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GENOMIC INSTABILITY MAY BE A SIGNAL OF HUMAN EMBRYONIC STEM CELL DIFFERENTIATION

By

Clara Ines Esteban-Perez

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Biology
in the Department of Biological Sciences

Mississippi State, Mississippi

April 2011

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By

Clara Ines Esteban-Perez

GENOMIC INSTABILITY MAY BE A SIGNAL OF HUMAN EMBRYONIC STEM CELL DIFFERENTIATION

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Embryonic stem (ES) cells have the ability to maintain pluripotency and self-renewal during *in vitro* maintenance, which is a key to their clinical applications. ES cells are a model in developmental biology studies due to their potential to differentiate *in vitro*. Understanding critical pathways of pluripotency, self-renewal, and differentiation during early embryonic development is important for the evaluation of the therapeutic potential of ES cells because of their ability for tumor transformation due to genetic and epigenetic instability acquired during *in vitro* culture maintenance. Single tandem repeats are sequences of DNA that have been implicated in the deregulation of gene expression in different human conditions. Understanding the origin of repetitive sequence instability and functions in the genome allow characterization of early genomic instability signals in ES cell pluripotency, differentiation, and tumor transformation pathways. The hypothesis of this study was that genetic stability, in repetitive sequences, located near embryonic developmental genes is responsible for pluripotency, self-renewal, differentiation, and chromatin assembly and could be a signal for adaptation, differentiation, or

transformation of ES cells *in vitro.* Our result showed instability in specific repetitive sequences which increased during ES cell passages and embryoid body differentiation *in vitro.* ES cells displayed significant mean frequencies of genomic instability in repetitive regions that lead to ES cells pluripotency, self-renewal maintenance, or cell lineage specialization. The present study reports potentially biomarkers for identifying accumulation of genomic instability in specific genes that may contributes to adaptation of ES cells and could be the switch that initiates early ES cell lineage commitment *in vitro*. Determining genetic and epigenetic modifications, including single tandem repeat instability, gene expression changes, and chromatin modifications, is essential for elucidating possible molecular mechanisms of genomic instability and determining novel molecular characterization for diagnostic purposes to ensure ES cell stability and integrity that could potentially lead to use of ES cell derivatives that could then be a safe source needed for regenerative medicine applications.

DEDICATION

Quiero dedicar este triunfo profesional a mis padres Carlos y Arcelia por sus consejos, por el amor que siempre me han brindado y por el sabio don de la paciencia y responsabilidad que me han inculcado. Mi triunfo es de ustedes. Los amo y agradezco a Dios la bendicion de poder a cada paso tenerlos conmigo, solo Dios sabe la dicha que es dedicarles este trabajo. A "bitos" Humberto y Odilia por su espíritu alentador que nos ayudo a continuar para lograr los objetivos propuestos. Quiero robarle minutos a las horas para que Dios nos permita recoger juntos en familia la cosecha de todos estos esfuerzos.

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"Desde lo alto de una montaña somos capaces de ver todo pequeño, nuestras glorias y nuestras tristezas dejan de ser importantes, aquello que conquistamos o perdimos esta abajo. Desde lo alto de la montaña, vemos que el mundo es grande y los horizontes infinitos…consiguiendo recordar que la vida sigue siendo una aventura" Paulo Coehlo.

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TABLE OF CONTENTS

Page

DEDICATION ii

ACKNOWLEDGEMENTS iv

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiii

CHAPTER

I. INTRODUCTION 1

1.1 Review of pertinent literature 7
1.1.1 Embryogenesis and embryonic stem cell origins 7
1.1.2 Pluripotency and self-renewal of embryonic stem cells 11
1.1.3 Differentiation of embryonic stem cells 13
1.1.4 Genomic instability in embryonic stem cells 14
1.1.5 Epigenetic instability in embryonic stem cells 17
1.1.6 Applications of embryonic stem cell 20
1.1.7 Cell transformation and tumorigenesis signaling 21
1.2 Significance of the research 22
1.3 References 24

II. DEVELOPMENT OF SINGLE TANDEM REPEAT SEQUENCE MULTIPLEXES FOR HUMAN EMBRYONIC STEM CELL EVALUATIONS OF GENOMIC AND EPIGENOMIC INTEGRITY DURING *IN VITRO* CULTURE 37

2.1 Abstract 37
2.2 Introduction 38
2.3 Methodology 39
2.3.1 DNA samples 39
2.3.2 Selection of tandem repeat sequences 39
2.3.3 Primer design 40
2.3.3.1 Software designer 40

2.3.3.2 Gene bank primer sequences 40
2.3.4 Singleplex PCR optimization....................................41
2.3.5 Multiplex PCR Optimization....................................42
2.3.6 PCR enhancers....................................43
2.3.7 Final PCR standardized conditions....................................43
2.3.8 Detection and analysis of PCR products....................................43
2.4 Results....................................44
2.4.1 Single tandem repeats are located near embryonic developmental genes....................................44
2.4.2 Size of repetitive markers 44
2.4.3 Optimization of PCR conditions in order to detect repetitive sequences 45
2.4.3.1 Primer concentration....................................45
2.4.3.2 PCR standard components 45
2.4.3.3 PCR standard conditions....................................46
2.4.4 PCR enhancers increased successful amplification of STR multiplexes....................................46
2.4.5 Residual dye artifacts....................................47
2.4.6 DNA concentration as a determinant factor on STR multiplexes validation....................................48
2.5 Discussion....................................49
2.6 Acknowledgements....................................64
2.7 References....................................65

