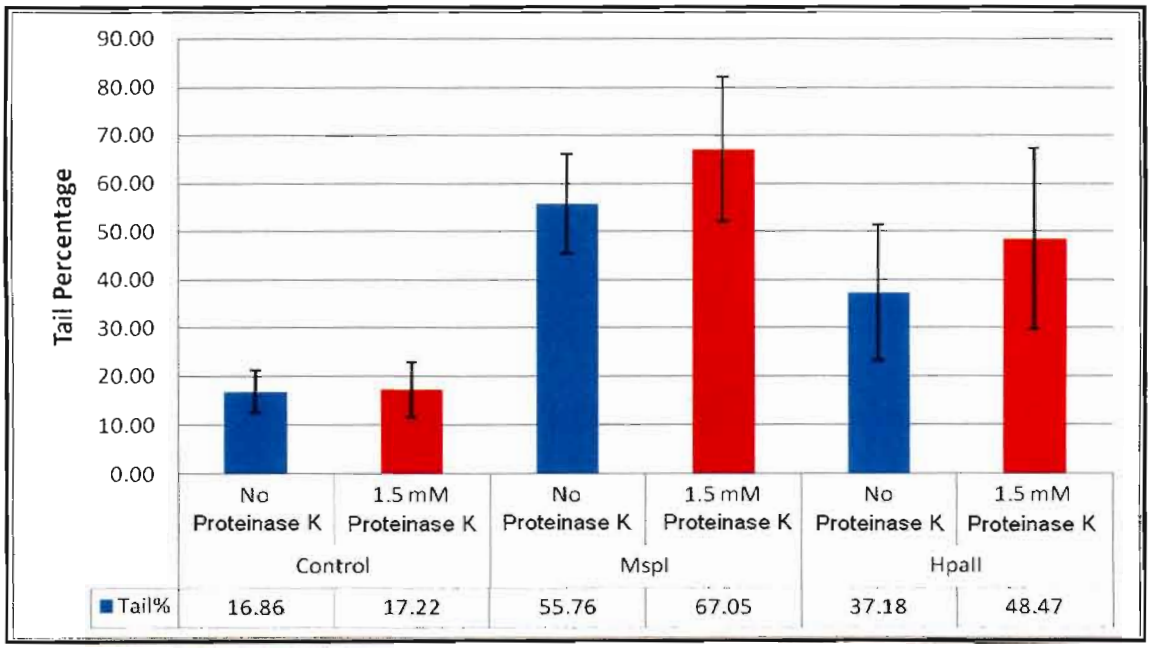

Figure 3.4 A comparison of the effectiveness of enzyme digestion between cells treated with Proteinase K (red) and untreated cells (blue).

Proteinase K acts on the nucleosomes causing the DNA to relax and unwind, making restriction enzyme recognition sites more accessible for *Msp*I and *Hpa*II (***Figure 3.4***). After proteinase K treatment there was a visible increase in the average tail percentage between the different enzyme digestions indicating the effectiveness of proteinase K in relaxing the nucleoids of HepG2tTS cells.

3.3.5 Electrophoresis, staining and quantification of nucleoids

After incubation the slides are placed in an electrophoresis tank and covered with electrophoresis buffer (5 mol/l sodium hydroxide (NaOH) and 0.4 mol/l EDTA). Electrophoresis was done for 45 minutes at a potential difference of 30V and a current of 300mA while the buffer is maintained at 4°C. Experiments showed that electrophoresis at 4°C are extremely crucial for optimum DNA fragment migration. After electrophoresis the slides are placed in a Tris-HCl neutralisation buffer for approximately 15 minutes. This buffer acts to neutralize the alkaline electrophoresis and prevents it from causing unwanted DNA damage. The slides are then stained with 10µg/ml ethidiumbromide for at least an hour at 4°C and rinsed afterwards with distilled water. Finally, the comet images were captured with an Olympus IX70 fluorescence microscope (x200 magnification) and scored using the Comet IV computer software (Perceptive Instruments Ltd). At least 100 comets were randomly scored per slide and the percentage of DNA migration from the head (tail intensity) was measured for each comet scored. All experiments were at least performed in duplicate.

3.4 Application of the methylation sensitive comet assay

After the comet assay was modified to measure global DNA methylation in cultured cells it was applied to cells treated with the demethylation agent 5-azacytidine. This application serves as the discussion and conclusion chapter of this study and is presented in the form of an article (**chapter 4**). The aim of this article was to determine whether the comet assay could be modified to detect changes in the levels of DNA methylation in single cells. Difference in methylation sensitivity of the isoschizomeric restriction endonucleases *Hpa*II and *Msp*I was used to demonstrate the feasibility of the comet assay to measure the global DNA methylation level in individual cells. The results were verified with the well established

cytosine extension assay. Finally cells were treated with the accumulating human Tyrosinemia type I metabolite succinylacetone to determine its affects on global as well as CpG island methylation.

CHAPTER FOUR

PAPER AS DISCUSSION AND CONCLUSION

Chapter four is in an article format and was written according to the instructions for authors of the scientific journal Analytical Biochemistry (http://ees.elsevier.com /yabio/default.asp) and serves as the discussion chapter of this study.

The article was submitted on the 2nd November 2009 and was accepted 9 February 2010.

