Arzu EROL

M.S. Thesis In Biology

August 2009

GENOMIC INSTABILITY EVALUATION IN DIFFERENT CELL-LINES BY RANDOM AMPLIFIED POLYMORPHIC DNA-PCR ANALYSIS

by

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Arzu EROL

A thesis submitted to

the Graduate Institute of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

August 2009
Istanbul Turkey

APPROVAL

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Assist. Prof. M. Serdal SAKÇALI
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assist. Prof. İ. İrem UZONUR
Supervisor

Assist. Prof. Sevim IŞIK
Coadviser

Examining Committee Members

Assist. Prof. Irem İ.UZONUR ………………………

Assist. Prof. Sevim IŞIK ………………………

Prof. Dr. Fahrettin GÜCİN ………………………

Assist. Prof. M. Fatih ABASIYANIK ………………………

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate Institute of Sciences and Engineering.

Assoc. Prof. Dr. Nurullah ARSLAN
Director

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Thesis Supervisor: Assist. Prof. İ. İrem UZONUR

ABSTRACT

Cancer results from a disordered and unstable genome. Such genomic instability appears to be subject to control by environmental factors as evidenced by the number of cancers that are either caused by specific environmental agents. Dietary factors might interact in several ways with the genome to protect against cancer.

An agent might interact directly with the genome and regulate expression (as a genetic or epigenetic regulator) or indirectly by influencing DNA repair responses and so improve genomic stability.

The development of widely applicable methods to monitor genomic instability gains importance nowadays. RAPD-PCR assay is a molecular method able to detect comparative DNA changes. This work is to show the applicability of the method to evaluate the ultimate changes caused in various cell-culture derived DNA which might be a model for carcinogenesis, genomic instability and routine cell-culture work.

Keywords: Genomic Instability, RADP-PCR, HEK cells, HUVEC cells, HELA cells, hMSC.

DEĞİŞİK HÜCRE HATLARINDA GENOMİK KARARSIZLIĞIN, RAPD-PZR ANALİZİ İLE DEĞERLENDİRİLMESİ

Arzu EROL

Yüksek Lisans Tezi: Biyoloji

Tez Yöneticisi: Yrd. Doç. Dr. İ. İrem Uzonur

ÖZ

Genom kararsızlığı başta kanser olmak üzere özellikle yaşlanma ile ilgili çeşitli hastalıklarda gözlenmektedir. Genomik kararsızlık farklı çevresel faktörlerin ve çevresel ajanların etki etmesi sonucu ortaya çıkan bir durumdur. Beslenme çeşitli şekillerde genom yapısını kansere karşı koruyucu etki sağlayacak şekilde tetiklemektedir. Gen ekspresyonu (genetik veya epigenetik düzenlemeler), doğrudan ya da dolaylı olarak DNA onarımı ve bunlara bağlı genom kararlılığı zamanla değişebilmektedir.

Genomik kararsızlığı tespit etmede çeşitli metotlar kullanılmaktadır. DNA'daki değişimi gözlememizi sağlayan bu metotlardan biri de RAPD-PCR analizidir. Bu çalışmada, çeşitli hücre hatlarınınn pasajlanmasıyla elde edilen genomik DNA'lar kullanılarak, oluşması beklenen değişimlerin tespiti RAPD profil değişimi olarak tespit edilmiştir. Bu değişimlerin mahiyeti: DNA hasarı ve/veya mutasyon olarak tespite çalışılmıştır.

Anahtar Kelimeler: Genomik karasızlık, RAPD-PZR, HEK hücreleri, HUVEC hücreleri, HELA hücreleri, insan mezenkimal kök hücreleri hMSC

Dedicated to my parents Mehmet EROL, Bahriye EROL, my brothers Kemal EROL, Senol EROL and my best friend Beyza S. GÖNCÜ.

ACKNOWLEDGEMENT

I express my sincere appreciation to my advisor Assist. Prof. Dr. İ. İrem UZONUR and co-advisor Assist. Prof. Dr.Sevim Işık for their guidance and insight throughout my research.

I express my thanks and appreciation to my family for their understanding, motivation and patience. The last, but not the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

TABLE OF CONTENTS

ABSTRACT.. iv
ÖZ.. v
DEDICATION... vi
ACKNOWLEDGMENT... vii
TABLE OF CONTENTS... viii
LIST OF TABLES... ix
LIST OF FIGURES.. x
LIST OF SYMBOLS AND ABBREVIATIONS.. xii
CHAPTER 1: NTRODUCTİON.. 1
1. Introduction……………………………………..………………………….............. 1
1.1. Genome………………………………..…………………………………….... 1
1.1.1. Nuclear DNA……………………………………………………………..... 1
1.1.2. Organelle DNA……………………...………………….......................... 2
1.2. Genome stability.. 2
1.3. Genomic stabililty protection mechanisms during replication…............................... 3
1.4. The mechanisms of cell cycle regulation and checkpoint control...……………… 3
1.5. Mechanisms of DNA repair……………………………………………………… 4
1.5.1. Mismatch Repair………………………………………………….............. 4
1.5.2. Base Excision Repair………………………………………………............ 5
1.5.3. Nucleotide Excision Repair……………………………………….............. 5
1.5.4. Double-strand Break Repair……………………………………………….. 6
1.6. Genomic Instability and DNA damage risk factors... 6
1.7. Epigenetic regulation of gene expression... 9
1.8. DNA Damage Quantification Techniques... 9
1.8.1. Comet Assay………………………………………………………………….. 10
1.8.2. Dicentric Chromosome Aberration Assay………………………………………. 11
1.8.3. Micronuclei Assay……………………………………………………………… 11
1.8.4. Translocation Frequency using FISH Technique……………………………….. 12
1.8.5. HPRT Mutant Frequency……………………………………………………... 13
1.8.6. Random Amplification of Polymorphic DNA-PCR…………………………….. 14
1.9. Cell Culture…………………………………………………………………… 16
CHAPTER 2: MATERIALS AND METHODS……………………………………... 18
2.1. Materials…………………………………………………………………….... 18
2.1.1. General Reagents…………………………………………………............ 18
2.1.2. Equipment…………………………………………………………………….. 18
2.1.3. Cell Culture.. 19
2.1.3.1. Cell types………………………………………………………………… 19
2.1.3.1. Cell culture chemicals and instruments……………………………………… 21
2.1.4. DNA Isolation and Quantification.. 21
2.1.4.1. DNA isolation Kits.. 21
2.1.4.2. DNA quantification Kits.. 21
2.1.5. PCR.. 22
2.1.5.1. Oligonucleotide primers... 22
2.1.5.2. PCR Chemicals and consumables... 22

2.1.6. Electrophoresis and Documentation.. 23
2.1.6.1. Agarose gel electrophoresis chemicals, buffers and instruments……………. 23
2.2. Methods.. 24
2.2.1. Cell Culture Techniques... 24
2.2.1.1. Growth Conditions………………………………………………………... 24
2.2.1.2. Thawing Cell Lines……………………………………………………….. 24
2.2.1.3. Sub-Culture of Cell Lines………………………………………………… 24
2.2.1.4. Cryopreservation of Cell Lines……………………………………............. 25
2.2.2. DNA Isolation... 25
2.2.3. DNA Quantification.. 26
2.2.4. RAPD-PCR Assay.. 27
2.2.5. Agarose Gel Electrophoresis.. 29
CHAPTER 3: RESULTS…………………….. 31
3.1. Morphological Changes Among Cell Subcultures In Different Cell Lines Under Inverted Phase Contrast Microscope………………………………………………… 31
3.2. Cell Quantification……………………………………………………………………. 33
3.3. Growth Curve Analysıs ………………………………………………………………. 34
3.4. DNA Quality And Quantity Assessments ……………………………………………. 36
3.5. RAPD-PCR Results …………………………………………………………………. 38
3.6. Primer Optimizations ..………………………………………………………………. 38
3.7. Repeatability And Reproducibility of Profiles ……………………………………. 41
3.8. RAPD Profiles of Each Cell Type and Subculture of Them……………………….. 42
3.9. Modified RAPD-PCR Results………………………………………………………. 44
3.10. RAPD to Detect DNA Damage and Genotoxicity………………………………… 46
CHAPTER 4: DISCUSSION…………………….. 49
CHAPTER 5: CONCLUSION...……………….. 63
REFERENCES………………………………………………………………………... 64

SAMPLE LIST OF TABLES

LIST OF TABLES

TABLE

1.1.Gene families likely or known to contain members contributing to genomic instability in human cancers………………………………………………………. 3
2.1. List of equipments and their brands and models……………….. 18
2.2. Human Embryonic Kidney 293 cells' (HEK) Information…………………….. 19
2.3. Human Umbilical Vein Endothelial Cells' (HUVEC) information……………. 20
2.4. Human epithelial carcinoma cell line (HELA) information…………………… 20
2.5. Base sequences of 10-mer primers.. 22
2.6. PCR kit components ………………………………………………………. 22
2.7. Agarose Gel Electrophoresis Chemicals, Buffers ....…………… 23
2.8. DNA Quantification Kit ingredients and amounts... 26
2.9. RAPD-PCR Solutions……………………………………………………......... 27
3.1. Growth curves for HEK, HUVEC, HELA cells……………………………….. 35
3.2. Information for the sample cell types, subculture names, counts prior to DNA extraction and DNA concentrations…………………………………………………. 36
3.3. Primer choice results for all cell types ……………………………………….. 40

SAMPLE LIST OF FIGURES

LIST OF FIGURES

FIGURE

1.1. Spectrum of DNA damage induced by physical and chemical agents………....... 8
1.2. DNA Damage and Quantification Techniques………………………………….. 10
1.3. Random Amplification of Polymorphic DNA-PCR……………………………….. 15
2.1. RAPD-PCR procedure…………………………………………………………... 28
2.2. RAMD-PCR procedure………………………………………………………….. 29
3.1.The comparative morphological appearances of the first and 23rd passages of HEK, HUVEC and HELA cells……………………………………………………… 32
3.2. Different DNA concentration for HUVEC……………………………………… 37
3.3. HEK primer gel experiment results…………………………………………….. 40
3.4 Detection of the first RAPD profiles in HEK, HUVEC, HELA and hMSCs with OPA7………………………………………………………………………………… 42
3.5. Comparative analysis of initial and final bands RAPD profiles produced using OPB7 primer for all cell types……………………………………………............... 43
3.6.a. Modified RAPD PCR results for HUVEC, HEK, HELA…............................... 43
3.6.a. Modified RAPD PCR results for HUVEC,HEK, HELA………………………… 44
3.7. Modified RAPD PCR results for Mesenchymal………………………………… 45
3.8. Glutaraldehyde's DNA effects have been assessed using RAPD assay and by cell cultures…………………………………………………………………………….. 47
3.9. Modified RAPD-PCR results for x8 %2 agarose gel of glutaraldehyde treated cells and DNA of harvested cells after removal of glutaraldehyde…………………….. 48
4.1. Genetic diversity as observed with OPB7 primer with different human cell types... 53
4.2. RAPD profiles with OPB7 primer for all cell types' all subculture DNA extracted. 56
4.3. Direct effects of DNA damage and mutation on RAPD profiles…………………... 57
4.4. An indication with arrows and boxes for the passages and RAPD profiles at which the most prominent changes first started for each cell type…………………………….. 57
4.5. Comparable picture of the first and last passages RAPD profiles, circles indicating a pattern homogenization in the last passages no matter what the cell type was……….. 58
4.6. RAPD profiles as detected with x8 RAMD using HEK 18th passage DNA, arrow indicates DNA instability caught either DNA damage or mutation……………………. 59
4.7. x8 RAMD-PCR profiles for starting and ending passages of HUVEC and HEK cells. Arrows indicating various profile changes that might be mutations…………….. 59
4.8. Two primer OPB8 and OPB7 x3 RAMD trials for the hMSC same passages' DNAs (1-7)…………………………………...………………………………… 60
4.9. Gluteraldehyde exposed DNA x8 RAMD profiles and after removal of gluteraldehyde…………………………………………………………………...... 61
4.10. A closer look at the types of changes in the reverted profiles still having changes indicated by arrows……………………………………………………………... 62

LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

DNA	Deoxyribonucleic acid
AP	Apurinic and Apyrimidinic sites
NER	Nucleotide excision repair
ds breaks	Double-strand breaks
HR	Homologous recombination
NHEJ	Non-homologous end joining
SSBR	Single-strand breaks
UV	Ultraviolet
FISH	Fluorescence in situ hybridization
HPRT	Hypoxanthine guanine phospho ribosyltransferase
RAPD	sRandom Amplification of Polymorphic DNA
RFLPs	Restriction fragment length polymorphism
hMSC	Human Mesenchymal Stem Cell
HEK	Human Embryonic Kidney C*ells*
HUVEC	Human Umbilical Vein Endothelial Cells
HELA	Human Cervical Carcinoma Cells

CHAPTER 1

1. INTRODUCTION

1.1. Genome

Every organism, including humans has a genome that contains all of the biological information needed to maintain a living example of that organism.

The biological information contained in a genome is encoded in its deoxyribonucleic acid (DNA) and is divided into discrete units called genes. Genes code for proteins by a series of reactions called gene expression.

1.1.1. Nuclear DNA

There is a nucleus inside each cell, a membrane-bounded region that provides a safe place for genetic information.

A DNA chain is made up of four chemical bases: adenine (A) and guanine (G), which are called purines, and cytosine (C) and thymine (T), referred to as pyrimidines. Each base has a slightly different composition, or combination of oxygen, carbon, nitrogen, and hydrogen. Every base in a DNA chain is attached to a sugar molecule (deoxyribose) and a phosphate molecule, resulting in a nucleic acid or nucleotide. Individual nucleotides are linked through the phosphate group, and it is the precise order, or sequence, of nucleotides that determines the product made from that gene (Calladine et al., 2003).

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1.1.2. Organelle DNA

Genetic information is not found only in nuclear DNA. Both plants and animals have an organelle called the mitochondrion. Each mitochondrion has its own set of genes. Plants also have a second organelle, the chloroplast, which also has its own DNA.

1.2.Genome Stability

Cancer results from a disordered and unstable genome. Such genomic instability appears to be subject to control by environmental factors as evidenced by the number of cancers that are either caused by specific environmental agents. Dietary factors might interact in several ways with the genome to protect against cancer. An agent might interact directly with the genome and regulate expression (as a genetic or epigenetic regulator) or indirectly by influencing DNA repair responses and so improve genomic stability (Boccia et al., 2007)

Preserving genomic integrity is obviously important, as manifested by our genome's investment of some 250 genes for purposes of DNA damage repair, more than 230 genes for high-fidelity DNA replication, and perhaps more than 500 for chromosome segregation, cell cycle checkpoints, telomeres, centromeres, damage sensing and the like (Burhansand et al., 2007) given in Table 1.1.

Table 1.1: Gene families likely or known to contain members contributing to genomic instability in human cancers

Number of known human genes families*

DNA repair	408
DNA replication	473
Chromosome segregation	344
DNA damage	575
Cell cycle checkpoint	227
DNAse	54
Recombinase	15

*Data are from the OMIM database 2009. Some overlap exists between families, although the potential number of genes involved in genomic instability remains relatively large.

1.3. Genomic Stability Protection Mechanisms During Replication

Endogenous and exogenous DNA damaging agents are constantly challenging the integrity of the genome. If DNA damage is repaired incorrectly, it can lead to genome instability, which is associated with tumor genesis in human. Eukaryotic organisms have evolved several repair and surveillance mechanisms that remove DNA damage and coordinate cell cycle progression.

1.4. The Mechanisms of Cell Cycle Regulation and Checkpoint Control

The cell cycle, or cell-division cycle, is the series of events that occur in a cell leading to its division and replication.

This cycle includes accurate duplication of the genome during the DNA synthesis phase (S phase), and segregation of complete sets of chromosomes to each of the daughter cells in M phase. The somatic cell cycle also contains "Gap” phases, known as G1, which connects the completion of M phase to initiation of S phase in the next cycle, and G2, which separates the S and M phases. Dependent on environmental and

developmental signals, cells in G1 may temporarily or permanently leave the cell cycle and enter a inactive or arrested phase known as G0 (Smith et al., 1973).

The cell cycle proceeds by a defined sequence of events where late events depend upon completion of early events (Hartwell et al., 1989). The aim of the dependency of events is to distribute complete and accurate replicas of the genome to daughter cells (Garrett, 2001). To monitor this dependency, cells are equipped with the checkpoints that are set at various stages of the cell cycle. When cells have DNA damages that have to be repaired, cells activate DNA damage checkpoint that arrests cell cycle.

According to the cell cycle stages, DNA damage checkpoints are classified into at least three checkpoints: G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint. Upon perturbation of DNA replication by drugs that interfere with DNA synthesis, DNA lesions, or obstacles on DNA, cells activate DNA replication checkpoint that arrests cell cycle at G2/M transition until DNA replication is complete.

1.5. Mechanisms of DNA Repair

Although it might seem that direct reversal of damage would be the simplest way to correct the damage, in most cases the reverse reaction is not possible due to the thermodynamic or kinetic reasons. In a few cases, the reaction is reversible, and in some of these cases mechanisms have been developed to take advantage of that reversibility.

Although all cells possess a large number of different types of repair system, each relatively specific for a certain type of DNA damage, those repair systems can be grouped into four major categories: Mismatch Repair, Base Excision Repair, Nucleotide Excision Repair, Double-strand Break Repair (Sancar et al., 2004).

1.5.1.Mismatch Repair

The mechanism of mismatch repair was first thoroughly studied in E. coli. As implied_most mismatches are due to replication errors. However, mismatches can also be produced by other mechanisms, for example, by deamination of 5-methyl cytosine to

produce thymidine improperly paired to G. Regardless of the mechanism by which they are produced; mismatches can always be repaired by the mismatch repair pathway. In cases where the appropriate DNA-N-glycosylase is available, mismatches can also be repaired by the base excision repair pathway (Sancar et al., 2004).

1.5.2.Base Excision Repair

The "pathway" most commonly employed to remove incorrect bases (like uracil) or damaged bases (like 3-methyladenine) is called "base excision repair". Actually, it's misleading to talk about this as a pathway, because there are numerous variations, each specific for a different type of incorrect base (Yoshida et al., 2004).