III. EMBRYONIC STEM CELL GENOMIC INSTABILITY RESULTING FROM CULTURE PASSAGES MAY BE A MECHANISM OF ADAPTATION AND PLURIPOTENCY MAINTENANCE 67

3.1 Abstract....................................67
3.2 Introduction....................................68
3.3 Materials and Methods....................................70
3.3.1 Embryonic stem cell maintenance 70
3.3.2 Mouse embryo fibroblast CF1 feeder layer 70
3.3.3 Embryonic stem cell passages 71
3.3.4 Immunohistochemical analysis....................................71
3.3.5 DNA isolation 72
3.3.6 Single tandem repeat markers selection and standardization 72
3.3.7 Genomic instability determination by single cell PCR....................................73
3.3.8 Statistical analysis of genomic instability....................................75
3.4 Results....................................75
3.4.1 Embryonic stem cell culture maintenance 75
3.4.2 Embryonic stem cells displayed morphological changes across passages....................................76
3.4.3 Genomic instability in single tandem repeat markers mediated embryonic stem cell culture adaptation....................................76

3.4.4 Genomic instability could be a signal of embryonic stem cell pluripotency and self-renewal loss during long term cell culture 77
3.4.5 Epigenetic changes that occur during embryonic stem cell *in vitro* culture could result from genomic instability 78
3.5 Discussion 79
3.6 Acknowledgements 103
3.7 References 104

IV. GENOMIC INSTABILITY DURING EARLY DIFFERENTIATION OF EMBRYONIC STEM CELLS 113

4.1 Abstract 113
4.2 Introduction 114
4.3 Materials and Methods 117
4.3.1 Embryonic stem cell culture conditions 117
4.3.2 Embryoid body formation 118
4.3.3 Histopathology 118
4.3.4 Immunohistochemical analysis 119
4.3.5 Germinal layer separation from embryoid bodies 119
4.3.6 DNA isolation 120
4.3.7 Determination of genomic instability with single cell PCR 120
4.3.8 Statistical analysis of genomic instability 121
4.4 Results 122
4.4.1 ES cells spontaneously differentiate into embryoid bodies *in vitro* 122
4.4.2 Genomic instability is present during ES cells differentiation *in vitro* 123
4.4.3 Single tandem repeat instability increased during EB formation over time 123
4.4.4 Embryoid bodies from H1 and H7 ES cell lines show different unstable marker profiles 124
4.4.5 Instability in repetitive regions related to differentiation genes coordinate cell fate decisions 125
4.4.6 Epigenetic modification during spontaneous EB differentiation a result from genomic instability 126
4.5 Discussion 127
4.6 Acknowledgements 151
4.7 References 152

V. UNSTABLE REPETITIVE SEQUENCES LOCATED NEAR GENE PROMOTER COULD BE A SIGNAL FOR REGULATION OF GENE EXPRESSION DURING TUMORIGENESIS 158

5.1 Abstract 158
5.2 Introduction 159

5.3 Materials and Methods 161
5.3.1 Cell culture 161
5.3.2 *In vitro* exposure to hydrogen peroxide 161
5.3.3 RNA isolation 162
5.3.4 Real time PCR 162
5.3.5 Real time gene expression statistical analysis 163
5.4 Results 163
5.4.1 Chromosome location of unstable markers and neighboring genes 163
5.4.2 Instability is a signal of gene expression changes in ovarian cells 164
5.4.3 DNA repair genes deregulate as a cause of instability of the upstream single tandem repeats 165
5.4.4 Genes involved in cell growth signals are expressed differentially in ovarian cancer cells 166
5.4.5 Instability is present in markers linked to deregulated genes involved in intracellular signals during tumorigenesis 166
5.4.6 AUTS2 gene is deregulated during ovarian cancer 167
5.5 Discussion 167
5.6 Acknowledgements 180
5.7 References 181

REFERENCES 185

APPENDIX

A TANDEM REPEAT MOTIF LOCATED IN PROXIMAL PROMOTER REGIONS OF PLURIPOTENCY, SELF-RENEWAL, AND DIFFERENTIATION GENES 208

B H1 (WA01), H7 (WA07) HUMAN EMBRYONIC STEM CELL LINES AND MEF CF-1 MOUSE FIBROBLAST CELL LINE INFORMATION AND CERTIFICATES OF ANALYSIS 213

C SP-PCR SOFWARE VERSION 2.0 FROM M.D. ANDERSON CENTER INTRUCTIONS AND MUTATION FREQUENCIES REPORTS (http://www.hkasoftware.com/index.php?object=SPPCR) 217

LIST OF TABLES

TABLE Page

2.1 Characteristics of single tandem repeats analyzed 51

2.2 List of single tandem repeat primers sequences 53

3.1 List of single tandem repeat markers 97

3.2 Mutation frequencies of five single tandem repeat markers located near genes related to pluripotency and differentiation 98

3.3 Mutation frequencies of three single tandem repeat markers located near genes related to chromatin assembly 99

3.4 Mutation frequencies of three single tandem repeat markers located near genes related to imprinting genes 100

3.5 List of unstable markers 101

4.1 List of single tandem repeat markers analyzed in samples of embryoid bodies from H1 and H7 ES cell lines 145

4.2 Mean mutation frequencies of eight unstable markers in embryoid bodies differentiated from H1 and H7 ES cell lines..146

4.3 Mean mutation frequencies of seven unstable markers that displayed statistically significant differences in EBs from the H1 ES cell line compared to EBs from the H7 ES cell line 147