Analytical Biochemistry 400 (2010) 190–194

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Assessing the DNA methylation status of single cells with the comet assay

Johannes F. Wentzel [a,*], Chrisna Gouws [a], Cristal Huysamen [a], Etresia van Dyk [a], Gerhard Koekemoer [b], Pieter J. Pretorius [a]

[a] Division for Biochemistry, School of Physical and Chemical Sciences, North-West University, Potchefstroom 2520, South Africa
[b] Statistical Consultation Services, North-West University, Potchefstroom 2520, South Africa

ARTICLE INFO

Article history:
Received 2 November 2009
Received in revised form 4 February 2010
Accepted 9 February 2010
Available online 13 February 2010

Keywords:
Comet assay
DNA methylation
HepG2
Cytosine extension assay
Tyrosinemia type I
Succinylacetone

ABSTRACT

The comet assay (single cell gel electrophoresis) is a cost-effective, sensitive, and simple technique that is traditionally used for analyzing and quantifying DNA damage in individual cells. The aim of this study was to determine whether the comet assay could be modified to detect changes in the levels of DNA methylation in single cells. We used the difference in methylation sensitivity of the isoschizomeric restriction endonucleases HpaII and MspI to demonstrate the feasibility of the comet assay to measure the global DNA methylation level of individual cells. The results were verified with the well-established cytosine extension assay. We were able to show variations in DNA methylation after treatment of cultured cells with 5-azacytidine and succinylacetone, an accumulating metabolite in human tyrosinemia type I.

Maintaining genome stability is crucial for the development and normal functioning of eukaryotic organisms. DNA methylation not only plays an important role in gene regulation but also is crucial for maintaining genome stability [1,2]. This epigenetic event can be described as the chemical modification of DNA in which a methyl group is attached to a cytosine base without changing the original DNA sequence. DNA methylation patterns can be established on a global or gene-specific level in accordance with regulatory needs [3]. Unmethylated CpG dinucleotides are present mainly in regions called CpG islands, which are characterized by relatively high CpG density [4]. If the epigenetic processes are not correctly regulated, it can lead to changes in DNA methylation and histone modification patterns that disrupt important cellular processes, including gene expression, DNA repair, and tumor suppression, which may lead to cancer [5–7]. Much progress has been made in using DNA methylation markers in cancer diagnosis and prognosis through the development of a wide range of methods to measure both global and gene-specific DNA methylation [8,9].

A variety of DNA lesions can be detected using the alkaline version of the single cell gel electrophoresis (comet)[1] assay, including DNA double- and single-strand breaks (DSBs and SSBs, respectively), as well as alkali-labile sites (ALSs) [10]. Modifications to the comet assay have allowed the use of lesion-specific endonucleases to detect specific base modifications as DNA SSBs [10,11]. For example, Fpg acts as both an AP lyase and an *N*-glycosylase, allowing it to release modified purines from double-stranded DNA [12,13]. It was shown that Fpg strongly enhances the detection of mitomycin C (MMC)- and ethylmethanesulfonate (EMS)-induced DNA modifications [14]. Damaged pyrimidines are removed in a similar manner from double-stranded DNA by the endonuclease EndoIII [15].

The isoschizomeric restriction enzymes HpaII and MspI recognize the same tetranucleotide sequence (5′-CCGG-3′) but display differential sensitivity to DNA methylation. HpaII is inactive when any of the two cytosines is methylated, but it digests the hemimethylated 5′-CCGG-3′ at a lower rate compared with the unmethylated sequences. On the other hand, MspI digests 5′-C^{m}CGG-3′ but not 5′-^{m}CCGG-3′ [16]. These features of the restriction enzymes have been used to assess the global DNA methylation status of DNA

* Corresponding author. Fax: +27 10 299 2316.
E-mail address: 20134045@puknet.ac.za (J.F. Wentzel).

[1] *Abbreviations used:* comet assay, single cell gel electrophoresis assay; DSB, double-strand break; SSB, single-strand break; ALS, alkali-labile site; MMC, mitomycin C; EMS, ethylmethanesulfonate; 5-Aza-dcR, **5-azacytidine;** LMPA, low-melting-point agarose; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; CEA, cytosine extension assay; dCTP, deoxycytidine triphosphate; PBS, phosphate-buffered saline; SA, succinylacetone; HTI, hereditary tyrosinemia type I; BSA, bovine serum albumin; dpm, disintegrations per minute.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2010.02.008

preparations [17,18]. We used this difference in the sensitivity to DNA methylation of the target sites of these enzymes to demonstrate that the comet assay can be modified to examine changes in the global DNA methylation status of cultured cells.

Materials and methods

HpaII and MspI were purchased from Fermentas. All other reagents were obtained from Sigma unless otherwise specified.

Cell line and culture conditions

HepG2 cells transfected with the G418-resistant tTS Neo vector (Clontech) were used in this study because they are cultured frequently in our laboratory. The cells were cultured in HyClone medium (Thermo Scientific) containing 10% fetal bovine serum, G418, penicillin/streptomycin amphotericin B (Lonza), and nonessential amino acids (Lonza). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

For azacytidine treatment, cells were seeded at 300,000 cells per 25-cm^2 flask and cultured for 48 h in normal conditions until confluent. The cells were subsequently cultured in the presence of 0.01 mM 5-azacytidine (5-Aza-dcR) for 24 h [19]. Following treatment, cells were trypsinized with 1× trypsin (Lonza) and counted after trypan blue staining.