Nevertheless, all of the variant pathways have features in common, and each of the pathways can be considered to consist of three steps, with steps two and three being common for all pathways:

1. Removal of the incorrect base by an appropriate DNA N-glycosylase to create an AP site.
2. Nicking of the damaged DNA strand by AP endonuclease upstream of the AP site, thus creating a 3'-OH terminus adjacent to the AP site.
3. Extension of the 3'-OH terminus by a DNA polymerase, accompanied by excision of the AP site.

1.5.3.Nucleotide Excision Repair

Although base excision repair is certainly important, it is insufficient to deal with all types of damage. There must be a DNA glycosylase capable of recognizing a specific damage to be corrected by base excision repair. The huge variety of DNA-reactive chemicals in our environment combined with various alterations that can be produced by radiation and by oxidative and free radical attack on DNA can generate so many types of damage that coping with all types of damage by development of damage-specific DNA glycosylases would be difficult if not impossible. Fortunately, a more flexible damage repair mechanism is present in living organisms, nucleotide excision repair (NER), which

recognizes damaged regions based on their abnormal structure as well as on their abnormal chemistry, then excises and replaces them (Sancar et al., 2004).

1.5.4. Double-Strand Break Repair

Double-strand breaks are repaired by two different types of mechanisms. The first type uses proteins that promote homologous recombination (HR) to obtain instructions from the sister or homologous chromosome for proper repair of breaks. The other type permits joining of ends even if there is no sequence similarity between them. This process is called non-homologous end joining (NHEJ). The process by which complex single-strand breaks (those that cannot be directly relegated) are repaired (SSBR) in some ways resembles NHEJ (Wood, 1997).

1.6. Genomic Instability, DNA Damage Risk Factors

The integrity of the genome of all living organisms is constantly threatened by exogenous and endogenous DNA-damaging agents. Exogenous DNA-damaging agents include physical agents, such as X-rays, oxidative stress, ultraviolet (UV) or ionizing radiation, and a wide variety of chemical agents, such as components of cigarette smoke, PAHs, numerous chemicals induce a wide variety of lesions in DNA. Obviously, this affects proper functioning of the DNA and can lead to cell death, cancer, inborn disorders, and overall functional decline contributing to aging. To counteract the gradual erosion of the vital genetic information and prevent its important detrimental consequences a complicated network of genome care-taking and protection systems has evolved. DNA repair pathways and cell cycle control mechanisms constitute an important component of this genome protection network.

DNA errors can take two forms: mutation and DNA damage. DNA damage tends to interfere with gene expression by preventing transcription of RNA from DNA, whereas mutation usually results in transcription that usually produces proteins with diminished or altered functionality. Mutations that are not lethal to a cell are more likely to be perpetuated in dividing cells. DNA damage rather than DNA mutation is posited as a cause of aging and cancer (Lengauer et al., 1998).

It is important to distinguish between DNA damage and mutation, the two major types of error in DNA. DNA damages and mutation are fundamentally different. Damages are physical abnormalities in the DNA, such as single and double strand breaks, 8-hydroxydeoxyguanosine residues and polycyclic aromatic hydrocarbon adducts (Wood 1996). DNA damages can be recognized by enzymes, and thus they can be correctly repaired if redundant information, such as the undamaged sequence in the complementary DNA strand or in a homologous chromosome, is available for copying.

If a cell retains DNA damage, transcription of a gene can be prevented and thus translation into a protein will also be blocked. Replication may also be blocked and/or the cell may die. In contrast to DNA damage, a mutation is a change in the base sequence of the DNA (Altonen et al.,1994).

A mutation cannot be recognized by enzymes once the base change is present in both DNA strands, and thus a mutation cannot be repaired. At the cellular level, mutations can cause alterations in protein function and regulation. Mutations are replicated when the cell replicates (Markowitz et al., 1995).

In a population of cells, mutant cells will increase or decrease in frequency according to the effects of the mutation on the ability of the cell to survive and reproduce. Although distinctly different from each other, DNA damages and mutations are related because DNA damages often cause errors of DNA synthesis during replication or repair and these errors are a major source of mutation (Sancar et al., 2004)

Given these properties of DNA damage and mutation, it can be seen that DNA damages are a special problem in non-dividing or slowly dividing cells, where unrepaired damages will tend to accumulate over time.

On the other hand, in rapidly dividing cells, unrepaired DNA damages that do not kill the cell by blocking replication will tend to cause replication errors and thus mutation.

The great majority of mutations that are not neutral in their effect are harmful to a cell's survival. Thus, in a population of cells comprising a tissue with replicating cells, mutant cells will tend to be lost.

However infrequent mutations that provide a survival advantage will tend to clonally expand at the expense of neighboring cells in the tissue. This advantage to the cell is disadvantageous to the whole organism, because such mutant cells can give rise to cancer.

Thus DNA damages in frequently dividing cells, because they give rise to mutations, are a prominent cause of cancer. In contrast, DNA damages in infrequently dividing cells are likely a prominent cause of aging (Lengauer et al., 1998).

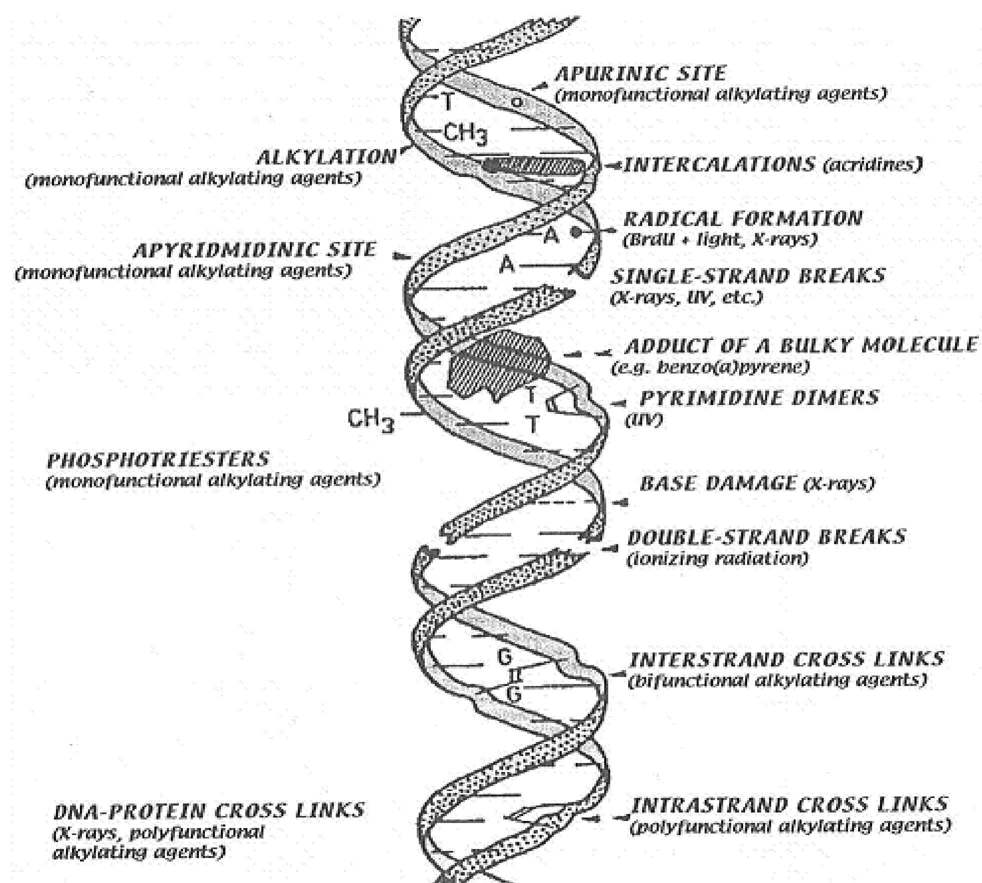


Figure1.1: Spectrum of DNA damage induced by physical and chemical agents.
(Casarett et al., 2001)

1.7. Epigenetic Regulation of Gene Expression

Cells of a multicellular organism are genetically homogeneous but structurally and functionally heterogeneous owing to the differential expression of genes. Many of these differences in gene expression arise during development and are subsequently retained through mitosis (Bird, 2007). Stable alterations of this kind are said to be “epigenetic”, because they are heritable in the short term but do not involve mutations of the DNA itself. Research over the past few years has focused on two molecular mechanisms that mediate epigenetic phenomena: DNA methylation and histone modifications (Dodd et al., 2007) Epigenetic effects by means of DNA methylation have an important role in development but can also arise stochastically as animals age. Identification of proteins that mediate these effects has provided insight into this complex process and diseases that occur when it is perturbed (Wolf, 2007). External influences on epigenetic processes are seen in the effects of diet on long-term diseases such as cancer.

Thus, epigenetic mechanisms seem to allow an organism to respond to the environment through changes in gene expression. The extent to which environmental effects can provoke epigenetic responses represents an exciting area of future research.

1.8. DNA Damage Quantification Techniques

Oxidative stress, radiation and other external insults have been shown to damage DNA molecules in cells derived from organisms as diverse as bacteria, yeast, drosophila, rodents and man (Kirsch-Volders et al. 2003). The presence of DNA damage may lead to cell cycle checkpoint arrest to allow time for DNA repair processes to occur. If however, the system becomes overwhelmed or the DNA repair mechanisms are impaired, the cell either enters the apoptosis pathway or become cancerous due to the accumulation of mutations resulting from replication of damaged DNA (Figure 1.2).

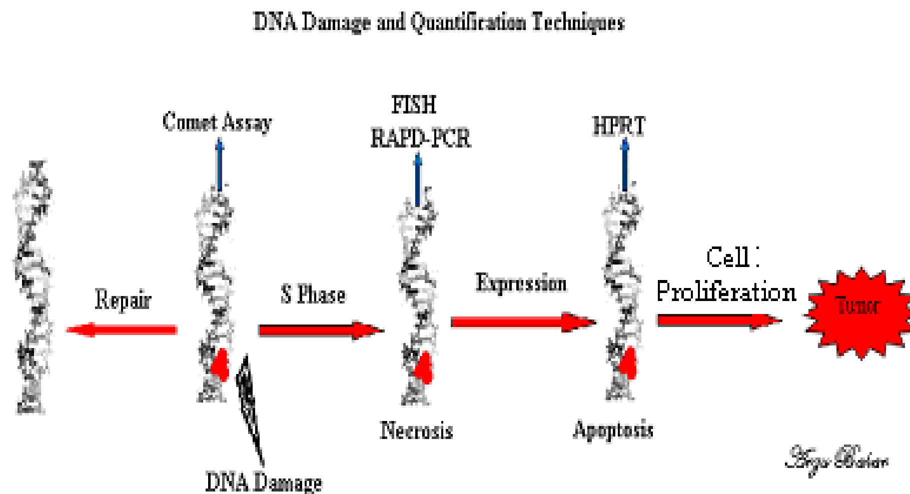


Figure 1.2: DNA Damage and Quantification Techniques

Therefore, a complete understanding of DNA repair mechanisms is of great interest in the study of cancer prevention and treatment. In addition, this process has been implicated in cellular senescence and aging (Kirsch-Volders et al. 2003).

1.8.1.Comet Assay

The Single Cell Gel Electrophoresis assay (also known as comet assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It has since gained in popularity as a standard technique for evaluation of DNA damage and repair, biomonitoring and genotoxicity testing. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline conditions, and electrophoresis of the suspended lysed cells (Klaude et al., 1996).

This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. This can be performed by manual scoring or automatically by an imaging software (Ollins et al., 1997).

The main advantages of the Comet Assay include: The collection of data at the level of the individual cell, allowing more robust statistical analyses; the need for a small number of cells per sample (<10,000); sensitivity for detecting DNA damage; and use of any eukaryote single cell population both in vitro and in vivo, including cells obtained from exposed human populations and aquatic organisms for eco-genotoxicological studies and environmental monitoring (Klaude et al., 1996).

1.8.2. Dicentric Chromosome Aberration Assay

When gamma radiation passes through the body cells it interacts with the various organelles present in the cell. Each cell has a nucleus. Chromosomes are present in the nucleus of the cell. The interaction of radiation with these chromosomes causes breaks in these chromosomes (Lloyd, 1984) .Generally the broken pieces of the individual chromosomes join back and revert to the original position. In the event of higher exposure to radiation such breaks may occur in more than one chromosome. During such period there is chance for misrepair, when the broken end of one chromosome may join with a broken end of another, resulting in the formation of a dicentric chromosome. A dicentric chromosome is one that contains two centromeres instead of one normally present in a single chromosome. The formation of dicentric chromosome is highly specific to ionizing radiation and its frequency serves as a measure of radiation exposure received by a person (Bender et al., 1988)

1.8.3. Micronuclei Assay

This assay is used when a large number of people happen to be exposed as in a disastrous situation as for instance an atom bomb is dropped as it was done at Hiroshima and Nagasaki in 1945. This assay is used as a screening procedure when a large number of people happen to be exposed as scoring of micronuclei under the microscope is much easier compared to scoring dicentric chromosomes. This assay is however comparatively less sensitive than dicentric measurement (Aardema et al., 2005)

Micronucleus is also induced by radiation exposure. The interaction of radiation with chromosomes will result not only in the formation of dicentric chromosomes but also acentric fragments. Since acentric fragments do not have a centromere, they are not pulled towards the daughter nuclei at the time of nuclear division. These acentric fragments are left in the cytoplasm which appear as micronuclei. The frequency of micronuclei provides an estimation of radiation exposure (Fenech, 2000)

1.8.4. Translocation Frequency Using FISH Technique

Fluorescence in situ hybridization (FISH) has found widespread application in the analysis of chromosomes and interphase nuclei fixed on slides (Anastasi et al., 1990). However, hybridization of specific DNA probes to isolated metaphase chromosomes in suspension offers a new approach to chromosome analysis and chromosome separation. So far the technique for FISH in suspension has been a modification of FISH techniques used for metaphase chromosomes and interphase nuclei fixed on slides. Formamide (and to some extent dextran sulfate) are obligatory components of this method. Thus, the technique requires a certain number of washing steps after hybridization. The washing steps for FISH in suspension, however, are based on centrifugal steps. These steps are responsible for a considerable reduction in the final amount of chromosomal material (Hausmann et al. 1991).

Fluorescence in situ hybridization has many advantages over conventional laboratory cultivation techniques. This method allows for the in situ localization and the study of spatial organization of cells as they occur in their natural habitat. This is important for studying the actual composition of a natural microbial community. FISH also allows for the detection of one to three orders of magnitude more cells in samples. On top of that, this method requires no cultivation of cells before analysis. Another added advantage is that for FISH, cells need not be alive. The intensity of the fluorescence is a

direct measure for the activity of the cells themselves. Inactive cells can be recognized by their low intensity fluorescence (Pinkel et al., 1988).

There are, of course, some disadvantages of fluorescence in situ hybridization. The process of preparing probes is complex due to the fact that it is necessary to tailor the probes to identify specific sequences of DNA. Also, it is difficult to count total numbers in probe-stained clusters of cells (Tanke et al., 1995).

1.8.5. HPRT Mutant Frequency

In recent years the measurement of radiation induced mutations is gaining importance from radiation protection point of view. This is because of the fact that mutations are now considered to be primary causes of cancer initiation. The estimation of mutant frequency in the reporter gene called hypoxanthine guanine phospho ribosyltransferase (HPRT) located on X chromosome can provide information on absorbed dose in accidental and occupational exposures and also serve as a parameter in determining risk factors in Radiation Protection. This feature makes it a useful biodosimetry tool and a good biomarker of exposure and effect (Rosa et al., 2007).

In order to measure mutant frequency at the HPRT locus, a T lymphocyte cloning assay has been established. This involves cloning (cloning here refers to growing a large number of cells from a single mutant cell) of human peripheral blood lymphocytes in a suitable medium in cell culture plates. Mutant cells are identified based on their ability to divide and form colonies in the presence of a toxic analogue introduced in to the medium (Sai-Mei Hou et al., 1999).

1.8.6. Random Amplification of Polymorphic DNA-PCR

RAPD stands for Random Amplification of Polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction (Atienzar et al., 2006).

No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared. Due to the fact that it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD is used to characterize, and trace, the phylogeny of diverse plant and animal species (Atienzar et al., 2005). Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains.

The above methods for utilizing RAPDs have played a very important role in the selection process for desired genotypic characteristics. Leading to the production of species-specific, genome specific and chromosome specific markers (Figure 1.3).