4.4 Mean mutation frequencies of three unstable markers that displayed statistically significant differences in EBs from the H7 ES cell line compared to with EBs from H1 ES cell line 148

4.5 List of genes associated with unstable markers 149

5.1 List of genes evaluated by real time PCR 179

5.2 Changes in gene expression for normal and cancerous ovarian cells 179

LIST OF FIGURES

FIGURE Page

1.1 Diagram of human embryonic development. 8

2.1 Examples of electropherograms. 56

2.2 Examples of Electropherograms 57

3.1 Flowchart of H1 and H7 ES cell line maintenance in culture 86

3.2 Examples of electropherograms 87

3.3 ES cell characterization 90

3.4 Morphologies of H1 ES cell colonies 91

3.5 Percentage of H1 and H7 ES colony morphology changes *vs* culture passages 92

3.6 Number of unstable markers across culture passages 93

3.7 Unstable markers related to genes involved in pluripotency and early differentiation 94

3.8 Unstable markers related to chromatin assembly genes....95

3.9 Unstable markers related to imprinting genes....96

4.1 Examples of electropherograms from mononucleotide unstable markers. 134

4.2 Histological characterizations of embryoid bodies from ES cells 138

4.3 Immunofluorescent characterization of embryoid bodies differentiated from ES cells 139

4.4 Mean mutation frequencies of unstable markers across EB samples at three points of time....140

4.5 Unstable markers in embryoid bodies differentiated from H1 and H7 ES cell lines....141

4.6 Unstable markers of embryoid bodies differentiated from H1 and H7 ES cell lines 142

4.7 Embryoid bodies from the H1 ES cell line differentiated into early neuroectodermal tissue after 30 days 143

4.8 Embryoid bodies from the H7 ES cell line differentiated into cardiac muscle tissue (early mesoderm) 14 days after in *vitro* differentiation induction 144

5.1 BAT 26 single tandem repeat marker 173

5.2 BAT 60 single tandem repeat marker 173

5.3 D7S3046 single tandem repeat marker 174

5.4 DXS9902 single tandem repeat marker 174

5.5 DXS6801 single tandem repeat marker 175

5.6 Changes in expression of DNA repair genes 176

5.7 Changes in expression of genes responsible for cell growth 177

5.8 Changes in expression of genes involved in tumorigenesis and one gene candidate for susceptibility of autism 178

LIST OF ABBREVIATIONS

BMP4	Bone Morphogenesis Protein 4
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridization
CI	Chromosome Instability
CPM	Contraction per Minute
DMEM	Dulbecco's Modified Eagle Medium
DMR	Differentially Methylated Regions
DMSO	Dimethylsulfoxide
DNMT	DNA (cytosine-5)-methyltransferase
EB	Embryoid Body
ES	Embryonic stem
FAM	Fluorescent Dye 6-Carboxyfluorescein
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GI	Genomic Instability
ICM	Inner Cell Mass
HEX	Fluorescent Dye Hexachlorofluorescein

H_2O_2	Hydrogen Peroxide
iPS	Induced Pluripotent Stem
LIF	Leukemia Inhibitor Factor
MEF	Mouse Embryonic Fibroblast
MEF-CM	Mouse Embryonic Fibroblast-Conditionated medium
MMR	Mismatch Repair
MSI	Microsatellite Instability
NANOG	Nanog Homeobox
OCT-4	Octamer-4 Transcription Factor
OS	Oxidative Stress
PCR	Polymerase Chain Reaction
POU5F1	POU class 5 homeobox 1
RFU	Relative Fluorescent Units
RNAi	RNA interference
ROS	Reactive Oxygen Species
SAS	Statistical Analyis Sofware
SNP	Single Nucleotides Polymorphism
SOX2	SRY-Box Containing Gene
STR	Single Tandem Repeat
STS	Single Tandem Sequences
UTR	Un-Translated Region
WNT	Wingless-Type MMTV Interaction Site Family
XIST	X (Inactive)-Specific Transcript

CHAPTER I

INTRODUCTION

Genetic and epigenetic developmental processes regulate pluripotency and differentiation in embryonic stem (ES) cells *in vivo* and *in vitro*. ES cells are pluripotent and are derived from the inner cell mass of blastocysts. ES cells are distinguished from other cell types by the following special characteristics: they can be maintained in an undifferentiated state during extended culturing over time, and they have the capacity to differentiate into every cell type in the body (Evans and Kaufman 1981). There have been remarkable breakthroughs in science over the last 20 years that have resulted in defined culture conditions for reproducible *in vitro* culture systems for ES cell maintenance (Thomson *et al.* 1998; Amit *et al.* 2000; Takahashi and Yamanaka 2006; Yu *et al.* 2007; Yu *et al.* 2009; Ohta *et al.* 2011). More recently, the developmental potential, including culture conditions and growth factors required to direct the *in vitro* development of these cells down tissue specific pathways for the purpose of regenerative medicine, have been under study (Xu *et al.* 2001; Ogawa *et al.* 2006; Bigdeli *et al.* 2008; Cordes *et al.* 2009; Takemoto *et al.* 2011). Initially, ES cells were established in co-culture with a mouse embryonic feeder layer (MEF) (Evans and Kaufman 1981; Thomson *et al.* 1998). Later, nutrient requirements for culture became more specifically characterized after the discovery of particular growth factors derived from the feeder layer. Leukemia inhibitory factor (LIF) is a growth factor derived from the feeder layer that maintains the characteristics of pluripotency and self-renewal in ES cell culture (Xu

et al. 2001; Ogawa *et al.* 2006). ES cells can now be maintained on a feeder layer in serum free medium supplemented with basic fibroblast growth factor (bFGF) and LIF (Brimble *et al.* 2004). ES cells can now also be cultured in the absence of feeder cells if they are cultured on matrigel or laminin coated plates in media supplemented with conditioned media from MEF (MEF-CM) (Bigdeli *et al.* 2008).