Modified comet assay

The comet assay was performed under alkaline conditions as described previously [20]. Modifications to suit local laboratory conditions were made [21]. Harvested cells were incubated in the same medium for 2 h at 37 °C in an orbital shaker to recuperate from the harvesting process. A 50-μl aliquot of the cell sample was carefully mixed with 100 μl of 0.5% low-melting-point agarose (LMPA) followed by the application of 100 μl of this solution to a frosted glass slide that had been precoated with a thin layer of 1% high-melting-point agarose. The slides were left at room temperature for the LMPA to set. The slides were subsequently submerged in lysing solution consisting of 5 mol/L NaCl, 0.4 mol/L ethylenediaminetetraacetic acid (EDTA), 10% dimethyl sulfoxide (DMSO), and 1% Triton X-100 at 4 °C for 16 h to prepare nucleoids. To ensure favorable conditions for enzyme digestion, the slides were soaked in restriction enzyme reaction buffer (10 mmol/L Tris–HCl, 10 mmol/L NaCl, 1 mmol/L mercaptoethanol, and 2 mmol/L EDTA) for 10 min. Separate enzyme mixtures consisting of HpaII or MspI (1.5 U/100 μl) in 1× Tango buffer (Fermentas) were prepared. Each slide was then overlaid with 100 μl of one of these enzyme mixtures and covered with a coverslip, followed by incubation in a preheated damp chamber at 37 °C for 1 h. After incubation and removal of the coverslips, the slides were put into the electrophoresis tank and covered with electrophoresis buffer (5 mol/L NaOH and 0.4 mol/L EDTA). Electrophoresis took place at 30 V and 300 mA for 45 min at 4 °C, after which a pH neutralization step was performed by soaking the slides in 0.4 M Tris–HCl buffer (pH 7.5) for 15 min. Finally, the nucleoids were stained with ethidium bromide (10 μg/ml) for 1 h at 4 °C and rinsed with distilled water. The comet images were captured with an Olympus IX70 fluorescence microscope (200× magnification) and scored using Comet IV computer software (Perceptive Instruments). At least 100 comets were randomly scored per slide, and the percentage of DNA migration from the head (tail intensity) was measured for each comet scored. All experiments were performed in duplicate.

Cytosine extension assay

Genomic DNA was isolated using the DNeasy (blood and tissue) kit (Qiagen) from the remaining HepG2tTS cells that were harvested for the comet assay. The isolated DNA was subsequently separately digested with MspI and HpaII. The restriction enzyme mixture consisted of 1 μl of 1× Tango buffer (per 5 U of enzyme), 500 ng/μl DNA, and 10 U of enzyme (MspI/HpaII) in a final volume of 20 μl. The enzyme reaction was performed at 37 °C for 1 h, followed by heat inactivation (65 °C for 20 min). The cytosine extension assay (CEA) reaction mixture consisted of 5× Taq buffer, 25 mM $MgCl_2$, 5 U of GoTaq enzyme (Promega), and 0.1 μl of [^{3}H] deoxycytidine triphosphate (dCTP) (GE Healthcare) in a final volume of 15 μl [18]. Then 5 μl of the digested DNA was added to the 15 μl of the CEA reaction mixture and incubated for 1 h at 56 °C for the cytosine incorporation. The samples were transferred to Whatman DE-81 ion exchange filters and washed three times with phosphate-buffered saline (PBS). The filters were air-dried at room temperature and processed for scintillation counting. Background counts were subtracted from enzyme-treated samples, and the results were expressed as relative [^{3}H] dCTP incorporation/0.5 mg of DNA and presented as percentage change from control samples. All samples were counted twice, and the average was calculated with sigma = 2%. The values were expressed as disintegrations per minute (dpm).

Succinylacetone treatment of cells

The comet assay was applied to HepG2 cells treated with succinylacetone (SA) to determine whether this hereditary tyrosinemia type I (HTI) metabolite has any effect on the global DNA methylation [22]. Cells were seeded at 300,000 cells per well of a six-well plate and given time to adhere. The cells were subsequently cultured in the presence of 0.05 mM SA for 24 h. Following treatment, cells were trypsinized and counted, and MspI and HpaII treatment was commenced as described earlier. To examine the DNA methylation within CpG islands, we used the methylation-sensitive endonuclease NotI [21]. The slides were soaked in restriction enzyme reaction buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, and 0.1 mg/ml bovine serum albumin [BSA]) for 10 min. An enzyme mixture consisting of NotI (1.5 U/100 μl) in 1× Buffer O (Fermentas) was prepared, and 100 μl of this enzyme mixture was applied to the slides and covered with a coverslip. The comet assay was then performed as described above.

Results and discussion

The aim of this study was to determine whether the comet assay could be modified to detect the level of global DNA methylation in single cells. Cells exposed to the demethylating agent 5-Aza-dcR were used to investigate whether it was possible detect differences in the degree of global DNA methylation with the comet assay. Nucleoids prepared from cultured cells were treated with restriction enzymes having different sensitivities to methylation of CpG dinucleotides in the target sites and were analyzed with the comet assay.

The modification of the comet assay described in this study is based on the difference in sensitivity to DNA methylation of the two isoschizomeric restriction endonucleases HpaII and MspI. When these restriction enzymes are used in the comet assay, one would expect that a higher level of methylation of the CpG dinucleotides in the recognition sites would result in a larger difference in the amount of DNA in the comet tails of HpaII-digested nucleoids versus MspI-digested nucleoids. It is evident from the results given in Fig. 1 that the treatment of agarose-embedded nucleoids on the