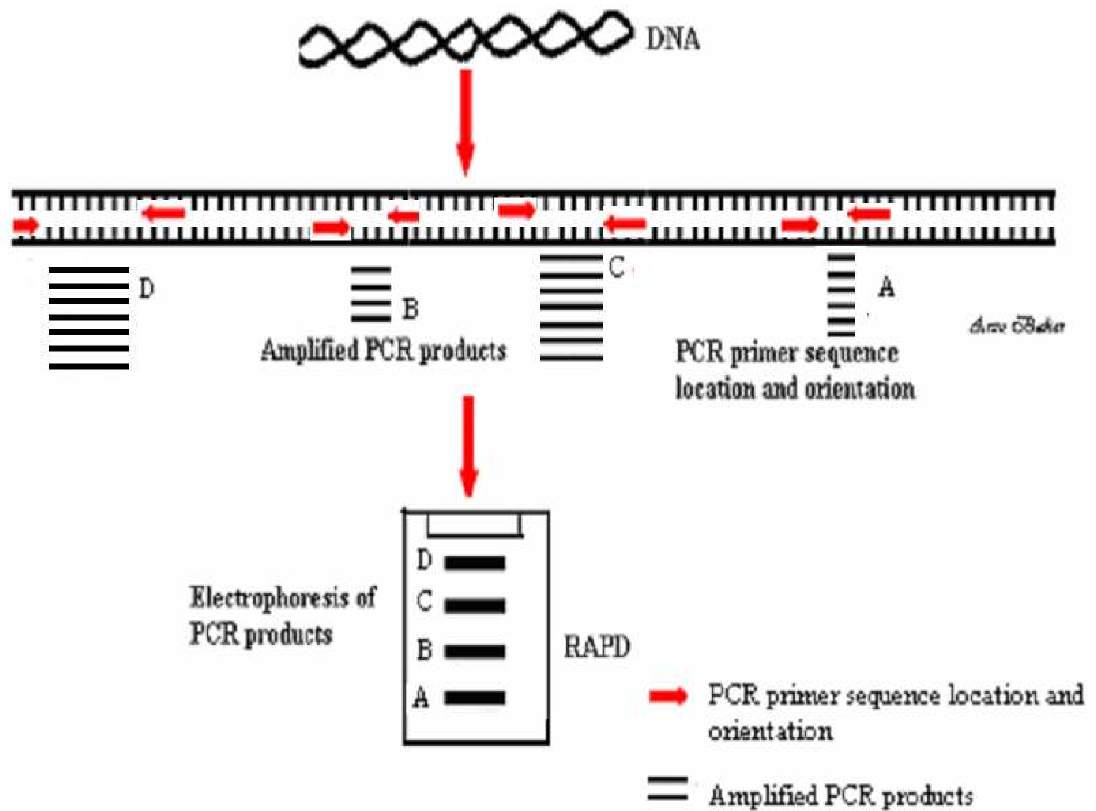


Figure 1.3 : Random Amplification of Polymorphic DNA-PCR

Advantages of using RAPD technology

1. More polymorphisms than RFLPs
2. Simple and quick
3. Selective neutrality
4. Option not to use radioisotopes
5. Differentially amplifies DNA samples based on mutations
6. DNA quality may be low (quick extraction possible)
7. A large number of bands are produced per primer
8. Primers are readily available

Disadvantages of using RAPD technology:

1.Detection of polymorphisms are still limited (similar to RFLPs)
2. Reproducibility of results is inconsistent
3.Poor profile resolutions of RAPDs on agarose gel resulting in very few bands
4.Only detects dominant markers

1.9.Cell Culture

Animal or plant cells, removed from tissues, will continue to grow if supplied with the appropriate nutrients and conditions. When carried out in a laboratory, the process is called Cell Culture. The culture process allows single cells to act as independent units, much like a microorganism such as a bacterium or fungus. The cells are capable of dividing, they increase in size and, in a batch culture, can continue to grow until limited by some culture variable such as nutrient depletion (Freshney, 1994)

There are a number of applications for animal cell cultures:

To investigate the normal physiology or biochemistry of cells. To test the effect of various chemical compounds or drugs on specific cell types. To study the sequential or parallel combination of various cell types. To synthesize valuable biologicals from large scale cell cultures. The biologicals encompass a broad range of cell products and include specific proteins or viruses that require animal cells for propagation (Mather et al., 1998).

The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The disadvantage is that, after a period of continuous growth, cell characteristics can change and may become quite different from those found in the starting population. Cells can also adapt to different culture environments by varying the activities of their enzymes (Rothblat et al., 1972).

Isolated cultures from mammalian tissues are known as primary cultures until sub-cultured. At this stage, cells are usually heterogeneous but still closely represent the

parent cell types as well as in the expression of tissue specific properties.Then several sub-cultures onto fresh media, the cell line will either die out or “ransform” to become a continuous cell line. Such cell lines show many alterations from the primary cultures including change in morphology, chromosomal variation and increase in capacity to give rise to tumors in hosts with weak immune systems.

CHAPTER 2

MATERIAL and METHODS

2.1 MATERIALS

2.1.1 General Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company (USA), Merck (Germany), Applichem (Germany) and Fluka (Germany).

2.1.2 Equipment

Table 2.1: List of equipments and their brands and models

Autoclave	**CERTO CLAW A-4050 Traun, Austria**
Balance	**Sartorius, Wender Landstrasse 94-108 D-37075 Goettingen,Germany**
Centrifuge	**Hettich, Mikro 22**
Electrophoresis Equipment	**Bio-Rad Sub Cell, GT**
Power Supplies	**Bio-Rad Power PAC**
Thermocyclers	**TECHNE TC-512**
Transilluminator	**Bio-Rad GelDoc 2000**
Vortex	**IKA LABOTECHNIK**
Water Purification System	**Millipore**
Inverted Light Microscope	**Zeiss**
Fluorescent Microscope	**Zeiss**
Laminar Flow Hood	**Esco, Kotterman**
CO_2 Incubator	**Thermo, Sanko**
Fluorometer	**Qubit,invitrogen**
PCR Machine	**Techne**

2.1.3. Cell Culture

2.1.3.1. Cell Types

Frozen cell lines (HEK293, HUVEC and HELA) were purchased from *ATCC (American Type Culture Collection)*. Collected cell-lines were stored in liquid nitrogen in Fatih University cell culture research laboratory. Mesenchymal stem cells' DNA were provided by my co-advisor Assist. Prof. Dr. Sevim Işık, Fatih University, Biology Department.

All cell line culture samples were collected by *ATCC (American Type Culture Collection)* laboratory and hMSCs were collected by Karadeniz Technical University. All the information about cell-lines are provided in Table 2.2, 2.3 and 2.4 from ATTC's web site.

Table 2.2: Human Embryonic Kidney 293 cells' (HEK) Information

ATCC Number	**CRL-2873**
Designations	293T/17
Organism	*Homo sapiens* (human)
Source	Organ: kidney
Morphology	Epithelial
Cell Type	Transformed with adenovirus 5 DNA
Propagation	Atmosphere: air, 95%; carbon dioxide (CO2),5% Temperature: 37.0°C
Subcultivation Ratio	1:10 to 1:20 weekly.
Medium Renewal	Every 2 to 3 days

Table 2.3: Human Umbilical Vein Endothelial Cells' (HUVEC) information

ATCC Number	CRL-1573
Designations	HUVEC-CS
Organism	*Homo sapiens* (human)
Source	**Organ:**umbilical vein **Tissue:** vascular endothelium **Disease:** normal **Cell Type:** endothelial
Morphology	Epithelial
Propagation	Atmosphere: air, 95%; carbon dioxide (CO_2), 5% **Temperature:** 37.0°C
Subcultivation Ratio	1: 3 to 1: 4
Doubling Time	about 36 hours

Table 2.4: Human epithelial carcinoma cell line (HELA) information

ATCC Number	**CCL-2**
Designations	HeLa
Organism	*Homo sapiens* (human)
Source	**Organ:**cervix **Disease:**adenocarcinoma **Cell Type:** epithelial
Morphology	Epithelial
Cell Type	Contain Papovavirus
Propagation	Atmosphere: air, 95%; carbon dioxide (CO2),5% Temperature**:** 37.0°C
Subcultivation Ratio	A subcultivation ratio of 1:2 to 1:6 is recommended.
Medium Renewal	2 to 3 times per week

2.1.3.2. Cell culture chemicals and consumables

Dulbeco's Modified Eagle's Medium (DMEM), RPMI 1640 Medium, essential amino acids, penicillin/streptomycin, tyripsin/EDTA, and fetal bovine serum (FBS and for mesenchymal cells: *hMSC qualified FBS*) were purchased from Biochrom, Berlin, Germany. Cell culture flasks: 15 and 50 ml polycarbonate centrifuge tubes with lids and cryotubes were from Grainer Bio-One Corp., Germany.

2.1.4. DNA Isolation and Quantification Materials

2.1.4.1. DNA Isolation

Macherey Nagel's MN Nucleospin Tissue Kit was used and the components are as follows.

Lysis Buffer, Buffer B1, Buffer B2, Wash Buffer B5, Wash Buffer BW, Elution Buffer BE, Proteinase K (lyophilized), Proteinase Buffer PB.

2.1.4.2. DNA Quantification

DNA quantification was done using Qubit fluorometer and its quantification kit components. Quant-it dsDNA BR Assay Kits Components are as follows: Working solution, Standard #1 Standard #2 and assay range is 2–1000 ng with sample starting concentration range: 100pg/µl-1µg/µl.

2.1.5. Polymerase Chain Reaction PCR

2.1.5.1.Oligonucleotide Primers

Table 2.5: Base sequences of 10-mer primers.

Primer	Sequence 5´to 3’	Primer	Sequence 5´to 3’
OPA 01	CAGGCCCTTC	OPB 01	GTTTCGCTCC
OPA 02	TGCCGAGCTG	OPB 02	TGATCCCTGG
OPA 03	AGTCAGCCAC	OPB 03	CATCCCCCTG
OPA 04	AATCGGGCTG	OPB 04	GGACTGGAGT
OPA 05	AGGGGTCTTG	OPB 05	TGCGCCCTTC
OPA 06	GGTCCCTGAC	OPB 06	TGCTCTGCCC
OPA 07	GAAACGGGTG	OPB 07	GGTGACGCAG
OPA 08	GTGACGTAGG	OPB 08	GTCCACACGG
OPA 09	GGGTAACGCC	OPB 09	TGGGGGACTC
OPA 10	GTGATCGCAG	OPB 10	CTGCTGGGAC

2.1.5.2. PCR Chemicals and consumables

Table 2.6: PCR kit components (Fermentas)

10X Taq Buffer with $(NH4)_2SO_4$	750mM Tris-HCl (pH 8.8 at 25°C), 200mM $(NH_4)_2SO_4$, 0.1% Tween 20. $MgCl_2$ 25mM $MgCl_2$
dNTP mix	1.0 µl of 2mM aqueous solution of each dGTP, dATP, dCTP, dTTP
$MgCl_2$	25 mM
Primers	10 pmol/reaction
Tag Polymerase	5U/µl in 20mM Tris-HCl (pH 8.0), 1mM DTT,0.1mM EDTA, 100mM KCl, 0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol.

2.1.6. Electrophoresis and Documentation

2.1.6.1. Agarose Gel Electrophoresis Chemicals and Buffers

Table 2.7 : Agarose Gel Electrophoresis chemicals, buffers and their components and composition concentrations.

10XTBE	For 1 Liter: 108g Tris base, 55g Boric acid,40mls 0.5M EDTA (pH 8.0), autoclave for 20 min
6XLoading Dye	10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA.
GeneRuler™ 100 bp DNA Ladder	100 µl (0.5µg/µl) 100 bp sized DNA fragments in 10mM Tris-HCl (pH 7.6), 1mM EDTA.
Etbr Staining Chemicals	0.5 µg/ml EtBr.

2.2.METHODS

2.2.2 Cell Culture Techniques

2.2.1.1 Growth Conditions

Cell lines were cultured at 37^0C under 5% CO_2 in culture medium unless otherwise specified.

2.2.1.2 Thawing Cell Lines

Previously frozen cells or cell line in liquid nitrogen was taken out from the tank, immediately soaked into pre-warmed water bath and incubated in water bath at 37^0C until totally thawed. Thawed cell gently mixed by pipetting and transferred to 15 ml tube which was containing 10 ml growth medium. Then cells were centrifuged at 1500 rpm for 10 min and supernatant carefully aspirated. Lastly cell pellet was resuspended in 1ml medium and cell number was counted. After this, cells were seeded in 25 cm^2 culture flask with 8 ml 10% FBS growth medium and were transferred to 25 cm^2 sterile culture flask. Culture flask size was determined according to total cell number. Culture flask was incubated at CO_2 incubator under humidified microaerofilic condition with 5% CO_2. Following day, cells were observed under invert microscope and growth medium was refreshed.

2.2.1.3 Sub-Culture of Cell Lines

Cultured cells were observed under microscope and if the confluency was about 90% and if there was no contamination or other abnormal formation, cells were determined to be sub-cultured. Culture medium was aspirated and cells were washed for once with pre-warmed PBS at 37 ^{0}C. After washing cells, they were detached by treatment with Tyripsin/ EDTA solution for 1-3 min until all cells were detached. 3 ml FBS for 25 cm^2 sterile culture flask was added to detached cells to stop the

Tyripsin/EDTA activity. Then cells were splitted or diluted depending on the purpose, transferred to new culture flasks, and incubated in 5% CO_2 incubator at 37 ^{0}C.

2.2.1.4 Cryopreservation of Cell Lines

Medium of the cells that are 80-90% confluent was aspirated, the cells were washed with 5 ml pre-warmed PBS in 25 cm^2 culture flask for once, treated with 1 ml Tyripsin/EDTA (4ml/25 cm^2), and incubated at 37 ^{0}C for 1-3 min to be detached. In order to inactivate Tyripsin/EDTA, 3 volume of 1ml FBS was added. FBS plus Tyripsin/EDTA mixture was transferred to 15 ml falcon tube and then was centrifuged at 1500 RPM for 5 minutes. After centrifugation, the cell pellet was resuspeded with FBS and counted with hemocytometer. About 1.5-2x10^6 cells were added to each tube with 10% DMSO on ice and was left at -20 ^{0}C for a while to cool down. Cryovials were transferred to -20 ^{0}C immediately and left for 2 hours, then transferred to -80 ^{0}C and left overnight. At last, cells were transferred into liquid nitrogen.

2.2.3. DNA Isolatıon

DNA extraction was performed according to MN-Nucleospin tissue kit protocol step by step as describe below:

1.0 x 10^6 cells were resuspended in a final volume of 200 µl Buffer T1. 25 µl Proteinase K solution and 200 µl Buffer B3 were added. Samples were incubated at 70°C for 10-15 min. In order to adjust binding condition 210 µl ethanol (96-100%) to the sample was added and vortexed vigorously. For each sample, one NucleoSpin® Tissue Column was placed into a collection tube. The sample was loaded onto the column, was centrifuged for 1 min at 11.000 *g*. Flow-through was discarded and the column was placed back into the collection tube. 500 µl Buffer BW was added onto the column, centrifuged for 1 min at 11.000 *g*. Flow-through was discarded and the column was placed back into the collection tube. 600 µl Buffer B5 was added onto the column, centrifuged for 1 min at 11.000 *g*. Flow-through was discarded and the column was placed back into the collection tube. In order to dry and remove the residual ethanol from

membrane empty column was centrifuged for 1 min at 11.000 *g*. NucleoSpin® Tissue Column was placed into a 1.5 ml microcentrifuge tube and 100 µl prewarmed Elution Buffer BE (70°C) was added onto the column, incubated at room temperature for 1 min, centrifuged 1 min at 11.000 *g*. Highly pure DNA is extracted at the end of procedure and eluted DNA concentration and purity is calculated using Qubit Fluorometer.

2.2.3. DNA Quantification

For the DNA quantification sample tubes were set up as follows and with the components as prescribed in Table 2.8.

Quant-iT Working Solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 µl of Working Solution are required for each sample and standard. Assay Tubes are prepared according to the table below.

Table 2.8: DNA Quantification Kit ingredients and amounts (µL) required for assay.

	Standard Assay Tubes	**User Sample Assay Tubes**
Volume of Working Solution to add	190 µL	180-199 µL
Volume of Standard to add	10 µL	—
Volume of Sample to add	—	1–20 µL
Total Volume in each Assay Tube	200 µL	200 µL

All tubes were vortexed for 2–3 seconds and incubated for 2 minutes at room temperature. And tubes were read in Qubit fluorometer. To determine concentration of the original samples, the instrument read values were multiplied by the dilution factor. Alternatively, Calculate sample concentration can be chosen to have the Qubit fluorometer perform this multiplication.

2.2.4. RAPD-PCR and RAMD-PCR Analysis

RAPD is different from conventional PCR as it needs one primer for

amplification. The size of primer is normally short (10 nucleotides), and therefore, less specific. The primers can be designed without the experimenter having any genetic information for the organism being tested. More than 2000 different RAPD primers can be available commercially.

The conditions of RAPD-PCR were optimized with some modifications stated in Figure 2.2 as RAMD-PCR (Random Amplified Mosaic DNA-PCR). RAPD-PCR reactions were performed in reaction mixture of 25 µl containing the components in Table 2.9 and according to the flow in Figures 2.1 and 2.2.

Table 2.9: RAPD-PCR solutions and their initial and final concentrations with final volumes calculated accordingly.

Reagent	Initial Concentration	Final Concentration	Final Volume
Taq Buffer	10X	1X	2.5 µl
dNTP	2 mM	0.2 µM	1.5 µl
$MgCl_2$	25 mM	2 mM	3 µl
Primers (x6)	25 pmol/µl	25 pmol	4 µl
ddH2O	-	-	11.9 µl
Taq DNA Polymerase	5 U/µl	1 U	0.2 µl
Template DNA			5 µl
Total Reaction Volume			25 µl

The RAPD and RAMD protocols consisted of an initial denaturing step of 5 min at 94^{0}C, followed by 45 cycles at 94 ^{0}C for 30 s (denaturation), 37 ^{0}C for 60 s (annealing) and 72^{0}C for 60 s (extention), with an additional extension period of 10 min at 72 ^{0}C.

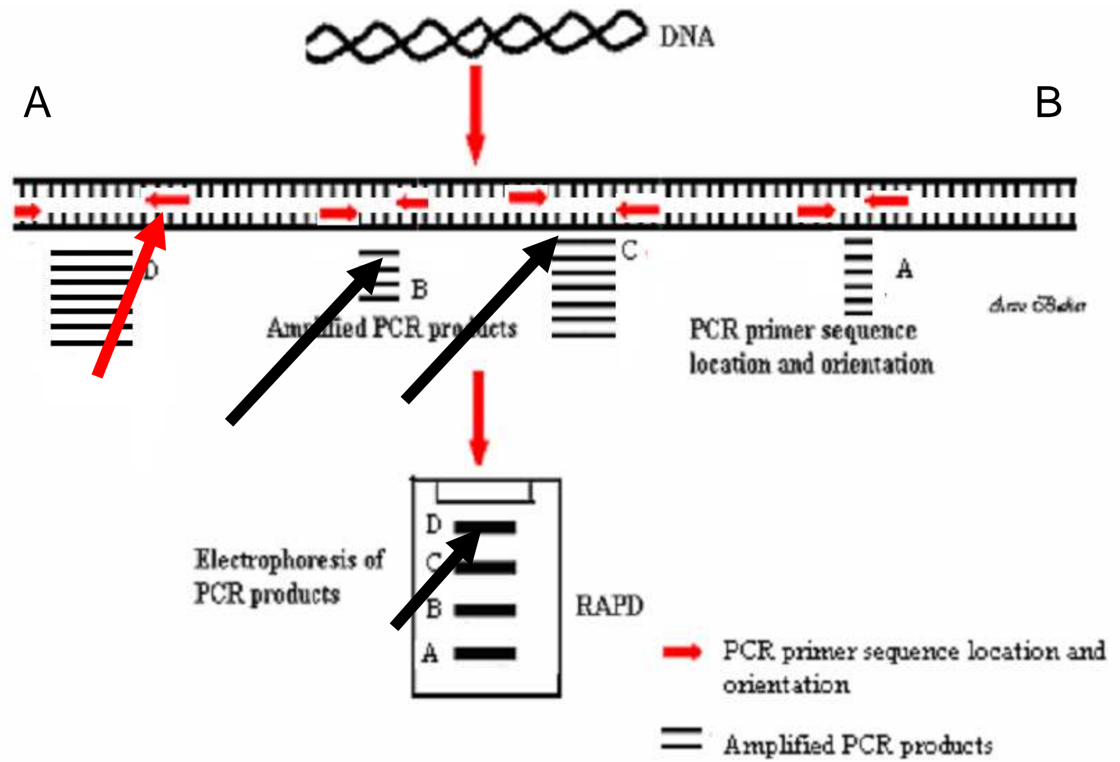


Figure 2.1: RAPD-PCR procedure. Red arrows are for the random primers, black lines are the chromosomes from A to B. Black ones are for the amplified DNA parts and A, B, C and D stand for the different sized PCR amplified products and below is the figure for electrophoresis of PCR products ordered according to their sizes.