Some reports show that ES cells cultured in these conditions for more than 100 passages are still able to maintain chromosomal stability and the capacity for differentiation into the three basic embryonic germ layers (mesoderm, ectoderm, and endoderm) *in vitro* as embryoid bodies (Amit *et al.* 2000) or *in vivo* as a teratoma (Evans and Kaufman 1981; Thomson *et al.* 1998; Mitalipova *et al.* 2005; Kamiya *et al.* 2011). Adaptation, survival, and growth of ES cells *in vitro* are facilitated by genomic instability. ES cells in culture, during late passages, show a higher genomic instability frequency than earlier passages. Genomic instability is characterized by overlapping in numerical chromosomal alterations (up to 45%), mitochondrial DNA mutations (up to 22%), and modifications on promoter gene methylation (up to 90%) (Maitra *et al.* 2005). Differences in the plasticity and ability for *in vitro* adaptation of ES cell lines is a result of incremental changes in genomic instability frequency leading to cellular and molecular modifications; this is frequently displayed as a proliferative advantage in late passages in contrast to early passages which are genetically and epigenetically stable (Inzunza *et al.* 2004; Allegrucci *et al.* 2007). Cellular adaptation resulting from genomic instability includes karyotype abnormalities, failure in X-inactivation, and epigenetic modifications that lead to imbalances between self-renewal and differentiation signals during *in vitro* culturing of ES cells (Enver *et al.* 2005; Shen *et al.* 2008; Bao *et al.* 2009).

ES cell research continues to face obstacles for clinical applications because of a wide range of variability in the maintenance of homogeneous and undifferentiated ES cells over time during culture passages (Toyooka *et al.* 2008; Ying *et al.* 2008). Furthermore, the signals or initial steps that originate deregulation of developmental gene expression and epigenetic changes still remain unknown. Transcription factors and the genetic network for pluripotency of ES cells have been widely described. POU class 5 homeobox 1(POU5F1, also known as OCT4), SRY-box containing gene 2 (SOX2), and Nanog homeobox (NANOG) are three key master transcription factors that have been identified and are responsible for the regulation and maintenance of pluripotency in ES cells. They regulate themselves through positive feedback expression and are responsible for the downstream transcriptional regulatory signals of more than 2,000 genes related to pluripotency, self-renewal, surveillance, and cell lineage commitment (Boyer *et al.* 2005; Loh *et al.* 2006; Chen *et al.* 2008).

ES cells that differentiate lose their pluripotency status and gain the lineage-specific signature via expression of their cell/tissue identity through gene and chromatin modifications in the promoter regions of developmental genes responsible for pluripotency and early cell differentiation (Mohn *et al.* 2008). Differentiation results from alterations in ES cell pluripotency and self-renewal. Maintenance of a differentiated state is a constant process of gene repression and/or activation coupled with chromatin modifications that modulate specific signals that induce morphological and functional characteristics in early cell progenitor derivatives during embryonic development (Niwa *et al.* 2005). These genetic and epigenetic modifications guarantee expression of genes involved in cell fate lineage and inactivation of developmental genes involved in pluripotency. Covalent histone acetylation and methylation, chromatin remodeling,

nucleosome assembly, and DNA methylation are all examples of epigenetic modifications that result in packaging DNA. Developmental gene sequences become inactivated; leading to their complete repression, avoidance of transcriptional protein complexes formations, and certification that cell/tissue specificity (differentiated state) would be maintained. Cell differentiation and tumor transformation both share several molecular signaling pathways, including gene expression and epigenetic modifications (Karakosta *et al.* 2005; Proia *et al.* 2011). Tumor cells display losses in genome integrity due to accumulation of DNA damage induced by oxidative stress (Fearon and Vogelstein 1990). Unrepaired instability in single tandem repeat sequences can induce frame-shift mutations in coding and non-coding regions of DNA, leading to failure in cellular regulatory pathways such as cell cycle control, apoptosis, and DNA repair which are needed in order to avoid cell transformation and maintain a differentiated state (Imai *et al.* 2008).

When developmental genes are deregulated during neoplastic transformation, it leads to cellular responses such as proliferation, de-differentiation, migration, invasion, and angiogenesis, which ensure and ideal environment for tumor transformation (Gupta *et al.* 2005; Ince *et al.* 2007). For example, aggressiveness and invasiveness are fundamental characteristics of ovarian and breast tumor progression. Several authors suggest that the ability of these cells to rapidly metastasize to different organs is due to cell signals that trigger reactivation of developmental genes containing genomic instability that may have originated during embryonic development; environmental "triggers" could deregulate these genes, acting as an on-switch for cell tumor transformation (Gupta *et al.* 2005). Genomic instability does not have to occur within a gene to affect its expression. Instability could occur in flanking regions of developmental

genes that are regulatory elements located upstream of promoters such as enhancer or repressor sequences that modulate transcription factor binding. Several studies reported the presence of regulatory elements localized in neighboring genes as enhancer or repressor sequences determined to be responsible for transcription modulation (Lettice *et al.* 2003; Kleinjan *et al.* 2006; Panne *et al.* 2007; Visel *et al.* 2009). Regulatory elements are located upstream or downstream of transcription start sites. Some authors report that they are within a 5 kb distance and others report distances up until 1 Mb (Lettice *et al.* 2003; Kleinjan *et al.* 2006; Visel *et al.* 2009). Several regulatory elements, or enhancers, are binding sites of specific gene regulatory protein complexes that define and allow for the sequential, specific development of embryos. Genetic control systems are established early in development and cell fate is determined; cell memory mechanisms maintain cellular specialization by remembering the early signals introduced during embryonic development (Alberts *et al.* 2008).