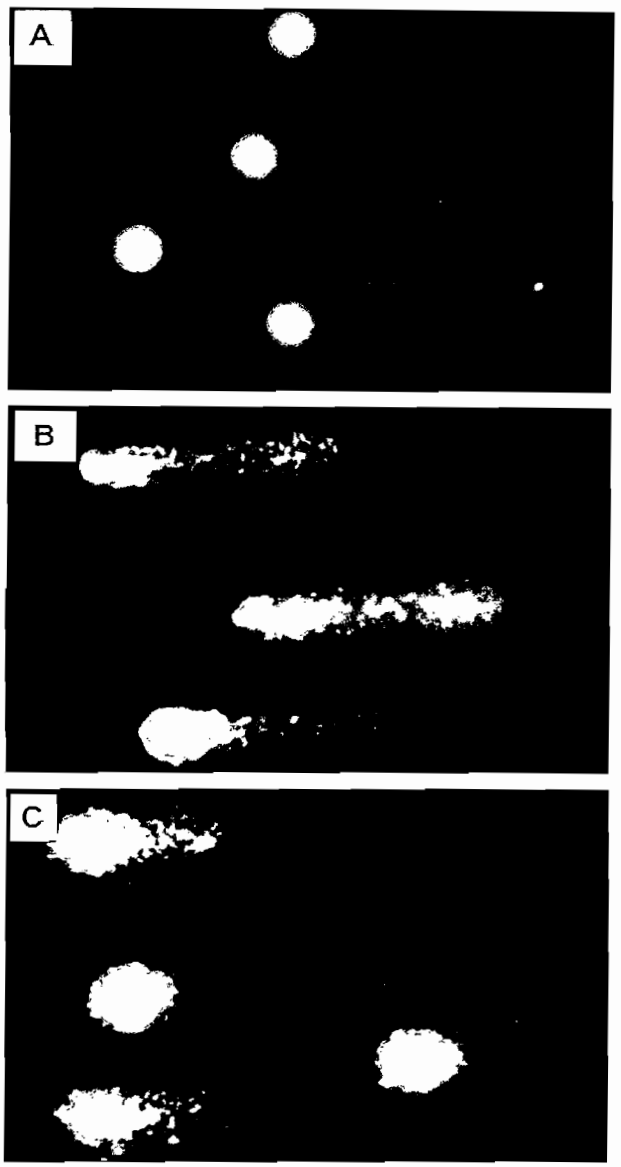

Fig. 1. Comets created by the treating nucleoids with the isoschizomeric enzymes MspI and HpaII: Nucleoids without enzyme treatment (A), with MspI treatment (B), and with HpaII treatment (C).

slides with MspI resulted in markedly more tail DNA in the comets relative to the undigested control. A significant, but smaller, increase in the tail DNA was observed following HpaII treatment. To ascertain whether differences in DNA methylation levels could be measured with the comet assay, cells were harvested following a 24-h exposure to 5-Aza-dcR, a known demethylating agent [20].

The results are expressed as percentage CpG methylation and are calculated using the ratio between the average percentage tail DNA of HpaII- and MspI-digested DNA, that is, $100 - \overline{\text{HpaII}}/\overline{\text{MspI}} \times 100$, where $\overline{\text{HpaII}}$ and $\overline{\text{MspI}}$ are the average percentage tail DNA of HpaII- and MspI-digested nucleoids, respectively. For the observed dataset, the calculated percentage CpG methylation is 61.6% for untreated cells and 44.0% for 5-Aza-dcR-treated cells. However, to ascertain the distribution properties of the percentage CpG methylation, the bootstrap can be employed. The bootstrap is a technique that can estimate population parameters and distribution properties of statistics by substituting the population mechanism used to obtain the parameter with and empirical equivalent. These estimates can be obtained analytically, but they are obtained mostly through the use of resampling and Monte Carlo methods carried out by a computer [23].

The bootstrap estimated distributions of percentage CpG methylation for both untreated and 5-Aza-dcR-treated cells are presented in Fig. 2. These results are based on a bootstrap replication number of 10,000. The 95% bootstrap percentile confidence intervals [23] for the percentage DNA CpG methylation are [58.76, 64.33] for untreated cells and [40.48, 47.48] for 5-Aza-dcR-treated cells. From these intervals, we can conclude that there exists a significant decrease in DNA methylation between the two groups studied.

Theoretically, the percentage tail DNA in the case of MspI treatment represents all of the CCGG sites in the DNA of the cells that were scored. The total global CCGG DNA methylation can then be calculated by the HpaII/MspI ratio. DNA damage prior to enzyme treatment is accounted for by subtracting the percentage tail DNA of the control sample from the values obtained for the digested sample. One should also bear in mind that the suppliers of the restriction endonucleases recommend strict guidelines for their use [16], whereas we have used them under rather robust conditions on the microscope slides in a damp sealable plastic container. In spite of this, both enzymes were active and a clear difference in percentage tail DNA was consistently observed between the control and experimental nuclei.

To validate the results obtained with the comet assay, we performed the CEA on the DNA isolated from the remaining cells of the same batch used for the comet assay (Fig. 3). The CEA is also based on the selective use of the methylation-sensitive restriction enzymes HpaII and MspI, both of which leave a 5′ guanine overhang after DNA cleavage followed by a single nucleotide extension with [^{3}H] dCTP. The level of radioactive cytosine incorporation is then determined by scintillation counting, and the absolute methylation percentage can subsequently be calculated [18].

A similar decrease in global DNA methylation (33%) as that observed with the comet assay was measured with the CEA. It should be kept in mind that the CEA measures DNA methylation of a pooled DNA sample, whereas the comet assay is used to quantify the DNA integrity of individual nucleoids. It is possible that in some cases not all of the restriction sites in the nucleoids may be accessible to the restriction enzymes leading to incomplete digestion, whereas the DNA is in solution in the CEA. Although a slightly larger decrease (29–33%) in global DNA methylation was observed with the CEA performed with DNA isolated from the same batch of cultured cells, the CEA largely supports the results obtained with the comet assay.

The slight difference in DNA methylation observed between the two assays used beckons the question of just how reliable restriction enzyme digestion is when applied in the comet assay. The concerns one may have when using restriction enzymes in the comet assay to show variation in global DNA methylation are also applicable to the use of other endonucleases in combination with the comet assay (e.g., Fpg, EndoIII). These enzymes are well established in the comet assay to detect the presence of different types of DNA adducts, thereby warranting the use of restriction enzymes as described here [14,15].