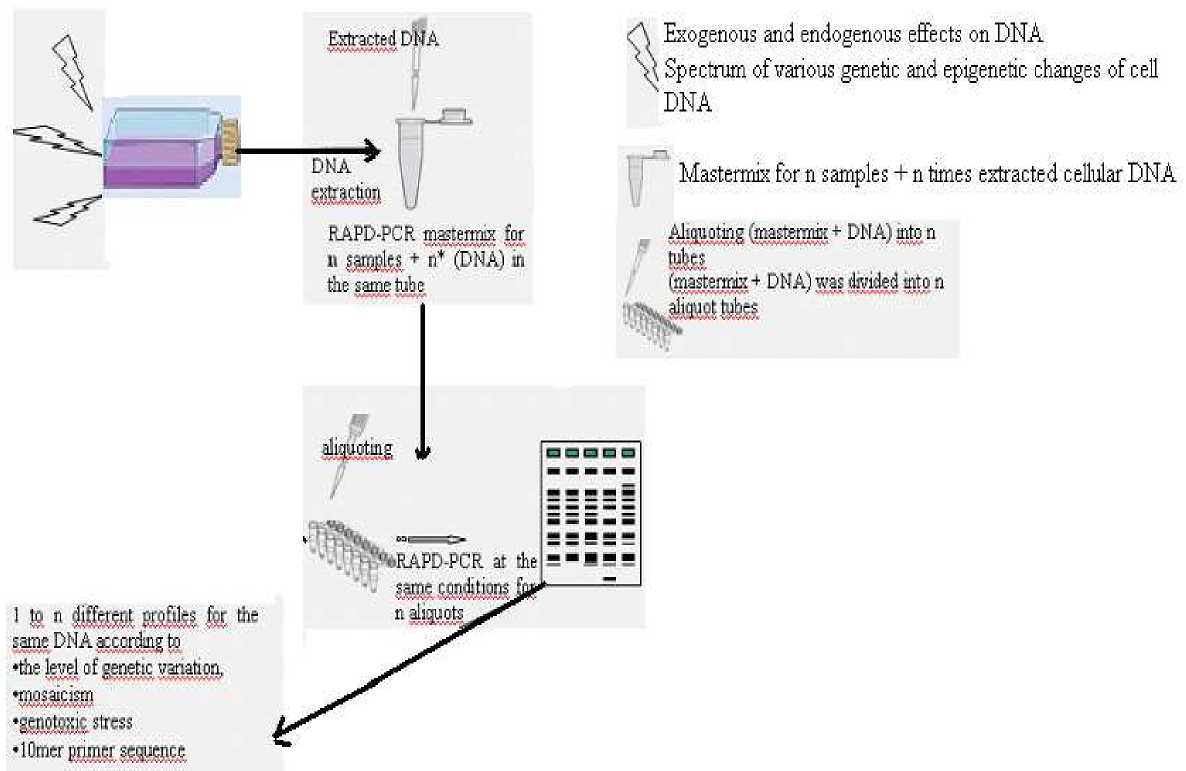


Figure 2.2: RAMD-PCR procedure that was used to detect the mosaic nature of subcultures due to various DNA damages.

2.2.4.Agarose Gel Electrophoresis

PCR products were resolved on 2% agarose gels. Gel is prepared adding 1.6 gram of powdered agarose gel into 80ml of 0.5 x TBE buffer solution and it is boiled until the agarose is completely dissolved in the buffer solution. Four µl of Ethidium Bromide was added when the boiled solutions began to cool down and reach approximately 55ºC. Solution is mixed homogenously by making hand-shaking. It is directly poured into horizontal agarose gel platform and the combs having either 8 or 22 wells are placed one side of the gel. Gels are let to solidify for at least 10 minutes, to confirm full polymerization 45 minutes.

Loading and visualization of the gels were done accordingly:

10 µl of PCR product was mixed with 2 µl bromophenolblue as loading dye/buffer. 12 µl PCR mixes were then loaded in each slot with appropriate micropipettes. 1 µl of a 100 bp DNA Ladder (MBI Fermentas, Hanover, MD, USA) was mixed with 1 µl deionized water and 1 µl bromophenolblue. Then 5 µl of this mix was put into usually the first slot as a molecular size marker. The gel was run at 95 V in 0.5X TBE buffer for 50 min. The gel was placed in Gel Doc 2000 (Bio-Rad, Milan, Italy) apparatus and the bands were detected under UV transilluminator.

CHAPTER 3

RESULTS

3.1. MORPHOLOGICAL CHANGES AMONG CELL SUBCULTURES IN DIFFERENT CELL LINES UNDER INVERTED PHASE CONTRAST MICROSCOPE

The modern compound microscope is a precision instrument, designed to perform particular functions in a particular way. When the microscope is used correctly, it will disclose many structures to the vision even of a non-expert on the field.

The morphology of cells is very important in many contexts. In culture the morphology indicates the status of the cells, both in terms of the health of the cells and in the case of primary isolates the differentiation state may be critical. These can be detected under light microscope.

Cells should be inspected visually frequently in order to get to know what makes the cells happy. Frequent feeding is important for maintaining the pH balance of the medium and for eliminating waste products. Cells do not typically like to be too confluent so they should be subcultured when they are in a semi-confluent state. In general, mammalian cells should be handled gently. They should not be vortexed, vigorously pipetted or centrifuged at greater than 1500 g. These are the prerequisites for further work which rely on visual inspection and morphology awareness.

Four different cells: HEK293, HUVEC, HELA, hHMSCs have been used in this work. The hHMSCs have not been worked directly, but the DNAs obtained from each subculture provided by my co-adviser Assist. Prof. Dr. Sevim Isik from Fatih University, Biology Department working group have been worked.

Figure 3.1 is for the observations of cells from various passages to make a comparison of the morphological changes.

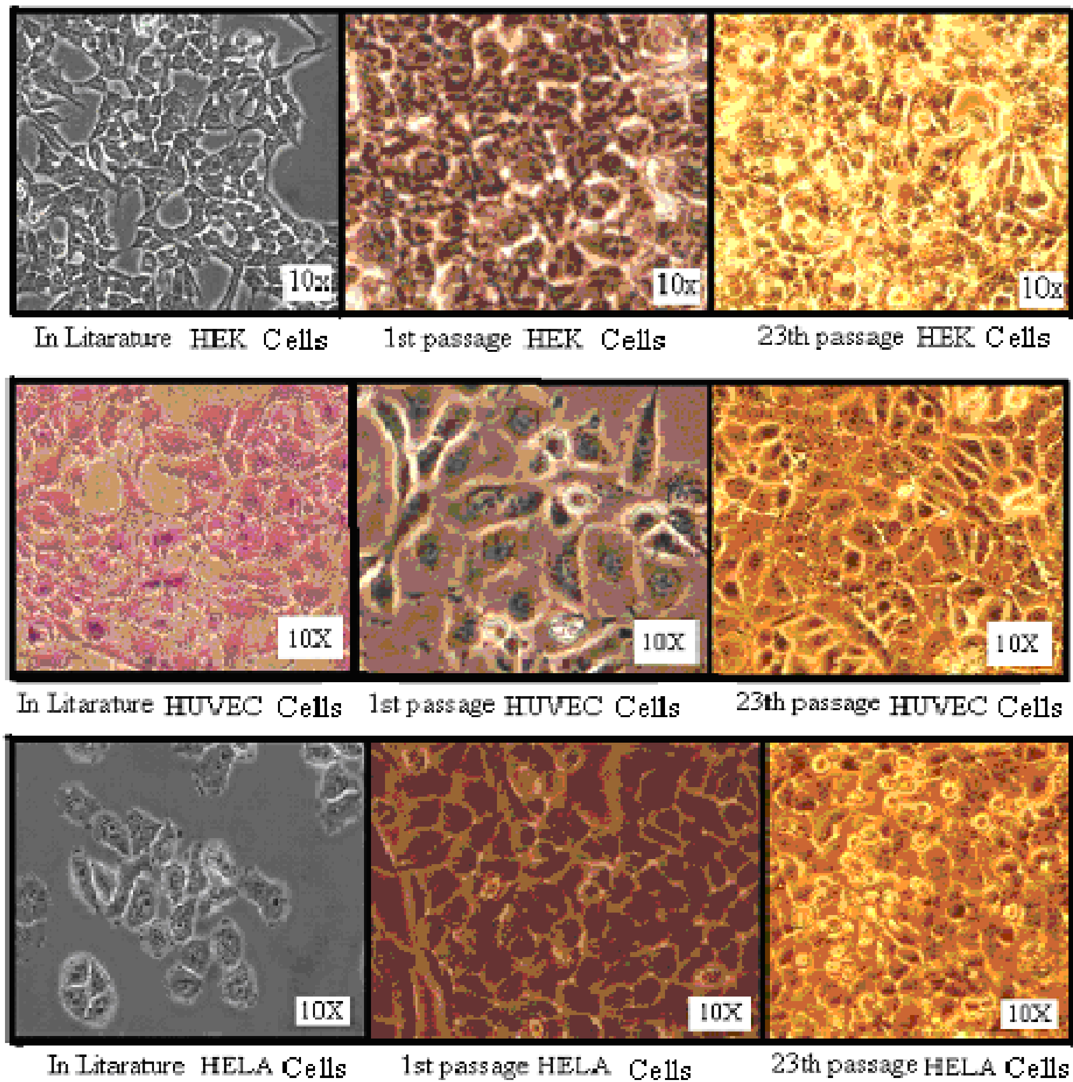


Figure 3.1 : The comparative morphological appearances of the first and 23rd passages of HEK293, HUVEC and HELA cells. The first photographs for each cell type are from pages of commercial cell suppliers unless otherwise stated.

3.2. CELL QUANTIFICATION

A single cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding. It is an essential step if qualitative estimates of cell proliferation or of plating efficiency are being made and if cells are to be isolated as clones.

Counting the cells with hemocytometer or an electronic particle counter and recording the cell counts are prerequisite work. Cell suspensions should be diluted to appropriate seeding concentrations. By adding appropriate volume of cell suspension to a pre-measured volume of medium in a culture flask or by diluting cells the total volume required and distributing that volume among several flasks is a procedure useful for routine subculture (Freshney, 2005).

In this work 2 different cell culture types. Primary cell culture and secondary cell culture or cell line have been used.

Primary culture, started from cells, tissues or organs taken directly from an animal. As primary cell culture human Mesenchymal stem cells have been used. Mesenchymal stem cells, or hMSCs, are multipotent stem cells that can differentiate into a variety of cell types; including osteoblasts, chondrocytes, myocytes, adipocytes, endotheliums, beta-pancreatic islets cells (*in vivo or in vitro*) (Freshney, 2005).

Cell line (or secondary cells), arises from the primary culture at the time of the first subculture. HEK293, HUVEC and HELA cells are cell line cultures. Secondary cells were originally explanted from a donor organism, and given the correct culture conditions, divide and grow for some time *in vitro*, e.g. 50-100 generations (Bodnar, et al., 1998). However, they do not continue to divide indefinitely and eventually, their physical characteristics may change, after which the cells will eventually senesce and die. The factors which control the replication of such cells *in vitro* are related to the degree of

differentiation of the cell - in general, terminally differentiated cells are harder to maintain than less specialized cells.

Cell count results are summarized in Tables 3.1 and 3.2 and will be discussed in 4th chapter, discussion part (Caputo, 1996).

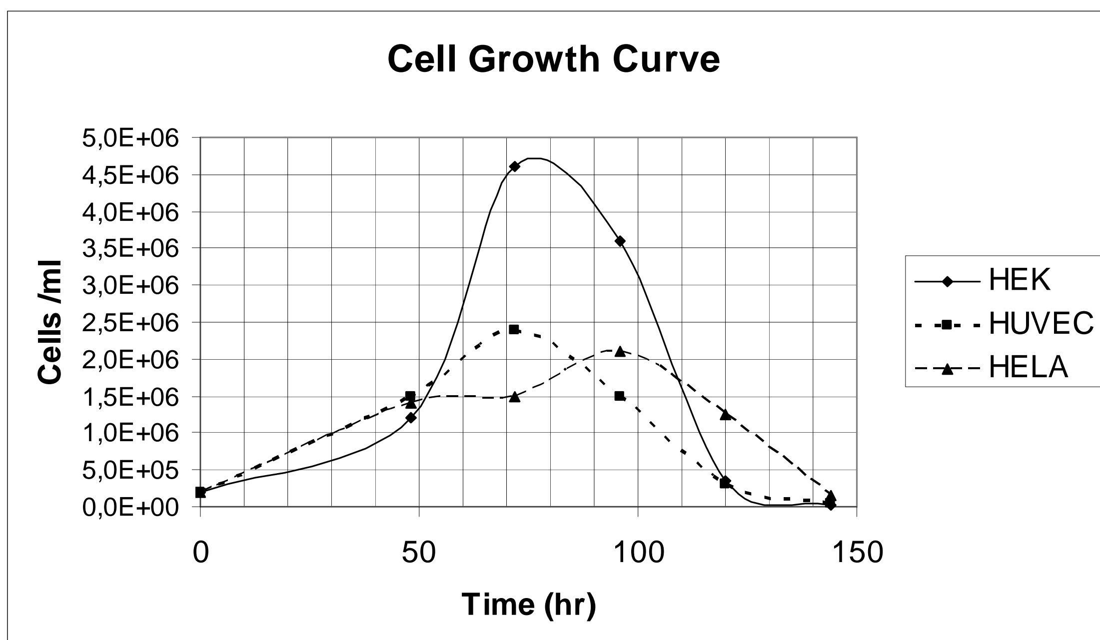
3.3. GROWTH CURVE ANALYSIS

Each time that a cell line is subcultured it will grow back to the cell density that existed before subculture (within the limits of its finite life span). A process which can be described by plotting a growth curve from samples taken at intervals throughout the growth cycle, which shows that the cells enter a latent period of no growth, called the *lag period*, immediately after reseeding. Lag period lasts from a few hours up to 48 h, but is usually around 12–24 h, and allows the cells to recover from trypsinization, reconstruct their cytoskeleton, secrete matrix to aid attachment, and spread out on the substrate, enabling them to reenter cell cycle. The cell population doubles over a definable period, known as the *doubling time* and characteristic for each cell line. As the cell population becomes crowded when all of the substrate is occupied, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle, entering the *plateau* or *stationary phase*, where the growth fraction drops to nearly zero. Some cells may differentiate in this phase; others simply exit the cell cycle into G0 but retain viability (Pretlow, 1989). Cells may be subcultured from plateau, but it is preferable to subculture before plateau is reached, as the growth fraction will be higher and the recovery time (lag period) will be shorter if the cells are harvested from the top end of the log phase. Reduced proliferation in the stationary phase is due partly to reduced spreading at high *cell density* and partly to exhaustion of growth factors in the medium at high *cell concentration*. These two terms are not interchangeable. Density implies that the cells are attached, and may relate to monolayer density (two-dimensional) or multilayer density (three-dimensional). In each case there are major changes in cell shape, cell surface, and extracellular matrix, all of which will have significant effects on cell proliferation and differentiation. A high density will also limit nutrient perfusion and create local exhaustion of peptide growth factors. In normal cell populations this leads to a withdrawal from the cycle, whereas in transformed cells, cell cycle arrest is much less

effective and the cells tend to enter apoptosis. Cell concentration, as opposed to cell density, will exert its main effect through depletion of nutrient and growth factors, but in stirred suspensions cell contact mediated effects are minimal, except where cells are grown as aggregates. High cell concentrations can also lead to apoptosis in transformed cells in suspension, notably in myelomas and hybridomas, but in the absence of cell contact signaling this is presumably a reflection of nutrient deprivation (Chen, 1977).

Growth curves were drawn for each cell line which is very important for proper subculturing and for obtaining maximum DNA extraction efficiency, because the DNA isolation kits have maximum yield for the cell count ranges 10^2-10^5cells/ml.

Table 3.1: Growth curves for HEK293, HUVEC, HELA cells



Time dependent, cell division rate is given in Table 3.1. According to the table at approximately 72nd hours, saturation density is reached for HEK293 and HUVEC cells.

3.4. DNA QUALITY AND QUANTITY ASSESSMENTS

Concentrations of DNA extracted for each subculture were measured with the Qubit fluorometer. The results obtained for each cell type as number of cells counted for each subculture DNA isolation, the concentration measured by Qubit fluorometer after DNA extraction are given in Table 3.2. A total of 19 passages have been done for each cell-line and from each subculture a total of 57 DNA samples were obtained for our work.

Table 3.2: Information for the sample cell types, subculture names, counts prior to DNA extraction and DNA concentrations.