We hypothesized that genomic instability in repetitive DNA sequences upstream or downstream of specific genes could be a signal that regulates their expression; our interest is in genes responsible for pluripotency, self-renewal, differentiation, or tumor transformation. This instability could lead to activation or repression of transcriptional regulatory elements in either normal ES cells or cancerous cells. Our objectives to test this hypothesis were to:

1) Identify single tandem repeat sequences located near promoters of developmental regulatory genes transcribed by the OCT4, NANOG, and SOX2 transcription factors responsible for pluripotency and self-renewal of ES cells,

2) standardize use of primers to detect single tandem repeats in single genome equivalent PCR amplifications via fragment analysis techniques to allow determination of the mean frequencies of instability present per marker,
3) determine the accumulation of instability in these single tandem repeats during ES cell culture of H1 and H7 ES cell lines by comparing mean frequencies of instability at three cell passage ages,
4) identify pluripotency genes located near significantly unstable single tandem repeats that could possibly be responsible for ES cell adaptation *in vitro*,
5) determine the accumulation of instability in single tandem repeats during ES cell differentiation into embryoid bodies (EBs) by comparing mean frequencies of instability in H1 and H7 ES cell lines at three different times post EB initiation,
6) identify differentiation and chromatin assembly genes located near significantly unstable single tandem repeats that could possibly be responsible for ES cell differentiation *in vitro*,
7) expression pattern of genes located near unstable single tandem repeats in cancerous ovarian cells in comparison with normal ovarian cells after 9 days post-H_2O_2 exposure,
8) determine and describe possible instability signals (transcriptional regulators) of gene expression in pluripotency, differentiation, chromatin assembly and imprinting genes during ES cell culture and cell differentiation *in vitro* or during cell transformation in ovarian cancer initiation and progression,

9) contribute to the identification of possible biomarkers that could be useful for screening and determining the quality of ES cells to be used for regenerative therapies,
10) and identify possible biomarkers that could be used as diagnostic or prognostic tests during cell transformation, progression, metastasis, or treatment of tumors.

1.1 Review of pertinent literature

1.1.1 Embryogenesis and embryonic stem cell origins

During mammalian ovulation and fertilization, once an oocyte is in the fallopian tube, it oocyte completes metaphase II after extrusion of the first polar body. Once fertilized with sperm the oocyte is activated, and the second polar body is then extruded. Immediately, sperm DNA remodeling is initiated and takes approximately 6-8 hours. This includes decondensation of sperm chromosomes, giving rise to the first pronucleus (male pronucleus), and also decondensation of oocyte chromosomes giving rise to the second pronucleus (female pronucleus). The pronuclei are haploid, each containing one set of chromosomes (Kiessling and Anderson 2007; Alberts *et al.* 2008). DNA synthesis is then initiated independently in both pronuclei. Next, pronuclei fusion occurs and the zygote is ready for the first cleavage which is an equal division into two daughter cells, each containing a diploid set of chromosomes; this occurs approximately 22 to 26 hours after fertilization (Braude *et al.* 1988; Alberts *et al.* 2008) (Figure 1.1).