Finally, we applied the methylation-sensitive comet assay to cultured cells that were treated with the metabolite SA (Fig. 4), which accumulates in HTI, because there is some evidence indicating that HTI-accumulating metabolites may alter DNA methylation [22]. This was done to see whether we could measure regional-specific DNA methylation with the modified comet assay. The cultured cells were exposed to 50 μM SA for a period of 24 h, and the comet assay was performed. Methylated cytosines outside the CpG islands were quantified using MspI and HpaII due to their tendency

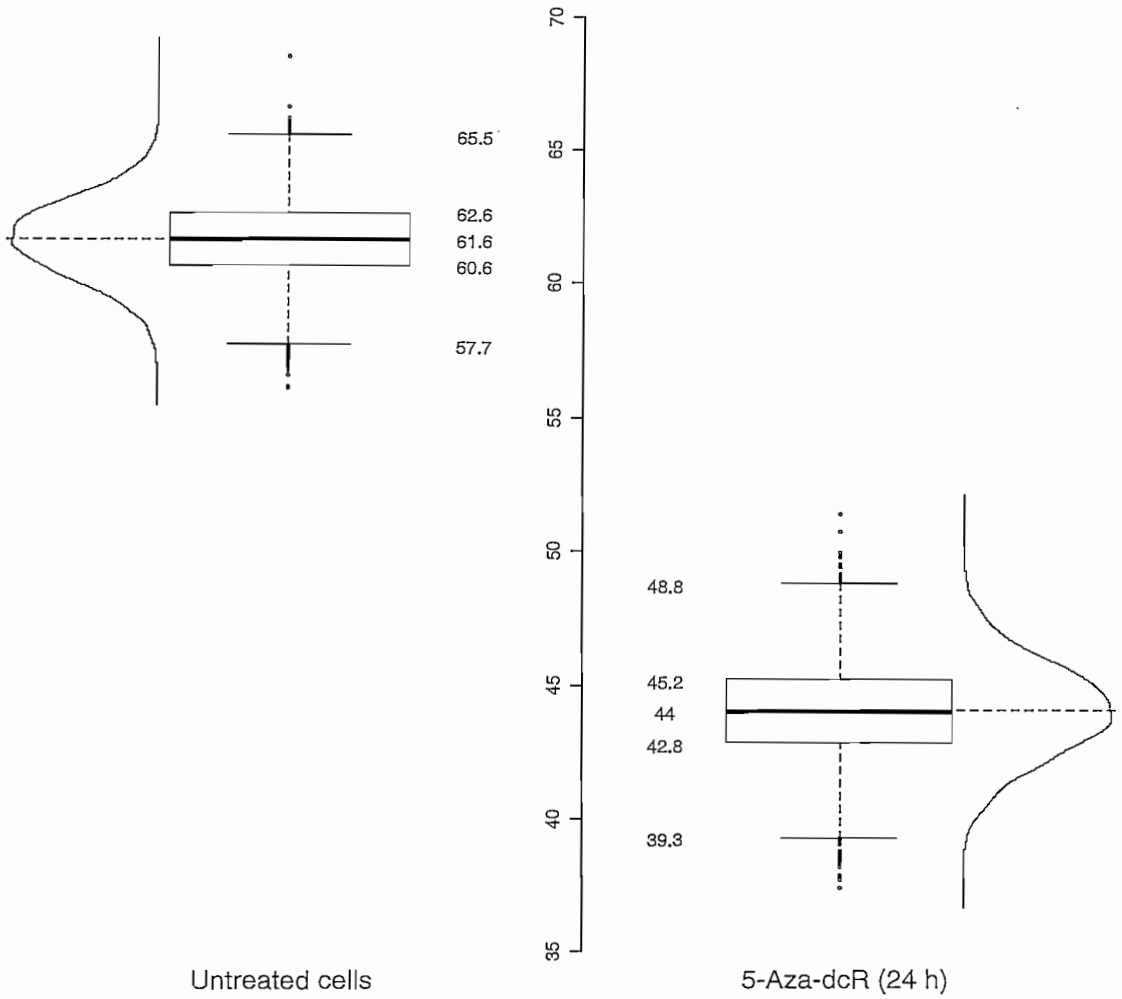

Fig. 2. Bootstrap distributions presenting the percentage CpG methylation of cells cultured in normal conditions in comparison with cells treated with the demethylation agent 5-Aza-dcR. The kernel density estimate is given next to the box plot as well as the summary statistics associated with the plot. The horizontal dotted line represents the average percentage CpG methylation obtained from the sample data.

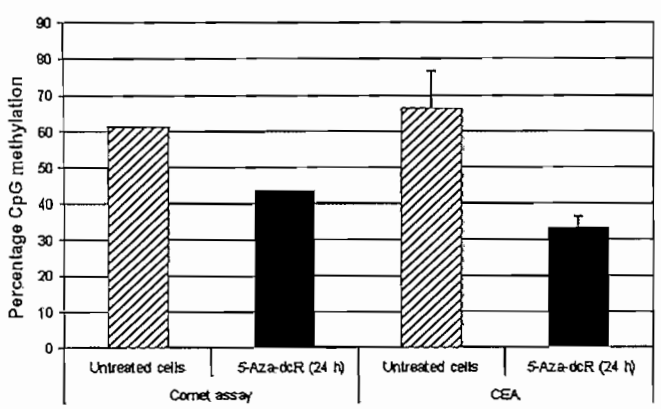

Fig. 3. Comparison between global CpG methylation of cells treated with the demethylation agent 5-Aza-dcR and cell culture in normal conditions using the comet assay and the CEA.