Passage# HEK293	**Number of cells ($x10^6$)**	**Conc. (ng/µl)**	**Passage # HUVEC**	**Number of cells $x10^6$**	**Conc. (ng/µl)**	**Passage #HELA**	**Number of cells ($x10^6$)**	**Conc. (ng/µl)**
HK1p	1.5	1.1	*HU1p*	1.5	1.7	*HL1p*	1.1	0.4
HK2p	0.36	0.3	*HU2p*	0.3	1.2	*HL2p*	1.4	1.4
HK3p	2.32	0.5	*HU3p*	2.3	0.6	*HL3p*	0.9	1.4
HK4p	1.1	0.8	*HU4p*	1.1	1.0	*HL4p*	2.8	1.1
HK5p	1.68	0.6	*HU5p*	1.6	0.6	*HL5p*	1.5	1.0
HK6p	1.01	0.7	*HU6p*	1.0	0.6	*HL6p*	1.6	1.0
HK7p	2.10	0.3	*HU7p*	2.1	2.6	*HL7p*	3.0	0.4
HK8p	2.10	0.6	*HU8p*	2.1	2.4	*HL 8p*	4.5	2.6
HK9p	2.4	1.7	*HU9p*	2.4	1.6	*HL 9p*	3.3	0.6
HK10p	1.23	1.2	*HU10p*	1.2	1.4	*HL10p*	2.4	1.0
HK11p	2.2	Too low	*HU11p*	2.2	1.4	*HL11p*	0.9	1.2
HK12p	1.5	1.1	*HU12p*	1.5	1.1	*HL12p*	1.0	1.1
HK13p	2.0	1.7	*HU13p*	2.0	5.1	*HL13p*	2.9	6.1
HK14p	1.8	0.6	*HU14p*	1.8	1.5	*HL14p*	1.5	2.7
HK15p	1.5	Too low	*HU15p*	1.5	2.3	*HL15p*	3.4	0.7
HK16p	1.2	0.6	*HU16p*	1.2	1.2	*HL16p*	1.1	0.5
HK17p	1.2	0.9	*HU17p*	1.2	1.2	*HL17p*	1.4	1.3
HK18p	1.56	1.1	*HU18p*	1.5	2.3	*HL18p*	0.9	1.5
HK19p	3.5	Too low	*HU19p*	3.5	Too low	*HL19p*	2.8	1.2

The quality of the extracted DNA plays a central role, and genomic DNA of poor quality will certainly lead to non-reproducible RAPD patterns. Similarly, the estimation of DNA concentration is important, because RAPD assays performed with very amounts of DNA will lead to different profiles majority of reproducible bands but some possible

differences in band intensity. In Figure 3.2, RAPD profiles of different DNA concentrations for the same DNA sample are compared. In our hands, very low DNA concentration can cause an overestimation of the DNA concentration and underestimation of the 260/280 nm ratio.

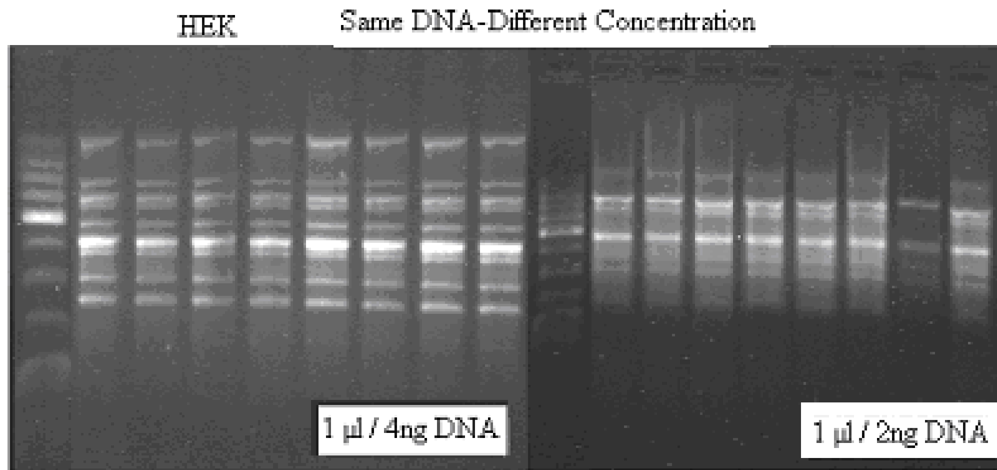


Figure 3.2: x8 RAMD-PCR profile differences using different DNA concentrations for HEK293, 18th passage.

3.5. RAPD-PCR OPTIMISATION RESULTS

The concentration of DNA is crucial in the production of reproducible genomic profiles, not only to ensure the largest number of amplified bands and therefore maximum discrimination, but also to confirm the fidelity of the PCR reaction condition. If profiles from the same genomic template, or from different individuals of the same species vary, the reproducibility of the assay should be confirmed by repeating the PCR reaction using two different template concentrations, differing by at least two-fold. If profiles still vary, then the results should always be treated with skepticism. To check the reproducibility of RAPD profiles, we generally used genomic DNA of concentrations ranging 0.05-100 ng [30, 32]. The results in Figure 3.2 are to show the effect of different DNA concentration on the repeatability of RAPD profiles.

3.6. PRIMER OPTIMIZATIONS

The DNA isolated from all cell types were tested using 21 10-mer primers (Table 3.3) were used to amplify genomic DNA samples from each cell type's subcultured cells. A total of 20 of 21 (95%) primers generated strong, scorable banding patters in all cell types tested; the other primer failed to amplified DNA (OPB03 for hMSC) or produced a non-scorable smear (OPA03 for HEK293 and HUVEC, OPB06 for HUVEC) as seen in Figure 3.3. A total of 84 amplification products (loci) from 21 primers for all cell types were identified, with an average of 96% products per primer.

Table 3.3: Primer choice results for all cell types. The sequences and the resulting bands of 21 primers used for RAPD amplification of DNA for all cell types, HEK293, HUVEC, HELA and hHMSC.

Primers	**Sequences (5'to 3')**	**Total bands HEK293**	**Total bands HUVEC**	**Total bands HELA**	**Total bands hMSC**
OPA 01	CAGGCCCTTC	4	8	5	4
OPA 02	TGCCGAGCTG	4	7	4	1
OPA 03	AGTCAGCCAC	Smear	Smear	2	5
OPA 04	AATCGGGCTG	2	7	7	4
OPA 05	AGGGGTCTTG	4	4	2	3
OPA 06	GGTCCCTGAC	3	Smear	4	2
OPA 07	GAAACGGGTG	6	6	3	4
OPA 08	GTGACGTAGG	5	5	1	2
OPA 09	GGGTAACGCC	4	1	3	2
OPA 10	GTGATCGCAG	1	9	4	4
OPA18	CCACAGCAGT	Smear	6	Not detected	Not detected
OPB 01	GTTTCGCTCC	4	5	Not detected	2
OPB 02	TGATCCCTGG	6	5	Not detected	1
OPB 03	CATCCCCCTG	1	7	Not detected	Smear
OPB 04	GGACTGGAGT	5	1	Not detected	3
OPB 05	TGCGCCCTTC	5	3	Not detected	2
OPB 06	TGCTCTGCCC	3	Smear	Not detected	2
OPB 07	GGTGACGCAG	3	6	Not detected	3
OPB 08	GTCCACACGG	4	6	Not detected	6
OPB 09	TGGGGGACTC	5	4	Not detected	2
OPB 10	CTGCTGGGAC	6	4	Not detected	3

Some primers were producing more polymorphic and informative bands in comparative profiling and most were monomorphic in profiling. OPA7 was a primer producing polymorphic and strong patterns whereas others were difficult to interpret less informative from repetitive perspective for our thesis. Monomorphic patterns gave patterns of almost that same intensity for all cell type's DNA like data shown in Figure 3.6.

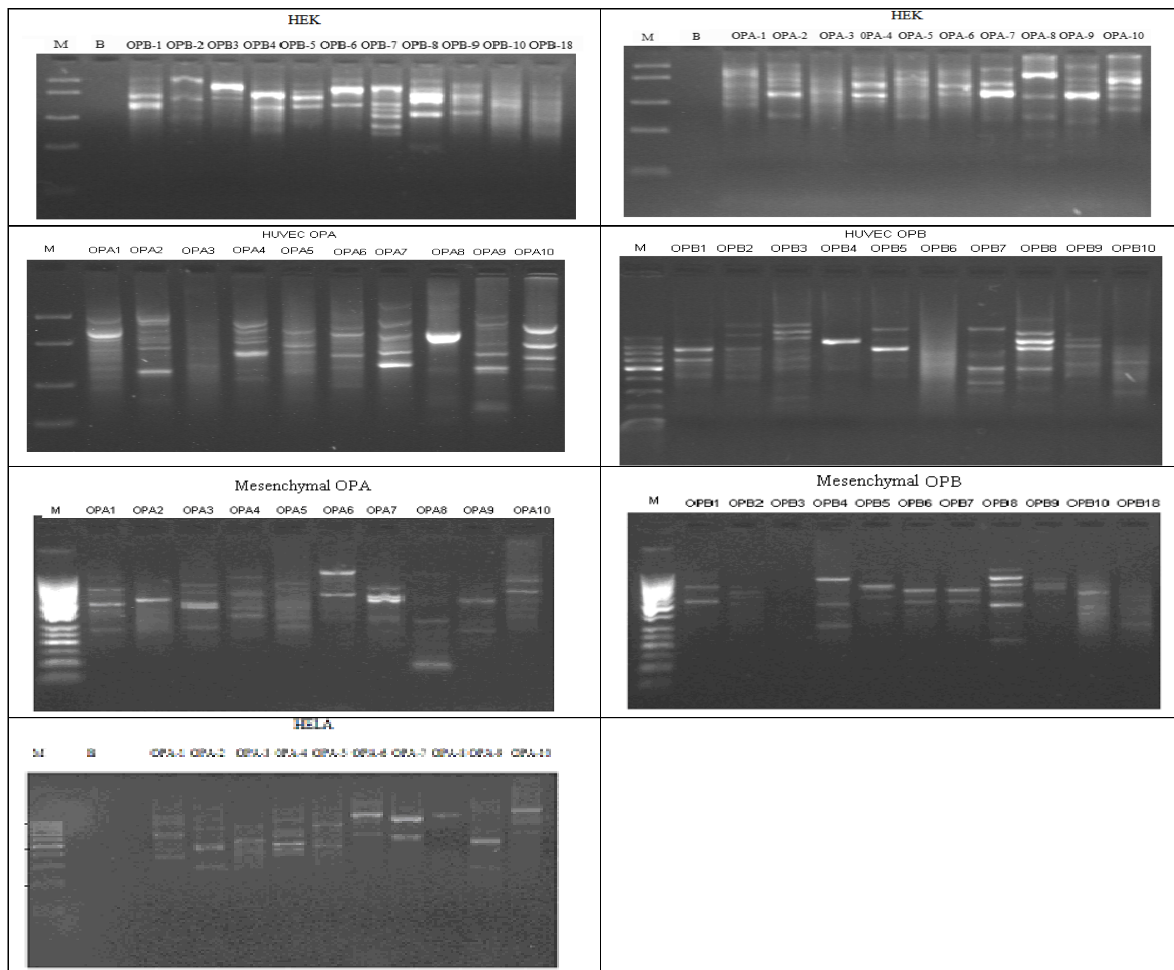


Figure 3.3: 10-mer primer trials for each cell type using the DNA extracted from the first subculture of HEK293, HELA, hHMSC and HELA cells, each lane indicating the amplification results of the denoted primer. M: size marker, B: no DNA, blank.

Different cell types exhibit different RAPD profiles for the same primer at their first subcultures, but after many subcultures (19-23) for cell line and (4-5) Mesenchymal cell the RAPD profiles with primer OPB07 the profiles become very homogeneous as seen in Figure 3.3, 3.4.

3.7. REPEATABILITY AND REPRODUCIBILITY OF PROFILES

Repeatability of profiles should be confirmed in the same laboratory among repeated, consequent experiments of the same conditions and reproducibility is also intended for the different laboratories that repeat the experiment with the same conditions.

RAPD is a reliable method, with practice and care, repeatability or reproducibility is actually high. However, it is also well established that many conditions of the RAPD reaction procedure may influence the results. For instance, the use of different thermostable DNA polymerase, or thermal cyclers can generate variable RAPD profiles. To perform RAPD reactions under strictly identical conditions was intended, but was really challenging and not thoroughly achieved. However to overcome the challenges in achieving strictly identical conditions in between different experiments that was not possible for most of our trials, we have tried to have strictly the identical conditions for the same experiment and to evaluate the results with the same experiment that is to be consistent and coherent without a further reproducible experiment need. In our approach we have prepared a mastermix for all the DNA samples in hand and than sub-divide the mastermix to a second mastermix for the individual DNA samples. After the addition of the individual DNA samples to the mastermix, the mastermixes are aliquoted into n PCR tubes and amplified under same conditions and than run on the same gel as if the same DNA has been worked for n times, as if the experiment has been performed n times under strictly the similar conditions. If there are prominent changes among the same DNA's RAPD profiles this should be further investigated and suspected to be due to an instability of genome. We have done our optimisations with enough high stringent conditions to be able to catch these genomic instabilities that we expect for the increasing passage numbers. The main advantage of high stringency conditions is that non-specific reactions are significantly reduced. Thus, protocols that use a high annealing temperature should always be preferred however we prefered to decrease the amounts of all PCR components to the lowest possible concentration including the DNA concentration assuming that this will also provide enough stringency for our intended RAPD profiles. This was an advantageous approach to economise our limiting materials.

3.8. RAPD PROFILES OF EACH CELL TYPE AND SUBCULTURE OF THEM

20 subcultures for all three cell lines and seven for hMScells have been done and using the most effective (i.e. reproducible and producing the most distinguishable banding profiles between controls and experimental group in our thesis flow) primer OPB7 RAPD profiles were obtained. Figure 3.4 shows us all the RAPD results obtained for all passages using OPB7 primer. Figure 3.5 shows us the first observed profile changes for each cell type's respective subcultures.

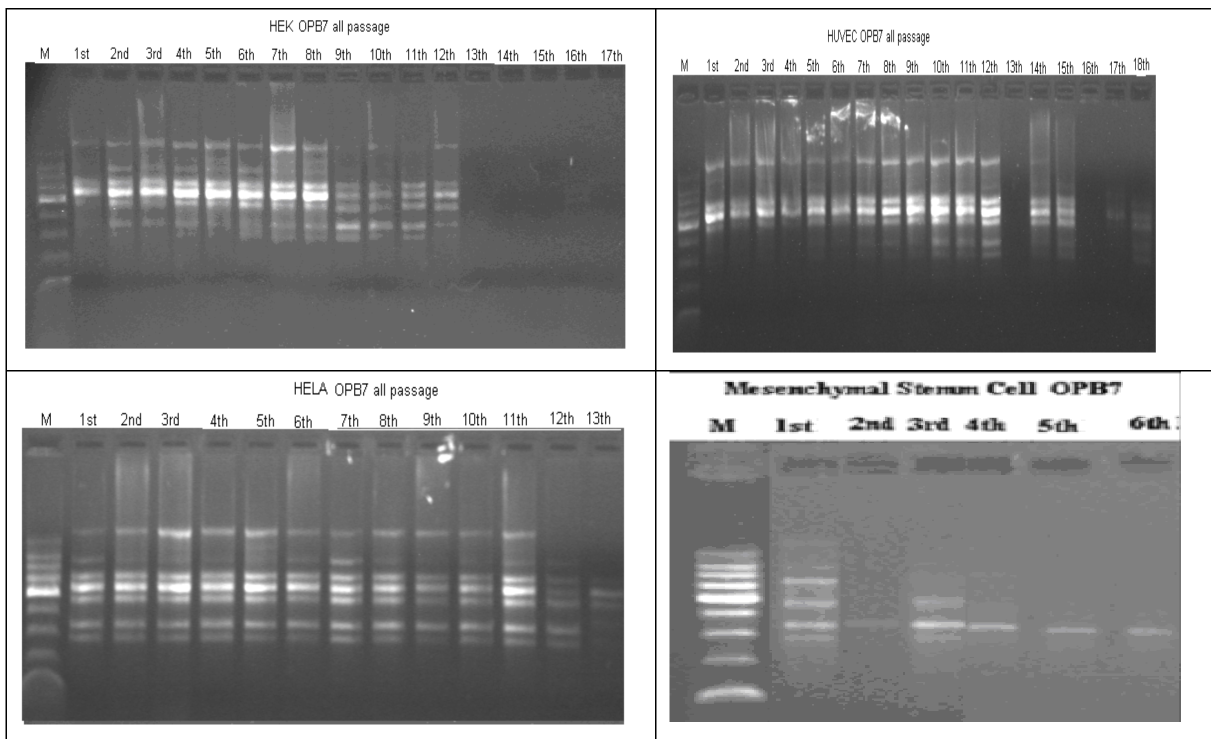


Figure 3.4: The RAPD-PCR profile results of each cell's all subcultures using OPB7 primer.

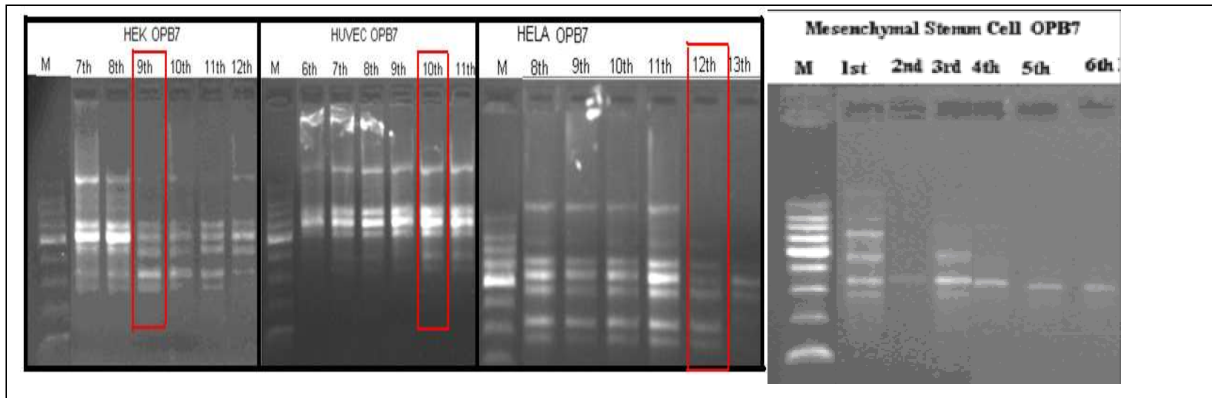


Figure 3.5: Detection of the first changes in RAPD profiles in HEK293, HUVEC, HELA and hMSCs with OPA7. It is 9th for HEK293, 10th for HUVEC, 12th for HELA and not sure for hMSCs.