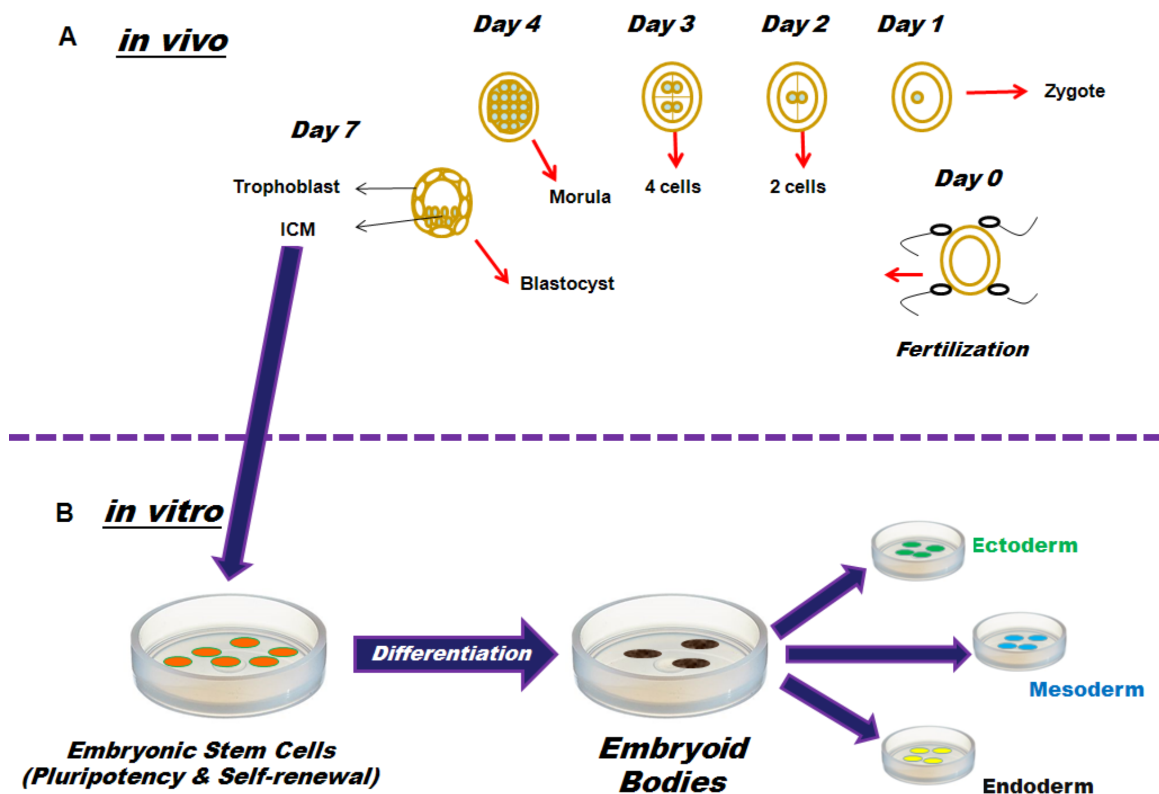

Figure 1.1 Diagram of human embryonic development.

Notes: (A) *In vivo* human embryonic development, starting with fertilization on day zero, goes through serial cleavages that occur starting at the two cell embryo stage at 2 days post-fertilization, until more than 200 cells are present in the morula stage at day 4. On day 7 post-fertilization, the first embryonic commitment signal for transformation into a blastocyst occurs. An external layer of trophoblast is differentiated and surrounds the ICM that will gives rise to the three germinal layers after implantation of the blastocyst (B) *In vitro* isolation of ES cell lines from the ICM of the blastocyst lead to the posterior differentiation of embryoid bodies, resulting in the generation of the three germinal layers.

These two daughter cells are known as a blastomere which is totipotent, a status defined by the capacity to derive a complete individual (placenta, extraembryonic membranes, and embryo). The second cleavage of the two cell blastomere results in four cells, and each successive cleavage results in the doubling of blastomere cells. At the fifth cleavage of 16 cells into 32 cells 3 days after fertilization, the blastomere is now at the

morula stage. (Braude *et al.* 1988; Kiessling and Anderson 2007; Alberts *et al.* 2008). At this stage, there is induction of the first embryonic commitment signal that transforms into a blastocyst, composed of an external layer of trophoblast that gives rise to the placenta and an internal group of cells called the inner cell mass (ICM). The ICM is what gives rise to the embryo (Kiessling and Anderson 2007; Alberts *et al.* 2008). ICM cells are pluripotent, meaning they can differentiate into any cell type from the three embryonic layers, but cannot become placental tissue (Thomson *et al.* 1998; Reubinoff *et al.* 2000). At this stage, the blastocyst arrives in the uterus, where the ICM has a second differentiation event, taking on a flat appearance and giving rise to the primitive endoderm. The primitive endoderm creates the extra embryonic membranes, including the amniotic sac that contains the fetus during development (Enders and King 1988).

Interaction of the blastocyst with the endometrium starts implantation on the day after fertilization; the trophoblast invades uterine epithelium and placenta formation begins (Georgiades *et al.* 2002). After implantation, the bulk of the embryonic stem cells begin undergoing differentiation events that commit them into the three germinal layers, the outer germ layer, and ectoderm, is the precursor for the epidermis and the nervous system. The inner germ layer, endoderm, is the precursor for the gut, lung, and liver. The middle germ layer, mesoderm between ectoderm and endoderm, is the precursor for muscle and other connective tissues (Pelton *et al.* 2002; Kiessling and Anderson 2007; Alberts *et al.* 2008).

In research, *in vitro* techniques have been used to develop embryos up until the blastocyst stage when the ICM can be isolated and maintained as an embryonic stem cell line in culture that preserves pluripotency and self-renewal features across passages (Thomson *et al.* 1998). Additionally, this preserves the ability *in vitro* to differentiate into

embryoid bodies (EBs) of any cell type from the three embryonic layers (mesoderm, ectoderm and endoderm) (Itskovitz-Eldor *et al.* 2000). When ES cells are injected into a mouse with immune suppression, the ES cells have the capacity to form teratomas *in vivo.* These teratomas can contain structures that resemble gut epithelium (endoderm layer), smooth and striated muscle (mesoderm layer), and neural epithelium (ectoderm layer) (Caricasole *et al.* 1998).

ES cells are classified into the following three categories according to their level of potency and plasticity: (1) totipotent cells have a capacity to give rise to an entire organism (e.g. blastomere), (2) pluripotent cells have the ability to give rise to the three embryonic layers but cannot develop extra embryonic tissue and a fetus (e.g. ICM/ES cells), and (3) multipotent cells have the ability differentiate into mature somatic cells for a specific tissue and have lost the ability to differentiate into any other tissue type (e.g. hematopoietic stem cells that differentiate in red and white blood cells and platelets). Currently, *in vitro*, human ES cells have been differentiated into neuroectoderm (Carpenter *et al.* 2001; Schulz *et al.* 2003; Ben-Hur *et al.* 2004), hematopoietic progenitors (Chadwick *et al.* 2003), endothelial cells (Gerecht-Nir *et al.* 2003), osteoblasts (Sottile *et al.* 2003), cardiac muscle (Kehat *et al.* 2001; Mummery *et al.* 2003; Mazhari and Hare 2007; Yang *et al.* 2008), pancreatic β cells (Assady *et al.* 2001; Burke *et al.* 2007), hepatic cells (Rambhatla *et al.* 2003), and skin cells (Green *et al.* 2003). ES cells differentiation into functional cell fate lineages of pancreas, heart, and neural systems are under constant scientific research.