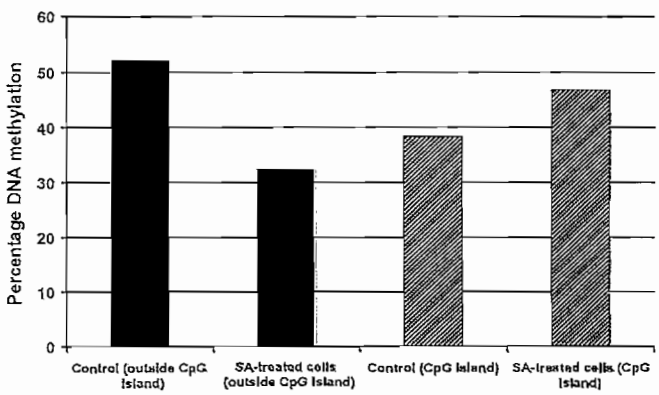

Fig. 4. DNA methylation percentage of cells treated with SA. Cells digested with MspI and HpaII give an indication of DNA methylation outside the CpG islands, whereas NotI is used to determine DNA methylation in CpG islands.

to digest mainly sequences outside the CpG islands. To examine methylation in CpG islands, we used the methylation-sensitive enzyme NotI, which has a 5′-GC^GGCCGC-3′ recognition site and acts mainly on target sites within CpG islands [22]. This enabled us to get an estimate of the percentage methylation of these CpG-rich islands: 100 − (NotI tail intensity/control tail intensity) × 100. In contrast to a notable decline in DNA methylation outside the CpG islands of approximately 20% after 24 h of exposure to SA,

an increase of approximately 8% in CpG island methylation was detected after SA treatment. This observation indicates that SA may indeed cause DNA hypermethylation in HTI, which may contribute to the initiation of hepatocarcinoma associated with this disease [22].

In conclusion, we have shown that the comet assay can be successfully modified to determine changes in the level of global and regional DNA methylation in single cells. This modification further expands the versatility of the comet assay by increasing the variety of observations that can be made in one experiment. Because DNA methylation was shown to be a tissue-specific event [18], this modification of the comet assay provides the opportunity to study the DNA methylation status of single cells that are prepared from different tissues under various physiological conditions [21].

Acknowledgment

The authors are grateful to the South African National Research Foundation (NRF) and North-West University for financial support of this project.

References

[1] A. Bird, DNA methylation Patterns and epigenetic memory, Genes Dev. 16 (2002) 6–21.
[2] R. Nag, M.J. Smerdon, Altering the chromatin landscape for nucleotide excision repair, Mutat. Res. 682 (2009) 13–20.
[3] D. Waggoner, Mechanisms of disease: epigenesis, Semin. Pediatr. Neurol. 14 (2007) 7–14.
[4] R.S. Illingworth, A.P. Bird, CpG islands: a rough guide, FEBS Lett. 583 (2009) 1713–1720.
[5] S.K. Patra, Ras regulation of DNA-methylation and cancer, Exp. Cell Res. 314 (2008) 1193–1201.
[6] B. Sadikovic, T.R. Haines, D.T. Butcher, D.I. Rodenhiser, Chemically induced DNA hypomethylation in breast carcinoma cells detected by the amplification of intermethylated sites, Breast Cancer Res. 6 (2004) 329–337.
[7] M. Szyf, Targeting DNA methylation in cancer, Ageing Res. Rev. 2 (2003) 299–328.
[8] M.J. Duffy, R. Napieralski, J.W.M. Martens, P.N. Span, F. Spyratos, F.C.G.J. Sweep, N. Brunner, J.A. Foekens, M. Schmitt, Methylated genes as new cancer biomarkers, Eur. J. Cancer 45 (2009) 335–346.
[9] S.-M. Ho, W.-Y. Tang, Techniques used in studies of epigenome dysregulation due to aberrant DNA methylation: an emphasis on fetal-based adult diseases, Reprod. Toxicol. 23 (2007) 267–282.
[10] A.R. Collins, I. Gaivão, DNA base excision repair as a biomarker in molecular epidemiology studies, Mol. Aspects Med. 28 (2004) 307–322.
[11] B. Epe, M. Pflaum, M. Häring, J. Hegler, H. Rüdiger, Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems, Toxicol. Lett. 67 (1993) 57–72.
[12] A.R. Collins, S.J. Duthie, V.L. Dobson, Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA, Carcinogenesis 14 (1993) 1733–1735.
[13] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobaysahi, Y. Miyamae, E. Rojas, J. Ryu, Y.F. Sasaki, Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.
[14] M.A. Andersson, B.E. Hellman, Different roles of *Fpg* and *EndoIII* on catechol-induced DNA damage in extended-term cultures of human lymphocytes and L5178Y mouse lymphoma cells, Toxicol. In Vitro 19 (2005) 151–158.
[15] G. Speit, M. Vasquez, A. Hartmann, The comet assay as an indicator test for germ cell genotoxicity, Exp. Cell Res. 681 (2004) 3–12.
[16] New England Biolabs Catalog and Technical Reference, New England Biolabs, Ipswich, MA, 2005–2006, pp. 266–267.
[17] H. Fujiwara, M. Ito, Nonisotopic cytosine extension assay: a highly sensitive method to evaluate CpG island methylation in the whole genome, Anal. Biochem. 307 (2002) 386–389.
[18] I. Pogribny, P. Yi, S.J. James, A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands, Biochem. Biophys. Res. Commun. 262 (1999) 624–628.
[19] H.L. Li, H.X. Wen, W.L. Wei, Effect of 5-2Aza-2′-deoxycytidine on the P16 tumor suppressor gene in hepatocellular carcinoma cell line HepG2, World J. Gastroenterol. 7 (2001) 131–135.
[20] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[21] K. Sekihahi, A. Yamumoto, Y. Matsumura, S. Ueno, M. Watanabe-Akanuma, F. Kassie, S. Knasmuller, S. Tsuda, Y.F. Sasaki, Comparative investigation of multiple organs of mice and rats in the comet assay, Mutat. Res. 517 (2002) 53–74.
[22] M.J. Fazzari, J.M. Greally, Epigenomics beyond CpG islands, Nature 5 (2004) 446–455.
[23] C. Hall, R.J. Tibshirani, An Introduction to the Bootstrap, Chapman & Hall, New York, 1992.

REFERENCES

Andersson, M.A. and B.E.Hellman. 2005. Different roles of Fpg and Endo III on catechol-induced DNA damage in extended-term cultures of human lymphocytes and L5178Y mouse lymphoma cells. *Toxicology in Vitro* 19:151-158.