When we do a comparative observation of the RAPD profiles for all cell types for initial (Figure 3.6 a) and final situations (Figure 3.6 b) using the same primer and conditions we saw a decrease in bands but reached a more common remaining bands for all cell types. These common bands corresponding to approximately 470bp and 180 bp on 2 % agarose gel electrophoresis (Figure 3.6 b).

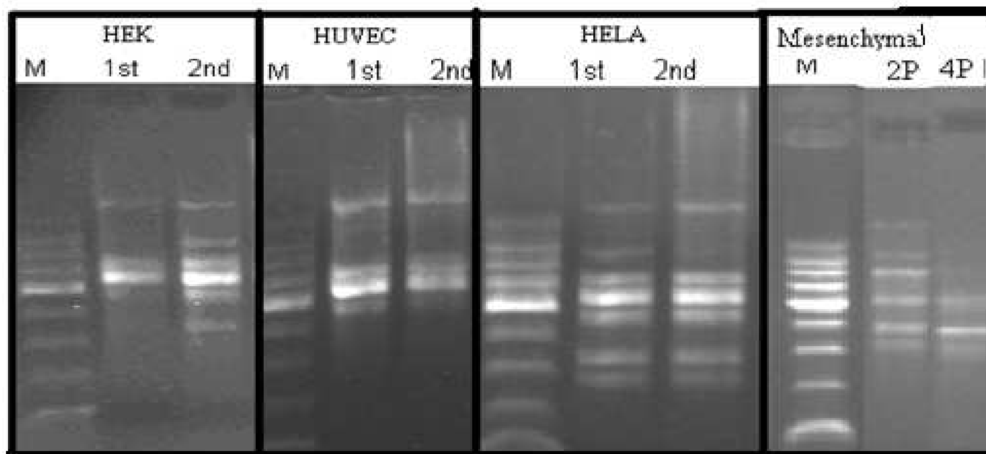


Figure 3.6 a: Comparative RAPD-PCR profile analysis of initial bands using OPB7 primer for all cell types.

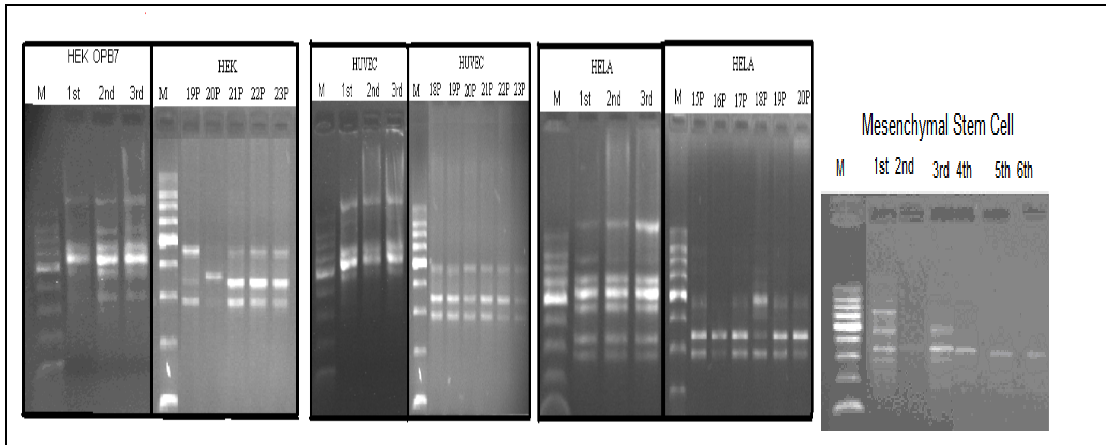


Figure 3.6 b: Comparative RAPD-PCR profile analysis of initial and final bands using OPB7 primer for all cell types.

3.9. MODIFIED RAPD-PCR RESULTS

RAPD-PCR assay and related methodologies have also proved useful to detect genomic instability.

RAPD-PCR technique has been modified to confirm the repeatability and to assess the variation in RAPD profiles that might be indicative for genomic instability, innate or acquired, genetic or epigenetic. For each DNA sample xn PCR mastermixes are prepared and also xn amount of same DNA added to the mastermix, producing completely the same, identical and homogeneous conditions. Aliquoted samples were amplified and run on the same gel, as if repeating the experiments n times with identical conditions which is nearly impossible to do in normal conditions. The results for 8x and 3x modified RAPD PCR are given in Figure 3.7 (for HUVEC, HEC293 (x8) and HELA and MSC (x3)).

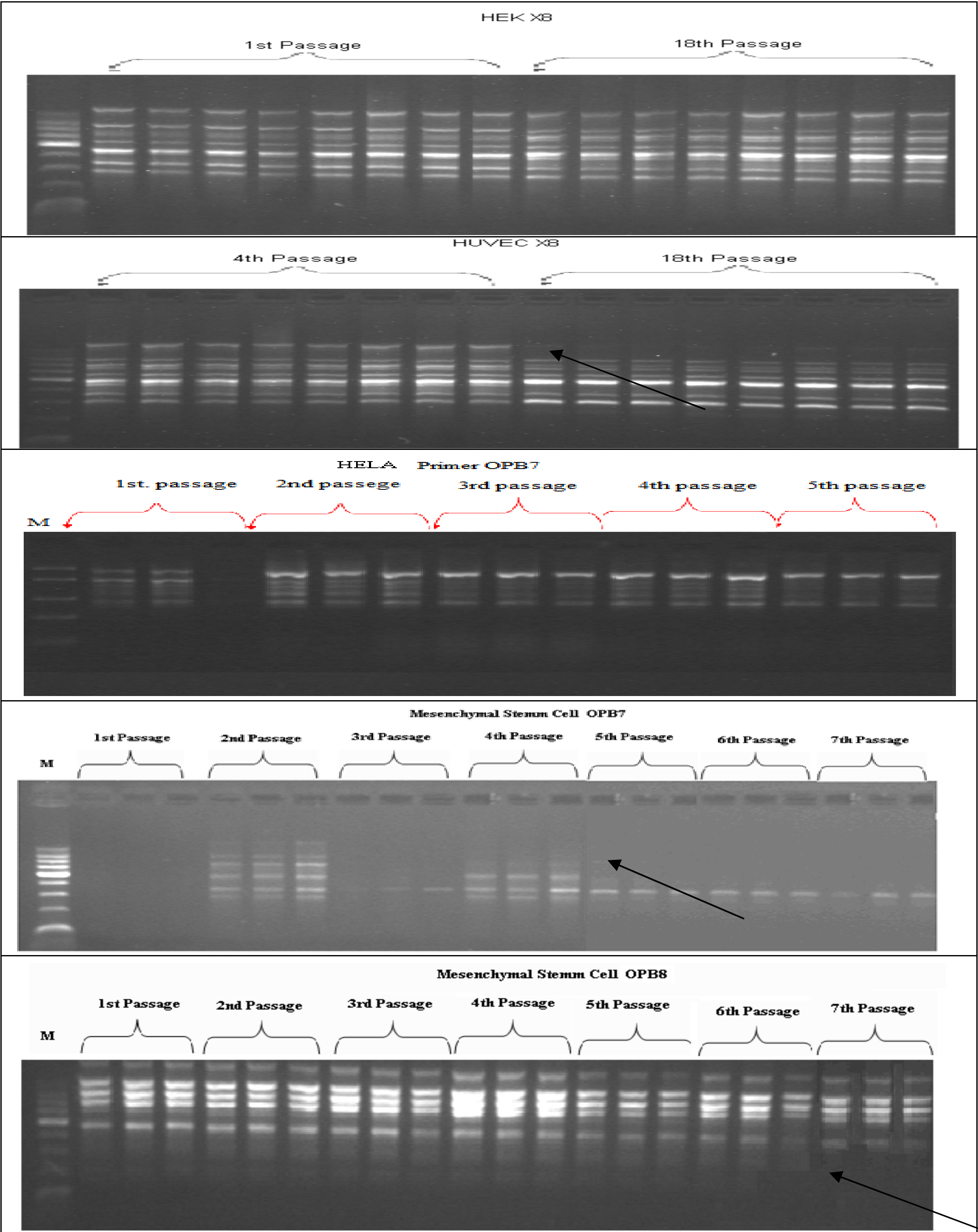


Figure 3.7: Modified RAPD PCR results for HUVEC, HEK293, HELA and HMCs. Modified RAPD PCR results for hMSCells using two different primers OPB7 and OPB8.

The fourth and fifth figures in Figure 3.7 are to show the meaning of informative RAPD profiles using different primers. In the Figure 3.7 part d, OPB7 primers produced more informative polymorphic patterns showing the extend of genome instability in various subcultures and in Figure 3.7 part e, OPB8 with that same set of DNA produced non-informative monomorphic RAPD profiles that do not show the extend of genomic instability in the same set of cells.

3.10. RAPD TO DETECT DNA DAMAGE AND GENOTOXICITY

DNA is the essential carrier of genetic information in all living cells. The chemical stability of the DNA molecule is not unusually great, DNA undergoes several types of spontaneous modifications, and it can also react with many physical and chemical agents, some of which are endogenous products of the cellular metabolism while others including ionizing radiation and ultraviolet light, are threats from the external environment. The resulting alterations of DNA structure are generally incompatible with its essential role in preservation and transmission of genetic information (Atienzar et al., 2006).

Damage to DNA can cause genetic alterations, and if genes that control cell growth are involved, these mutations may lead to the development of cancer. Of course, DNA damage may also result in cell death which can have serious consequences for the organism of which the cell is a part; for example, loss of irreplaceable neurons in the brain. Accumulation of damaged DNA has also been considered to contribute to some of the features of aging. It is not surprising that a complex set of cellular surveillance and repair mechanisms has evolved to reverse the potentially deleterious damage that would otherwise destroy the precious blueprint for life. Some of these DNA repair systems are so important that life cannot be sustained without them (Atienzar et al., 2006).

Glutaraldehyde is a known DNA-protein cross linker and a high production volume chemical with many medical, scientific and industrial uses. Humans are mainly exposed via inhalation but the exposure is not widespread. Glutaraldehyde has been

extensively tested for toxic and genotoxic activity but there is still disagreement in the literature with regard to its genotoxic potential.

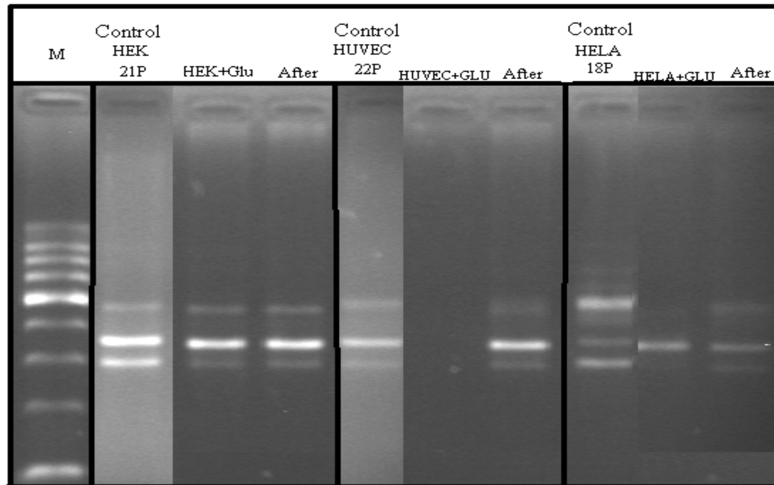


Figure 3.8: Glutaraldehyde's DNA effect results using RAPD assay for three cell-types. Controls are before starting the experiment 21st passage for HEK, 22nd for HUVEC and 18th for HELA, than gluteraldehyde added experiment group results shown with HEK+Glu, HUVEC+Glu and HELA+Glu and After means, the next passage for all cells after removal of gluteraldehyde from the cell culture mediums.

The same RAPD profiles comparison strategy this time to show genoxicants effects on genomic instability detection in different cell types has been employed. The 21st, 22nd and 18th subcultures cells for HEK293, HUVEC and HELA cells respectively were exposed to glutaraldehyde. Effects of glutaraldehyde have been assessed using various approaches modified RAPD-PCR with preparation of x1 RAPD-PCR and x8 master mix for HEK293, HUVEC and HELA cells (Figures 3.8 and 3.9).

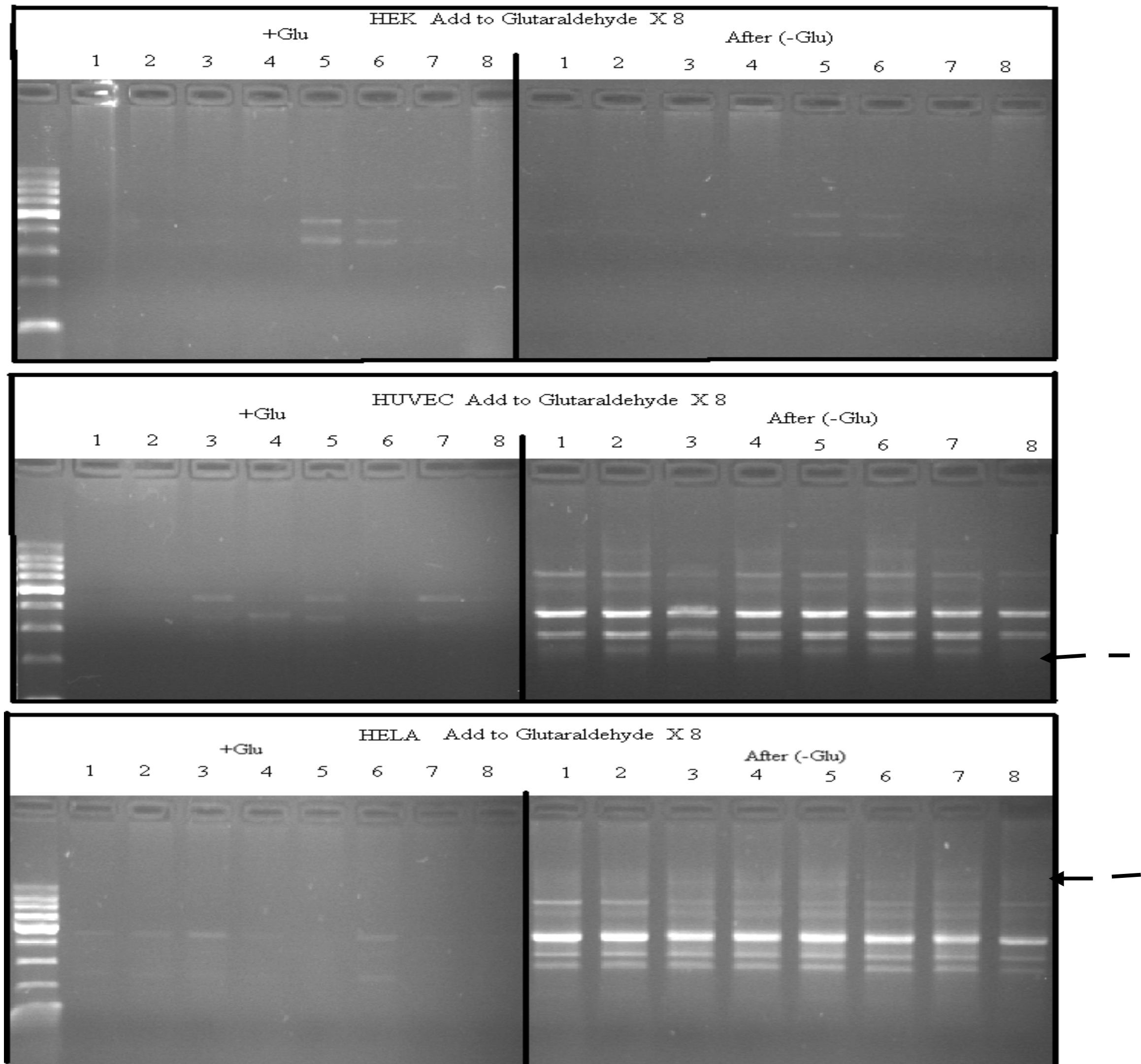


Figure 3.9: Modified RAPD-PCR results for HEK293(a), HUVEC(b) and HELA(c) cells x8 %2 agarose gel runs of glutaraldehyde treated cells' DNA and DNA of harvested cells after removal of glutaraldehyde.

The effects are much more prominent for HUVEC and HELA cells in Figure 3.8. Whereas with RAMD PCR Figure 3.9 shows prominent variation for HEK293 cells when compared with HUVEC and HELA cells and they are stable changes even after removal of Gluteraldehyde from the medium. It is obvious that the effects are reversible from the comparison of reverted profiles after removal of glutaraldehyde from the medium.

The other effects after removal of glutaraldehyde for HUVEC and HELA cells are loss of RAPD-PCR bands indicated with arrow and a (-) sign in the figure 3.9 for HEK293 and HELA cells respectively.

CHAPTER 4

DISCUSSION

The thesis we put forward here is to our knowledge a novel approach in assessment of various DNA effects; either DNA damages or mutations caused by endogenous or exogenous exposures in routine cell-culture work (Keshava, Keshava et al. 1999; Keshava, Zhou et al. 2001; Lery, LaRue et al. 2003; Lee, Yang et al. 2007) The various cell types either cell-lines or primary cells are for the sake of modeling various aspects of a cell's life-time exposures and events and in this context to discuss the events like genomic instability, DNA damage, mutation, DNA repair, mutagenesis, carcinogenesis, ageing and related disorders. Below is a brief information before discussion of the results obtained that is to clarify the events underlying the work starting with the importance of genomic stability, how to maintain it, what happens when it is lost, what are the causes of genomic instability, what it may lead to in a cell's life-time exposures. Next a brief introduction to the cell-types used is given so that the cell's different behaviors can be better understood although they are all human cells that shouldn't have theoretically that many differences genetically. Some are cancer cells, some are stem cells and some are terminally differentiated cells all having the same genetic make-up but completely different genes at work which can to an extend be visualised in this thesis. Next thing is the RAPD-PCR and its RAMD approach, again a novel approach for both repeatability assessments and to see the behavior of the intra-passage cells' DNA.