ES cell embryologic development research has evolved for different tissue types to determine and validate of possible tissue engineering techniques for potential clinical applications. For example, the pancreas is an organ that plays an important role in

glucagon production which is essential for glucose regulation. Diabetes type I is caused by the disruption of pancreatic β cells in the langerhans islets, allowing deregulation of glucose levels (Burke *et al.* 2007). Diabetes is treated by exogenous insulin injections. However, pancreatic cell transplantation can offer a better permanent solution, but insufficient numbers of compatible cells prevent this from being a successful therapeutic approach (Korsgren *et al.* 2005). Instead, ES cells differentiated and derived into the pancreatic cell lineage could be a solution for this disease that affects approximately 5 million people worldwide (Lu *et al.* 2007). Myocardial infarction is another example where ES cells could be derived into cardiac muscle cells as a treatment to replace damaged cardiac tissue, and in patients with potential heart failure; use of ES cell transplants as a source of cardiac cell remodeling could be part of cardio protective therapy (Mazhari and Hare 2007; Mazhari and Hare 2007; Yang *et al.* 2008). A final example is use in traumatic spinal cord injury that usually results in irreversible damage and disability. Transplanting ES cells derived into neuroprogenitors could be a solution for regenerating and repairing this damage, and, after supportive physical therapy, could offer recovery from that disability (Ben-Hur *et al.* 2004).

ES cell studies continue to evolve with the development of better protocols to direct differentiation and ensure genomic stability of specific, functional cell lineages derivatives to be used in cell transplantation and tissue regeneration applications.

1.1.2 Pluripotency and self-renewal of embryonic stem cells

As mentioned, pluripotency of early embryonic stem cells is maintained through key transcription factors, including OCT4, NANOG, and SOX2. These are considered to be the three master regulatory genes that control pathways of pluripotency, self-renewal,

surveillance, and cell lineage determination (Loh *et al.* 2006). They function as transcription factors that bind downstream target sequences of pluripotent genes, including fibroblast growth factor-4 (FGF4), undifferentiated embryonic cell transcription factor 1(UTF1), F-box protein 15 (FBXO15), and left-right determination factor 1 (LEFTY1) (Vallier *et al.* 2005). OCT4, NANOG, and SOX2 regulate their expression directly via positive feedback loops. Several reports have established that OCT4, NANOG, and SOX2 are the main transcription factors responsible for progression of early embryonic development *in vivo* and ES cell maintenance *in vitro* (Abeyta *et al.* 2004; Boyer *et al.* 2005; Babaie *et al.* 2007; Masui *et al.* 2007). Recently, Yu & Thomson *et al.* 2007 and Takahashi *et al. 2006* have demonstrated that these three master genes work together in concert with two additional transcription factors, namely *c-myc* and *Klf4*, to reprogram adult cells into induced pluripotent stem cells (iPS) from both embryonic and adult fibroblasts. Although it is known that both *c-myc* and *Klf4* function either directly or indirectly as oncogenes, their roles in early embryonic development *in vivo* relative to OCT4, NANOG, and SOX2 are less defined.

The OCT4, NANOG, and SOX2 transcription factors are expressed in undifferentiated ES cells (Boyer *et al.* 2005; Lee *et al.* 2006; Creyghton *et al.* 2010). Experiments which use interference RNA (RNAi) to selectively turn off each one of these genes, one or two at a time, provide clues as to their function during early embryonic development. For example, when both OCT4 and NANOG are silenced, cells lose their pluripotency and show inappropriate differentiation to inner cell mass, trophectoderm, and extra embryonic endoderm (Chambers *et al.* 2003; Alon 2007; Hu *et al.* 2009). The promoter region of 623 genes contains the target sequence (ATGCAAAT) for OCT4, 1,271 genes contain target sequences for NANOG and 1,687 genes contain

target sequences for SOX2. Also, OCT4, NANOG, and SOX2 are co-involved and overlap in promoter regions of at least 353 coding genes (Boyer *et al.* 2005; Chia *et al.* 2010; Fernandez-Tresguerres *et al.* 2010).

1.1.3 Differentiation of embryonic stem cells

Essential cellular processes ensure correct body formation during early embryonic development. First, cells proliferate by embryonic cleavage stages. All cells in the body originate from one cell (egg after fertilization). Second, cell specialization from the ICM to all the cells in the body is produced with their specific features. Third, cell interactions coordinate signals between cells and the surrounding environment. Fourth, cell migration causes cell assembly during embryonic development into tissues and organs (Alberts *et al.* 2008).