Bartel, D.P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* 116(2):281-297.

Biolabs, N.E. 2005-2006. New England biolabs catalog and technical reference.266 – 267.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes & Development* 16:6-21.

Bordwell, F.G. 1988. Equilibrium acidities in dimethyl sulfoxide solution. *Chemical Research* 21:456-463.

Brenner, C., Deplus, R., Didelot, C., Loriot, A., Vire, E., De Smet, C., Gutierrez, A., Davoni, D., Bernard, D., Boon, T., Giuseppe, P.P., Amati, B., Kouzarides, T., De Launoit, Y., Di Croce, L. and Fuks, F. 2005. Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO* 24:336-346.

Chakrabarti, R. and Schutt, C.E. 2001. The enhancement of PCR amplification by low molecular-weight sulfones. *Gene* 274(1-2):293-298.

Collins, A.R., Dobson, V.L., Duinská, M. and Kennedy, G. R. 1997. The comet assay: what can it really tell us? *Mutation Research* 375(2):183-193.

Collins, A.R., Duthie, S.J.and Dobson, V.L. 1993. Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14:1733-1735.

Collins, A.R.and Gaivão, I. 2004. DNA base excision repair as a biomarker in molecular epidemiology studies. *Molecular Aspects in Medicine.* 28(3-4):307-322.

Cottrell, S.E. 2004. Molecular diagnostic applications of DNA methylation technology. *Clinical Biochemistry* 37(7):595-604.

Ding, X.C., Weiler, J. and Großhans, H. 2009. Regulating the regulators: mechanisms controlling the maturation of microRNAs. *Trends in Biotechnology* 27(1):27-36.

Dobrovic, A. and Kristensen, L.S. 2009. DNA methylation, epimutations and cancer predisposition. *The International Journal of Biochemistry & Cell Biology* 41(1):34-39.

Duffy, M.J., Napieralski, R., Martens, J.W.M., Span, P.N., Spyratos, F., Sweep, F.C.G.J., Brunner, N., Foekens, J.A. and Schmitt, M. 2009. Methylated genes as new cancer biomarkers. *European Journal of Cancer* 45(3):335-346.

Ehrlich, M. 2002. DNA methylation in cancer: too much, but also too little. *Oncogene* 21:5400-5413.

Epe, B., Pflaum, M., Häring, M., Hegler, J.a nd Rüdiger, H. 1993. Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems. *Toxicology Letters* 67(1-3):57-72.

Escargueil, A.E., Soares, D.G. Salvador, M. Larsen , A.K. and Henriques, J.A.P. 2008. What histone code for DNA repair? *Mutation Research* 658:259–270.

Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. 1995. The comet assay: a comprehensive review. *Mutation Research* 339(1):37-59.

Fan, S., Zhang, M.Q. and Zhang, X. 2008. Histone methylation marks play important roles in predicting the methylation status of CpG islands. *Biochemical and Biophysical Research Communications* 374(3):559-564.

Fan, S. and Zhang, X. 2009. CpG island methylation pattern in different human tissues and its correlation with gene expression. *Biochemical and Biophysical Research Communications* 383(4):421-425.

Franco, R., Schoneveld, O., Georgakilas, A.G. and Panayiotidis, M.I. 2008. Oxidative stress, DNA methylation and carcinogenesis. *Cancer Letters* 266(1):6-11.

Fuks, F. 2005. DNA methylation and histone modifications: teaming up to silence genes. *Current Opinion in Genetics & Development* 15(5):490-495.

Gardiner-Garden, M.A.F.1987. CpG islands in vertebrate genomes. CpG islands in vertebrate genomes. *Journal of Molecular Biology* 196:261-282.

Gopalakrishnan, S., Van Emburgh, B.O. and Robertson, K.D. 2008. DNA methylation in development and human disease. *Mutation Research* 647(1-2):30-38.

Gräff, J. and Mansuy, I.M. 2008. Epigenetic codes in cognition and behaviour. *Behavioural Brain Research* 192(1):70-87.

Guil, S.and Esteller, M. 2009. DNA methylomes, histone codes and miRNAs: Tying it all together. *The International Journal of Biochemistry & Cell Biology* 41(1):87-95.

Guyton, A.C., and Hall, J.E. 2006a. Textbook of Medical Physiology. 11th Edition. W.B. Saunders, Philadeiphia, pp. 429 – 430.

Guyton, A.C., and Hall, J.E. 2006b. Textbook of Medical Physiology. 11th Edition. W.B. Saunders, Philadeiphia, pp. 440.

Haines, T.R., Rodenhiser, D.I. and Ainsworth, P.J. 2001. Allele-Specific Non-CpG Methylation of the *Nf1* Gene during early mouse development. *Developmental Biology* 240(2):585-598.

Herman, J.G. and Baylin, S. B., DNA Methylation and Epigenetic Silencing of Genes in Cancer, in: C.R. Sciver ALB, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Diseases, eigth New York, 2001. Chapter 8

Hirst, M. and Marra, M.A. 2009. Epigenetics and human disease. *The International Journal of Biochemistry & Cell Biology* 41(1):136-146.

Ho, S.M. and Tang, W.-y. 2007. Techniques used in studies of epigenome dysregulation due to aberrant DNA methylation: An emphasis on fetal-based adult diseases. *Reproductive Toxicology* 23(3):267-282.

Illingworth, R.S. and Bird, A.P. 2009. CpG islands - `A rough guide'. *FEBS Lett.* 583(11):1713-1720.

Kalda, A., Heidmets, L.T., Shen, H.Y., Zharkovsky A. and Chen, J.F. 2007. Histone deacetylase inhibitors modulates the induction and expression of amphetamine-induced behavioral sensitization partially through an associated learning of the environment in mice. *Behavioural Brain Research* 181:76-84.