The human genome is exposed to potentially deleterious genotoxic events during every cell division cycle. The endogenous sources of DNA damage result from cellular metabolism or routine errors in DNA replication and recombination. In addition, cellular and organismal exposure to exogenous genotoxic agents such as ultraviolet light, oxidative stress, and chemical mutagens, can lead to a variety of nucleotide modifications and DNA strand breaks. In order to fight these attacks to the genome, the cell has a response system that induces cell cycle arrest, allowing sufficient time for specialized

groups of proteins to repair the incurred damage. The DNA damage response system activates the appropriate DNA repair pathway or in the case of irrepairable damage, induces apoptosis.

Some cancer types arise as a consequence of the accumulation of genetic alterations (gene mutations, gene amplification, and so on) and epigenetic alterations (aberrant DNA methylation, chromatin modifications, and so on) that transform different cells into cancer cell types.

The loss of genomic stability and resulting gene alterations are key molecular pathogenic steps that occur early in tumorigenesis; they permit the acquisition of a sufficient number of alterations in tumor suppressor genes and oncogenes that transform cells and promote tumor progression.

Genomic instability encompasses molecular events such as point mutations, genomic and chromosomal rearrangements, deletion, and insertions. Of special interest regarding the application of these techniques to cancer is that the amplified bands usually originate from unique sequences rather than from repetitive elements. RAPD is very likely to detect genomic instability as the malignant cell will produce a clone of dividing daughter cells. Thus, the proportion of cells presenting the same genomic instability is high and easy to detect. Finally, further analysis of the relevant bands allows not only to identify some of the molecular events (e.g. allelic losses and gains) implicated in the genomic instability, but also to discover genes playing key roles in the initiation and development of malignancy (e.g. oncogenes, DNA repair genes, anti-oncogenes).

In addition to genomic instability, epigenetic instability results in the aberrant methylation of tumor suppressor genes. Roles of genomic and epigenomic instability in different tumor formation has the potential to yield more effective prevention strategies and therapeutics for patients with cancer.

Cancer results from a disordered and unstable genome. Such genomic instability appears to be subject to control by environmental factors as evidenced by the number of

cancers that are either caused by specific environmental agents. Dietary factors might interact in several ways with the genome to protect against cancer.

An agent might interact directly with the genome and regulate expression (as a genetic or epigenetic regulator) or indirectly by influencing DNA repair responses and so improve genomic stability.

The design of new therapies for cancer depends on first understanding the molecular events that cause the disease.

DNA damage has been studied in a variety of organisms such as bacteria, cyanobacteria, phytoplankton, macroalgae, plants, animals and humans. It may be spontaneous or environmental that affects all living cells in a number of ways (Horio, Miyauchi-Hashimoto et al. 2007).

The development of widely applicable methods to monitor genomic instability gains importance nowadays (Nachtsheim, Vogel et al. 1960; Mendelsohn 1989; Kondrashov and Crow 1993; Honma, Mizusawa et al. 1994; Morley 1996; Henke and Henke 1999; Atienzar, Evenden et al. 2002; Castano and Becerril 2004; Araten, Golde et al. 2005; Lee, Yang et al. 2007). RAPD-PCR assay is a molecular method able to detect comparative DNA changes. This work is to show the applicability of the method to evaluate the ultimate changes caused in various cell-culture derived DNA which might be a model for carcinogenesis, genomic instability and routine cell-culture work.

HEK293 cells were generated by transformation of cultures of normal human embryonic kidney epithelial cells. As an experimentally transformed cell line, HEK293 cells are not a particularly good model for normal cells, cancer cells, or any other kind of cell that is a fundamental object of research. However, they are extremely easy to work with, being straightforward to culture.

HUVEC are Human Umbilical Vein Endothelial cells that are widely used in research to study vascular endothelium in a cell culture model. primary human umbilical

endothelial cells in culture have a finite proliferative lifespan. They undergo permanent growth arrest, known as replicative senescence.

When replicative senescence is bypassed by transformation with viral oncogene with telomerase reverse transcriptase, HUVEC are immortalized. The HUVECells, we used in our thesis are cell-lines like the HEK293 and HELA cells.

A HELA cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer on October 4, 1951. Horizontal gene transfer from human papillomavirus 18 (HPV18) to human cervical cells created the HeLa genome which is different from either parent genome in various ways including its number of chromosomes.

Tissue-specific stem cells produce differentiated cells through a series of increasingly more committed progenitor intermediates. In hematopoiesis (blood cell formation), the process begins with long-term hematopoietic stem cells that self-renew and also produce progeny cells that upon further replication go through a series of stages leading to differentiated cells without self-renewal capacity. In mice, deficiencies in DNA repair appear to limit the capacity of hematopoietic stem cells to proliferate and self-renew with age (Rossi, Bryder et al. 2007; Rossi, Seita et al. 2007). (Sharpless and DePinho 2007) reviewed evidence that hematopoietic stem cells, as well as stem cells in other tissues, undergo intrinsic aging. They speculated that stem cells grow old, in part, as a result of DNA damage like some others who work in the field (Cervantes, Stringer et al. 2002; Hong and Stambrook 2004; Hong, Cervantes et al. 2006; Hong, Cervantes et al. 2007; Stambrook 2007; Pearson 2008; Hodgkinson, Ladoukakis et al. 2009).

Growth curves (Table 3.1) were obtained for each cell type and calculated doubling times were calculated to decide for the optimum subculture timing and the cell types nature of profileration capacity and the nature of the cell whether it is a dividing or non-dividing cell. When working with concepts like DNA damage, mutation, genomic instability, cell division, mutagenesis, carcinogenesis, ageing, the cell’s nature of division capacity gains utmost importance. The end of log phase is nearly 72 hours for HEK and

74 for HUVEC cells and 96 hours for HELA cells, so for the time of doubling for each cell type which is an indication for their proliferation potency were calculated. We expect to see more change in HEK cells' DNA, because it replicates faster than the others. Saturation density is the highest for HEK, the second one is HUVEC and the third one is HELA and the results were all in compromise with the expectations.

RAMD-PCR procedures (Uzonur 2004) were used to detect the mosaic nature of subcultures due to various DNA damages and/or mutations. The choice of primers were done comparatively for all cell types. OPB7 was chosen because it produced most prominent and distinguishable and repeatable banding profiles for all cell types. It is also interesting to see the completely different profiles for all primers for human DNA. OPB7 amplification results for HEK HUVEC and hMSCs to draw attention to the completely different patterns obtained for all used DNA. How instability might be causing genetic diversity can be discussed with the below Figure 4.1.

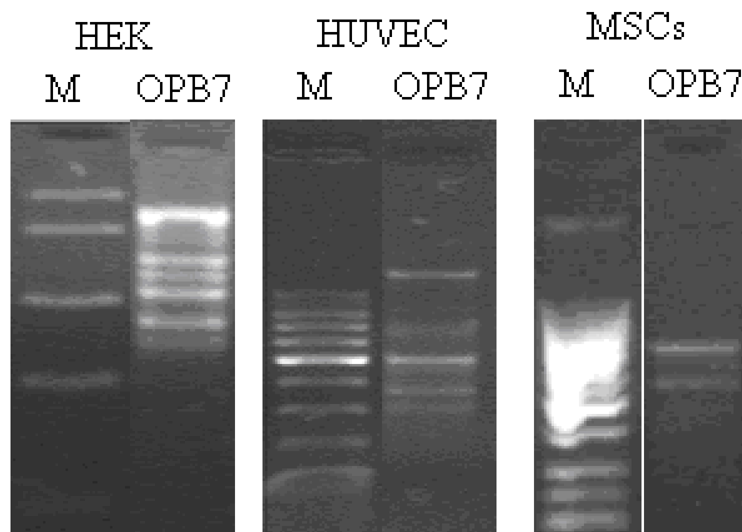


Figure 4.1: Genetic diversity as observed with OPB7 primer with different human cell types.

DNA concentration is very important for optimizations of RAPD-PCR (Atienzar, Evenden et al. 2000; Atienzar and Jha 2006). Figure 3.2 shows how the concentration can change the profiles obtained. The first part of the figure is for 4ng/µl and the second for

2ng/μl DNA. It is the same DNA and completely the same PCR conditions for both figures. X8 mastermixes for RAPD-PCR have been prepared and the same DNA, HEK cell DNA has been added to the mastermix eight times. The repeatability is confirmed in this way. The lost bands are prominent in second figure which is an indication for DNA variation and instability. The HEK DNA used in this experiment belongs to 18th passage.

RAPD is very likely to detect genomic instability, because the cancerous cell and the cells in our cell culture work will produce a clone of dividing daughter cells. This won't be the case, when an organism is exposed to a genotoxin or when the % of altered cells is low and may not be detectable if below about 2% (Jones 2000; Atienzar and Jha 2006) which is a very important fact about RAPD-PCR detection capacity of genomic instability.

It is important to distinguish between DNA damage and mutation, the two major types of error in DNA. DNA damages and mutation are fundamentally different. Damages are physical abnormalities in the DNA, such as single and double strand breaks, 8-hydroxydeoxyguanosine residues and polycyclic aromatic hydrocarbon adducts. DNA damages can be recognized by enzymes, and thus they can be correctly repaired if the undamaged sequence in the complementary DNA strand or in a homologous chromosome, is available for copying. If a cell retains DNA damage, transcription of a gene can be prevented and thus translation into a protein will also be blocked. Replication may also be blocked and/or the cell may die. In contrast to DNA damage, a mutation is a change in the base sequence of the DNA. A mutation cannot be recognized by enzymes once the base change is present in both DNA strands, and thus a mutation cannot be repaired. At the cellular level, mutations can cause alterations in protein function and regulation. Mutations are replicated when the cell replicates. In a population of cells, mutant cells will increase or decrease in frequency according to the effects of the mutation on the ability of the cell to survive and reproduce. Although distinctly different from each other, DNA damages and mutations are related because DNA damages often cause errors of DNA synthesis during replication or repair and these errors are a major source of mutation.

Given these properties of DNA damage and mutation, it can be seen that DNA damages are a special problem in non-dividing or slowly dividing cells, where unrepaired damages will tend to accumulate over time.

On the other hand, in rapidly dividing cells, unrepaired DNA damages that do not kill the cell by blocking replication will tend to cause replication errors and thus mutation. The great majority of mutations that are not neutral in their effect are deleterious to a cell's survival. Thus, in a population of cells comprising a tissue with replicating cells, mutant cells will tend to be lost. However infrequent mutations that provide a survival advantage will tend to clonally expand at the expense of neighboring cells in the tissue. This advantage to the cell is disadvantageous to the whole organism, because such mutant cells can give rise to cancer. Thus DNA damages in frequently dividing cells, because they give rise to mutations, are a prominent cause of cancer. In contrast, DNA damages in infrequently dividing cells are likely a prominent cause of aging.

The DNA damage theory of aging proposes that aging is a consequence of unrepaired DNA damage accumulation. Damage in this context includes chemical reactions that mutate DNA and/or interfere with DNA replication. In humans, DNA damage occurs frequently and DNA repair processes have evolved to compensate. On average, approximately 800 DNA lesions occur per hour in each cell, or about 19,200 per cell per day (Vilenchik and Knudson, 2000). In any cell some DNA damage may remain despite the action of repair processes. The accumulation of unrepaired DNA damage is more prevalent in certain types of cells, particularly in non-replicating or slowly replicating cells, which cannot rely on DNA repair mechanisms associated with DNA replication such as those in the brain, skeletal and cardiac muscle.

In view of these knowledge let's go over the results. The figure 4.2 is for all cell types' nearly all passages to see the total DNA variation profile. For HEK cells especially after 9th passage the changes are more prominent. The arrows show the types of changes observed, the cumulative changes when the passages increase indicate that these are

mutations. Loss of bands, appearance of bands, decrease and increase in band intensities all detectable DNA effects have been detected for all cell types, but more prominent in HEK cells, they are epithelial cells that are having the least doubling time, and reaching the highest number two times the other cell types at 72 hours, highest saturation density. Some are DNA damages because reverted back in increasing number of passages, some are mutations because were stabilized in increasing number of passages, in long-term.

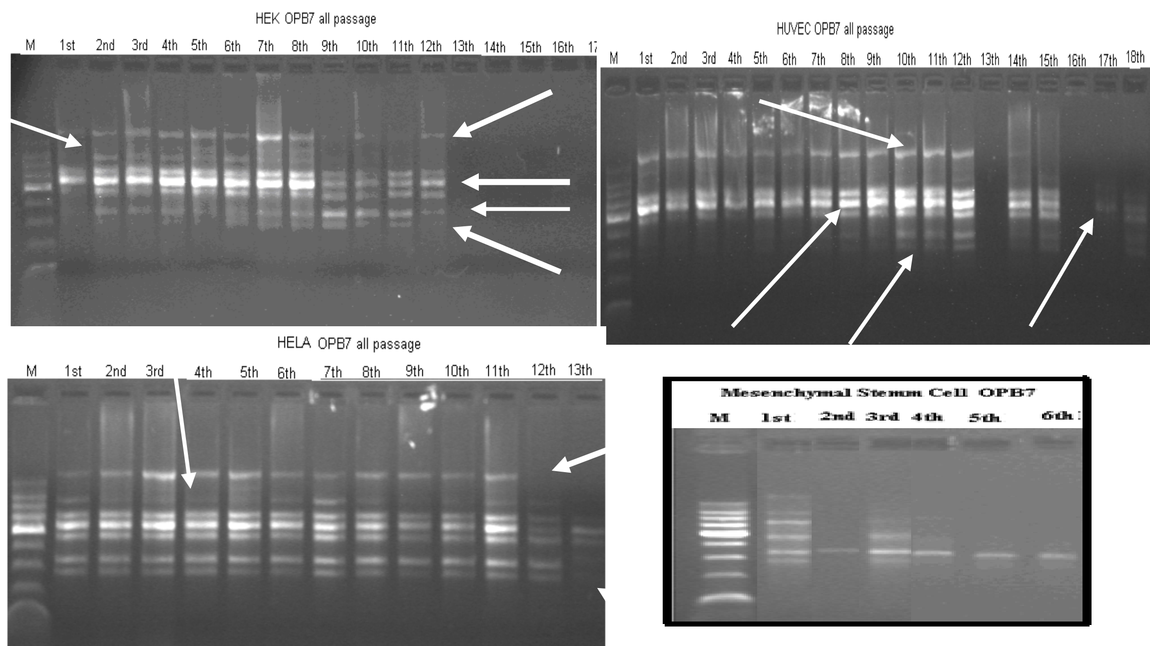


Figure 4.2: RAPD profiles with OPB7 primer for all cell types' all subculture DNA extracted. M: DNA size marker. Arrows indicate the most prominent changes of RAPD profiles: loss of bands, appearance of bands, decrease in band intensity and increase in band intensity.

Either DNA damage or mutation the effects on RAPD profiles can be seen as loss of bands, appearance of bands, decrease in band intensity and increase in band intensity. The Figure 4.3 from the review of Atienzar (Atienzar and Jha 2006) about RAPD assay makes the discussions of profile changes highly clear.

Direct effect of DNA damage and mutation on RAPD profiles

DNA alterations	Consequences	Effects on RAPD profiles	Probability to detect the lesion by RAPD
DNA adduct	→By pass → processivity affected	→Decrease in band intensity/band loss	Medium
	→By pass → processivity not affected	→No effect	
	→Block → dissociation enzyme adduct	→Band loss	Low
	→Block → dissociation → more free Taq	→Increase in band intensity	Medium
	→Block → no dissociation	→Band loss	Low
	→No primer/DNA association (adduct within the priming site)	→Band loss	Low
DNA breakage	→Block → dissociation enzyme DNA	→Band loss	Low
Point mutation	→No primer/DNA association	→Band loss	Low
Annealing site	→Creation of a new annealing site	→Appearance of band	Low
Rearrangement	→Loss of pre-existing annealing site	→Band loss	Low
	→New priming site	→Appearance of band	High

For more details please refer to the text. ●: DNA adduct; ⊐ ⊏: DNA breakage; ◖: Taq DNA polymerase; — — — ▶: DNA synthesis; ×: point mutation; ⊥⊥⊥⊥⊥⊥: 10-mer primer.

Figure 4.3: Direct effects of DNA damage and mutation on RAPD profiles.

Figure 4.4: An indication with arrows and boxes for the passages and RAPD profiles at which the most prominent changes first started for each cell type.

The first stable changes start at the 9th passage for HEK cells whereas in 10th and 12th in HUVEC and HELA cells respectively.