Kanai, Y., Ushijima, S., Nakanishi, Y., Sakamoto, M. and Hirohashi, S. 2002. Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. *Cancer Letters* 129:75-82.

Kangaspeska, S., Stride, B., Metivier, R., Polycarpou-Schwarz, M., Ibberson, D., Carmouche, R.P., Benes, V., Gannon, F. and Reid, G. 2008. Transient cyclical methylation of promoter DNA. *Nature* 452(7183):112-115.

Keverne, E.B. and Curley, J.P. 2008. Epigenetics, brain evolution and behaviour. *Frontiers in Neuroendocrinology* 29(3):398-412.

Klein, C.B. and Costa, M. 1997. DNA methylation and gene expression: Introduction and overview. *Mutation Research* 386(2):103-105.

Klose, R.J. and Bird, A.P. 2006. Genomic DNA methylation: the mark and its mediators. *Trends in Biochemical Sciences* 31(2):89-97.

Kondo, T., Bobek, M.P., Kuick, R., Lamb, B., Zhu, X., Narayan, A., Bourc'his, D., Viegas-Pequignot, E., Ehrlich, M. 2000. . Human Molecular Genetics 9: 597-604. 2000. Whole-genome methylation scan in ICF syndrome: Hypomethylation of non-satellite DNA repeats D4Z4 and NBL2. *Human Molecular Genetics Review* 9:597-604.

Lambert, M.P. and Herceg, Z. 2008. Epigenetics and cancer, 2nd IARC meeting, Lyon, France, 6 and 7 December 2007. *Molecular Oncology* 2(1):33-40.

Lennartsson, A. and Ekwall, K. 2009. Histone modification patterns and epigenetic codes. *Biochimica et Biophysica Acta* 1790(9):863-868.

Liu, L., van Groen, T., Kadish, I. and Tollefsbol, T.O. 2009. DNA methylation impacts on learning and memory in aging. *Neurobiology of Aging* 30(4):549-560.

Lovell, D.P. 2008. Statistical issues in the use of the comet assay. *Mutagenesis*:1-12.

Lujambio, A. and Esteller, M. 2007. CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle* 6(12):1455-1459.

Métivier, R., Gallais, R., Tiffoche, C., Le Péron C., Jurkowska, R.Z., Carmouche, R.P., Ibberson, D., Barath, P., Demay, F., Reid, G., Benes, V., Jeltsch, A.,Gannon, F. and Salbert, G . 2008. Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 452(6):45-50.

Moazed, D.N.p. 2009. Small RNAs in transcriptional gene silencing and genome defence. *Nature* 457(22):413-420.

Mohn, F.and Schübeler, D. 2009. Genetics and epigenetics: stability and plasticity during cellular differentiation. *Trends in Genetics* 25(3):129-136.

Moss, T.J. and Wallrath, L.L. 2007. Connections between epigenetic gene silencing and human disease. *Mutation Research* 618(1-2):163-174.

Nag, R. and Smerdon, M.J. 2008. Altering the chromatin landscape for nucleotide excision repair. *Mutation Research* 682(1):13-20.

Ohgane, J., Yagi, S. and Shiota, K. 2008. Epigenetics: The DNA methylation profile of tissue-dependent and differentially methylated regions in cells. *Placenta* 29(Supplement 1):29-35.

Okano, M., Bell, D.W., Haber, D.A. and Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99(3):247-257.

Patra, S.K. 2008. *Ras* regulation of DNA-methylation and cancer. *Experimental Cell Research* 314(6):1193-1201.

Progribny, I.P., James, S.J., Jernigan, S. and Pogribna, M. . 2004. Genomic hypomethylation is specific for preneoplastic liver in folate/methyl deficient rats en does not accur in non-target tissues. *Mutation Research 658* (548):53-59.

Prokhortchouk, E. and Defossez, P.-A. 2008. The cell biology of DNA methylation in mammals. *Biochimica et Biophysica Acta* 1783(11):2167-2173.

QIAGEN. 2005. Introducing QIAGEN Proteinase K- the robust proteinase. https://www.roche-applied-science.com/pack-insert/3115887a.pdf. 4 November 2009

Rojas, E., Lopez, M.C. and Valverde, M. 1999. Single cell gel electrophoresis assay: methodology and applications. *Journal of Chromatography B: Biomedical Sciences and Applications* 722(1-2):225-254.

Sawan, C., Vaissière, T., Murr, R. and Herceg, Z. 2008. Epigenetic drivers and genetic passengers on the road to cancer. *Mutation Research* 642(1-2):1-13.

Speit, G., Vasquez, M. and Hartmann, A. 2004. The comet assay as an indicator test for germ cell genotoxicity. *Experimental Cell Research.* 681(1):3-12.

Spencer, H.G. 2000. Population genetics and evolution of genomic imprinting. *Annual Research in Genetics* 34:457-477.

Sweatt, J.D. 2009. Experience-dependent epigenetic modifications in the central nervous system. *Biological Psychiatry* 65(3):191-197.

Szyf, M. 2003. Targeting DNA methylation in cancer. *Ageing Research Reviews* 2(3):299-328.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobaysahi, H., Miyamae, Y., Rojas, E., Ryu, J. and Sasaki, Y.F. 2000. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environmental Molecular Mutagenics* 35:206-221.

Vaissière, T., Sawan, C. and Herceg, Z. 2008. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutation Research* 659:40-48.

Waddington, C.H. 1939. Preliminary notes on the development of the wings in normal and mutant strains of *drosophila. Proceedings of the National Academy of Sciences* 25:299-307.

Waggoner, D. 2007. Mechanisms of Disease: Epigenesis. *Seminars in Pediatric Neurology* 14(1):7-14.

Wilson, A.S., Power, B.E. and Molloy, P.L. 2007. DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1775(1):138-162.