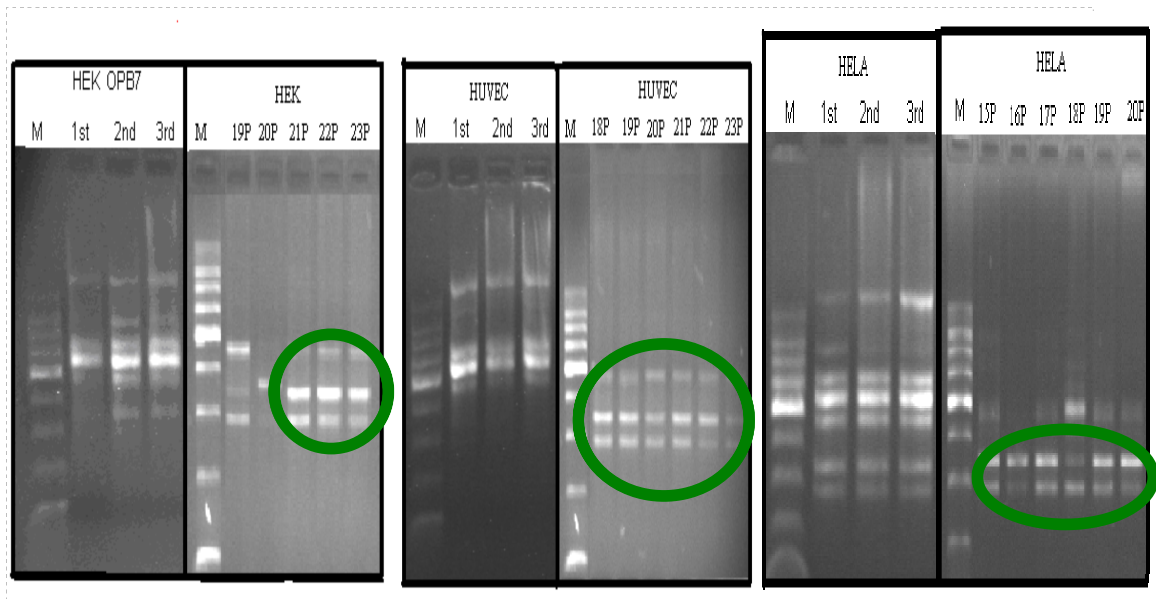


Figure 4.5: Comparable picture of the first and last passages RAPD profiles, circles indicating a pattern homogenization in the last passages no matter what the cell type was.

In different cell types the first passages and the last passages are compared in the above Figure 4.5. The stabilization can be easiy seen for the last passages. The homogeneity of band profiles are prominent. In all cell types the remaining bands were quite the same, monomorphic, although the starting profile bands were much more different, polymorphic. The remaining bands at the last passages were having band sizes in between 270 bp and 470 bp.

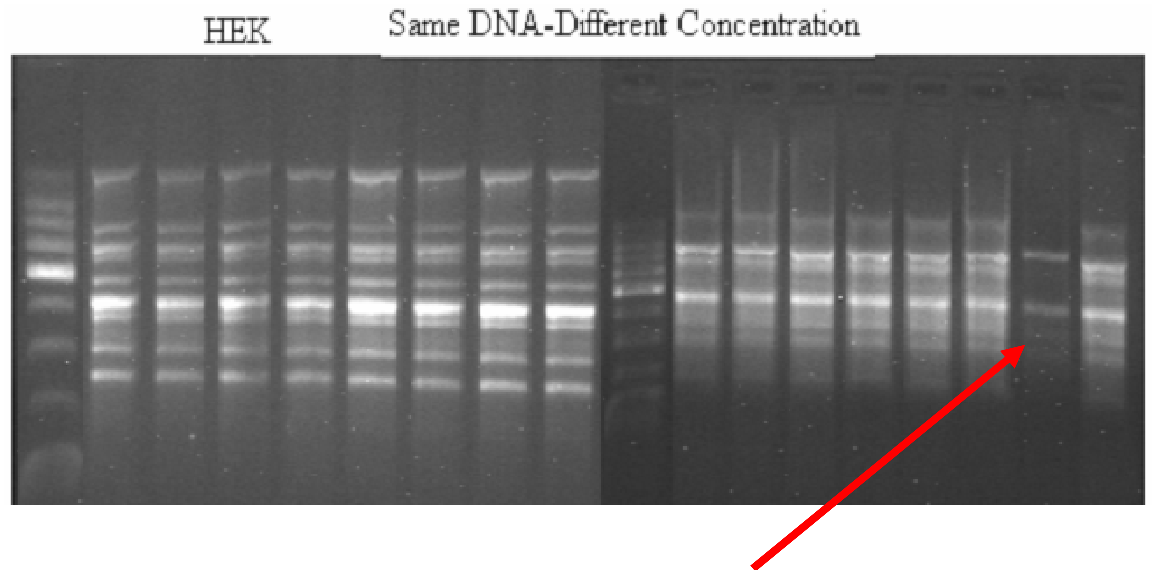


Figure 4.6: RAPD profiles as detected with x8 RAMD using HEK 18th passage DNA, arrow indicates DNA instability caught either DNA damage or mutation.

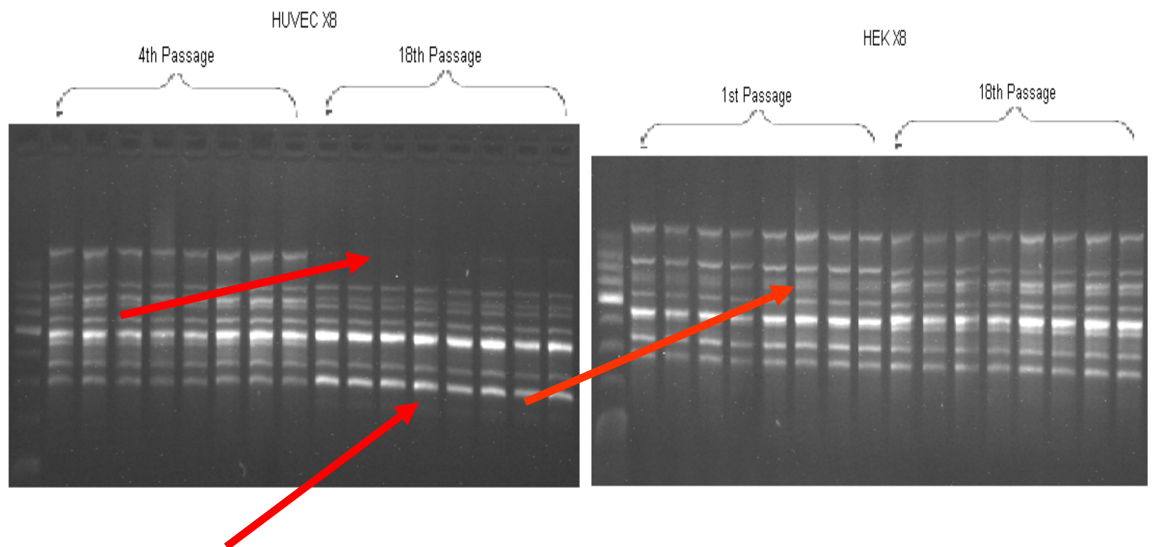


Figure 4.7: x8 RAMD-PCR profiles for starting and ending passages of HUVEC and HEK cells. Arrows indicating various profile changes that might be mutations.

The loss of band and intensity changes are very prominent for the long term comparisons shown in Figure 4.7, 4th and 18th passages for HUVEC and 1st and 18th passages for HEK

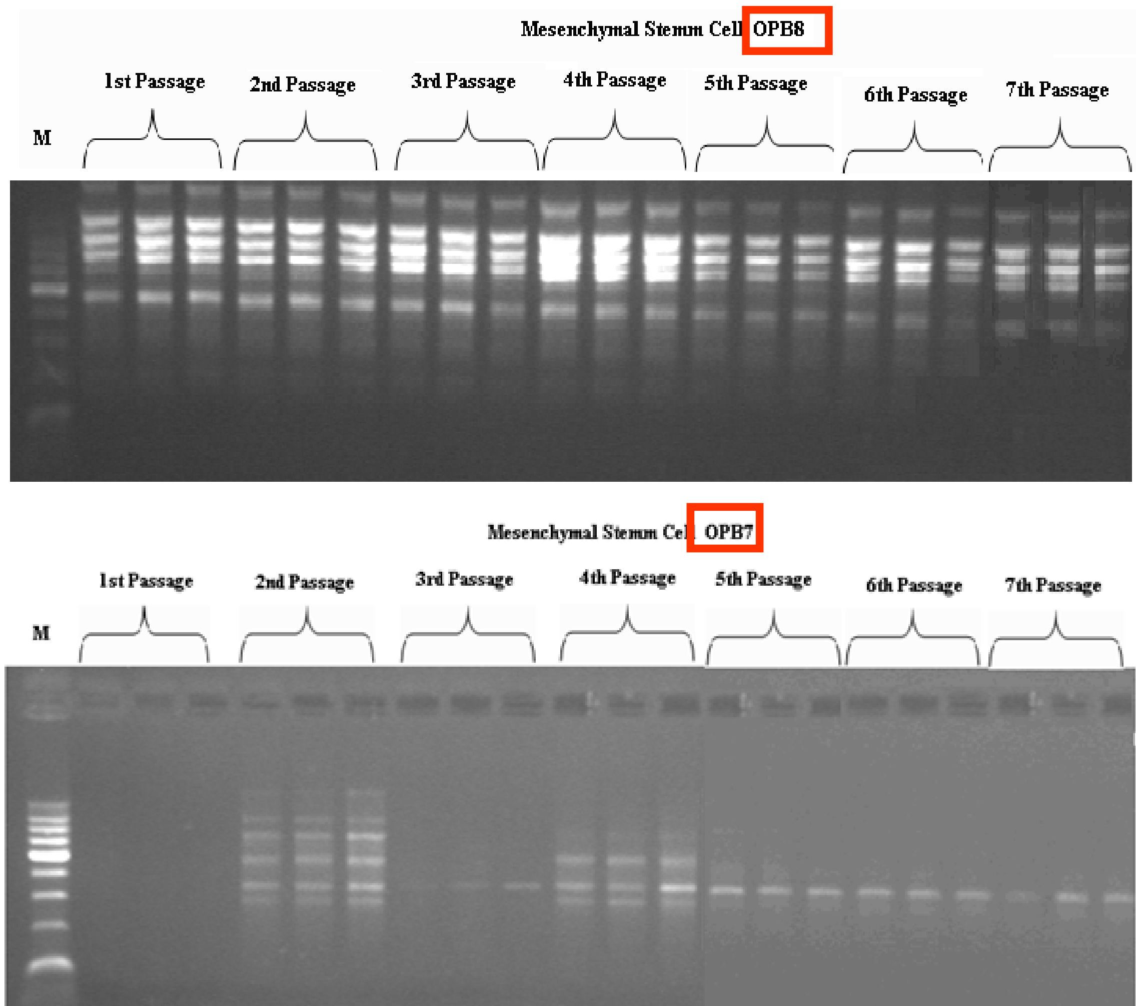


Figure 4.8: Two primer OPB8 and OPB7 x3 RAMD trials for the hMSC same passages' DNAs (1-7).

The Figure 4.8 shows the importance of primer choice and importance of looking at the genome using more primers, it is like the windows of a building if their number is high than you can see more of your environment. We looked at our hMSCs passage DNAs from two different views by using OPB8 and OPB7 primers and have seen a monomorphic pattern for OPB8. Changes were hardly detectable whereas with the other primer OPB7 many profile changes could be detected.

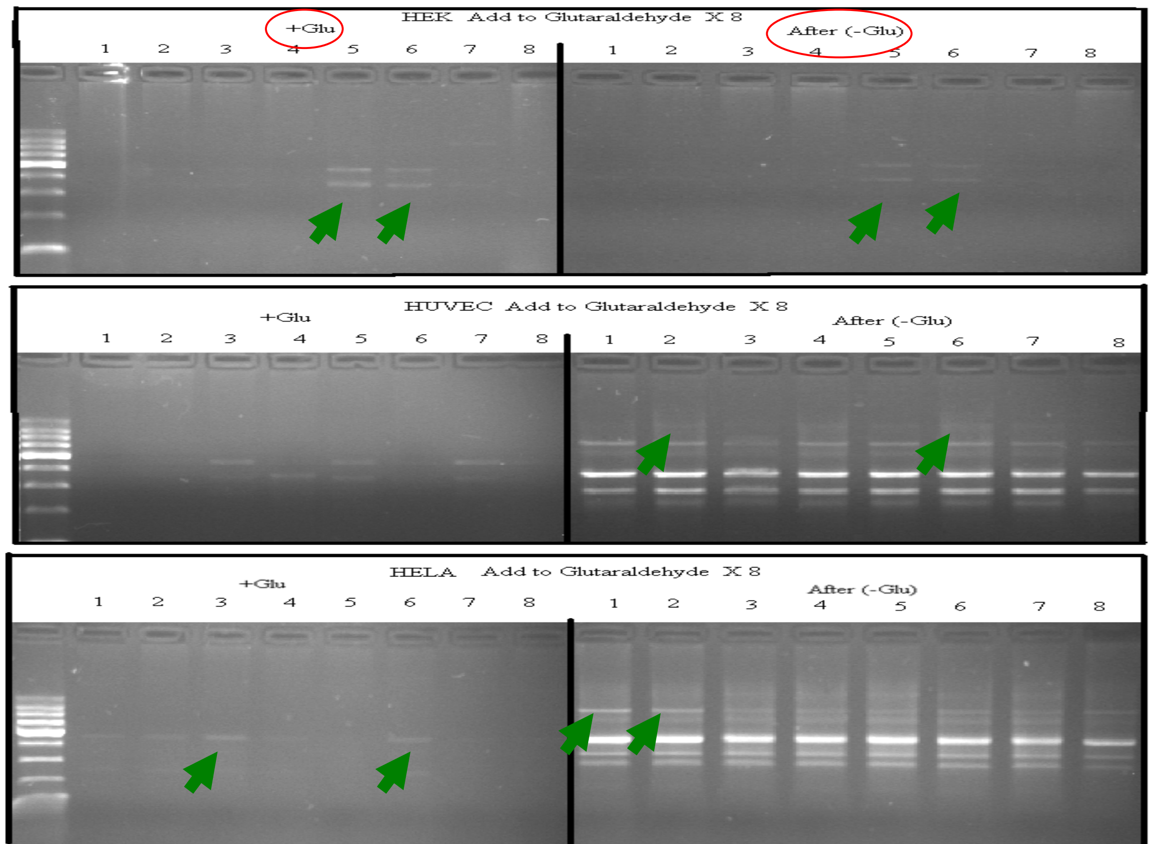


Figure 4.9: Gluteraldehyde exposed DNA x8 RAMD profiles and after removal of gluteraldehyde, the next passages' DNA x8 RAMD profiles. The arrows indicate the initial and final profile changes in all cell types. +Glu is for gluderaldehyde added ones and after –glu is after removal of gluteraldehyde from the medium.

The Figure 4.9 shows the DNA damaging effect of gluteraldehyde which is a DNA-protein crosslinker on our different cell types.The first pictures are the Gluteraldehyde exposed cells' profiles. Profiles are for 8 times RAMD-PCR of 21st passages for exposed and 22nd for the gluteraldehyde removed cell samples.

For HUVEC and HELA cells the DNA effects are reverted back indicating that the changes are DNA damages, but for the more dividing cell, HEK cells the effects are not reverted back, indication of mutation. The repair mechanisms can not work properly for HEK cells and this might be an indication that the fast dividing cells can have diffuculties in repair processes which might be causing further pathology.

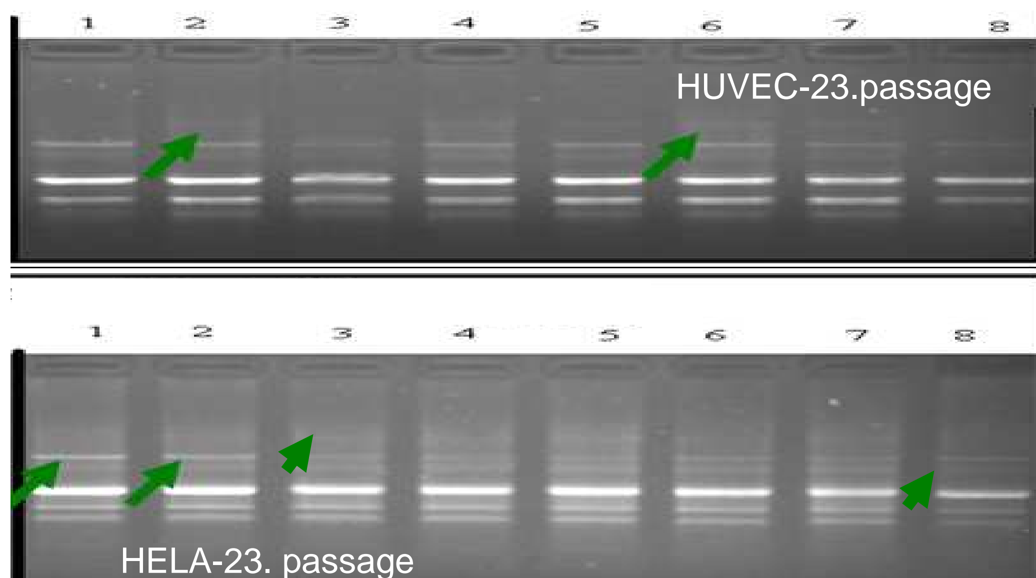


Figure 4.10: A closer look at the types of changes in the reverted profiles still having changes indicated by arrows.

Let us look more closely to the reverted profiles, but still there exists changes that are not reverted back they may become mutations or still they are DNA damages waiting to be repaired.

The effects on DNA should be endogenous, because in cell culture we aim to have a controlled environment for the cells to continue with the least change. There is a change, a continuous change, but it is detectable in long term rather than changes observed in each passage. The RAPD profiles obtained are in agreement with this. The RAMD-PCR profiles that can detect the intra-specific DNA variation, mosaicism of the individual passage DNA are very homogenous, monomorphic indicating there is a change and if there is a change that is DNA damage.

We can not really discuss any results related to ageing because ageing is more related with the non-dividing cells and in cell culture it is not really possible to show the accumulation of DNA damages in non-dividing cell types, because the cells continue to divide and we could extract the DNA and work with it only in some ranges but not below that range otherwise our system fails.

CHAPTER 5

CONCLUSION

In conclusion, while RAPD and related techniques have been used extensively for diverse studies, application of these techniques has also attracted criticisms with respect to its reproducibility that we have overcame in this thesis by RAMD-PCR approaches. However, it emerges that most of the criticism relates to lack of proper optimisation and validation of the techniques in different cell types and species, prior to their applications under *in vivo* or *in vitro* conditions. Nevertheless, the RAPD assay and related assays offer great promise especially for the determination of genetic damage under *in vivo* conditions in wild species and for the evaluation of genomic instability in the process of carcinogenesis. While a large number of new technologies and assays are developing to profile the gene expression pattern either following exposure to environmental contaminants or during the process of malignant development, the RAPD based techniques offer great promise for future and would continue to complement other new and well-established techniques in population genetics, genotoxicity and carcinogenesis studies and cell-culture work in view of this thesis.

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