Functional genomics studies aimed at identifying key regulatory genes involved in the initiation of differentiation events have shown that LIF, bone morphogenesis protein 4 (BMP4), wingless-type MMTV interaction site family (WNT), and Fibroblast growth factor- beta (FGF-β) are all factors that play important roles in differentiation signaling pathways (Niwa *et al.* 1998; Ying *et al.* 2003; Goldstein *et al.* 2005). Initially, ES cells were isolated and maintained on mouse feeder layers that supplied critical growth factors for ES cell survival. Individual growth and inhibitory factors have been identified from these early experiments and have led to the discovery that ES cells can be maintained without feeder layers if the cultures are supplemented with LIF. When cultured ES cells are deprived of LIF, differentiation to primitive ectoderm occurs (Chen *et al.* 2008). Therefore, LIF has the ability to maintain ES cells in a stable pluripotent state *in vitro*. Also, the differentiation into skin and neural ectoderm is mediated by

BMP4 and WNT, respectively (Goldstein *et al.* 2005). BMP4 is also a regulator of germ cell development in the mouse embryo (Lawson *et al.* 1999) and acts to inhibit neuro-ectoderm development while allowing differentiation to mesoderm (Ying *et al.* 2003). WNT is a secretory protein that controls the cell cycle during mouse and human embryogenesis. The WNT pathway is activated during ES cell culturing in absence of MEF and retains undifferentiated characteristics for short periods of time (5-7 days). During embryoid body formation, WNT inhibits the secreted frizzled-related protein 2 (SFRP2) signal and leads to neural development (Sato *et al.* 2004).

1.1.4 Genomic instability in embryonic stem cells

DNA genomic instability is an accumulative process that leads to gene expression deregulation as a mechanism of ES cell culture adaptation *in vitro* or tumor transformation *in vivo*. Genomic instability is originated by different changes on single tandem repeat sequences, accumulation of point mutations, deletions, insertions, non-sense mutations, and numerical and structural rearrangements in the chromosomes (Niwa 2006; Imai *et al.* 2008; Martinez and Kolodner 2010). Genomic instability can lead to the disruption of gene expression network modulators that govern cell survival and growth advantages favoring adaptation during *in vitro* culturing (Niwa 2006).

Chromosomal instability (CI) displays disruption of DNA replication, telomere maintenance, DNA repair, chromosome condensation, sister chromatid cohesion, and cell cycles (Wang *et al.* 2004; Weaver and Cleveland 2007; Barber *et al.* 2008). Unrepaired genetic alterations have been shown to lead to oncogenesis, and these genetic changes mainly affect self-renewal, cell differentiation, apoptosis, and cell cycles, resulting in uncontrolled increases of cell growth. In this way, ES cells have a growth advantage *in*

vitro (Brimble et al. 2004; Maitra et al. 2005; Olariu et al. 2010). Karyotype abnormalities determined by cytogenetic analysis, comparative genomic hybridization (CGH), or single nucleotide polymorphisms (SNP) have been reported by many ES cells laboratories (Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005; Mitalipova *et al.* 2005).

Initially, the most frequent karyotype alterations showed in ES cells *in vitro* is gains of chromosomes 12 [isochromosome 12p (i12p)], 17q and X (Summersgill *et al.* 2001). Trisomies in chromosomes 1, 2, 3, 7, 8, 9, 11, 13, 14, 18, and 20 have also been reported but at a lower frequency (Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005; Mitalipova *et al.* 2005). These abnormalities have been observed during the oncogenesis process for tumors such as testicular germ cell tumors, seminomas, and choriocarcinomas (Abeyta *et al.* 2004; Mitalipova *et al.* 2005). Similar to tumor cells, the unstable chromosomes of ES cells carry genes involved with cell growth, self-renewal, and pluripotency. It is well established that CI occurs during later passages as a signal of adaptation in ES cells *in vitro* (Maitra *et al.* 2005; Mitalipova *et al.* 2005).

Interestingly, CI occurs in the key pluripotent gene NANOG that is located in chromosome 12p13.31 (Lindgren *et al.* 2011). Overexpression of this gene has been observed to promote self-renewal, prevent differentiation, and give advantages to the *in vitro* adaptation mechanism (Chambers *et al.* 2003). Other associated gene such as the developmental pluripotency associated 3 (DPPA3 also known as STELLA) gene located in the 12p13.31 region, codes for a protein that functions as a transcriptional repressor and is in charge of maintaining cell pluripotency (Nakamura *et al.* 2007). The growth differentiation factor 3(GDF3) gene, located in the 12p13.1 region, is a member of the bone morphogenetic protein (BMP) family and the transforming growth factor-beta

(TGF-β) superfamily that regulates cell growth and differentiation during embryogenesis (Levine and Brivanlou 2006). The Cyclin-D2 (CCND2) gene, located in 12p13 region, is a regulator of CDK kinases that regulate cell cycle G1/S transitions (Mai *et al.* 1999). Indeed, the 12p12.1 region has been reported to be a critical region for mutations and instability because it contains the oncogene vi-ki-ras 2 kirsten rat sarcoma viral oncogen homolog (KRAS) that is involved in tumorigenesis (Tol *et al.* 2010) and the SRY sex determining region Y-box5 (SOX5) gene which is responsible for determination of cell fate during embryogenesis (Martinez-Morales *et al.* 2010).

Another unstable chromosome found in ES cells lines is chromosome 17. Several groups have reported that ES cells tend to gain material from chromosome region 17q (17q21 and 17q23.2) (Thomson *et al.* 1998; Azuhata *et al.* 2001; Draper *et al.* 2004; Maitra *et al.* 2005; Mitalipova *et al.* 2005). Interestingly, the antiapoptotic gene baculoviral IAP repeat containing 5 (BIRC5) is located in this region. Also, genes abnormally up-regulated in ES cell lines with 17p11.2 aberrations are the topoisomerase DNA III alpha (TOP3A) gene located in the 17p12 region, mitogen-activated protein kinase 7 (MAPK7), and growth factor receptor bound protein 2 (GRB2) are both located in 17q24 region. These three genes are responsible for maintaining cells in an undifferentiated state and reducing apoptotic signals through transcription regulation during proliferation, differentiation, and embryonic development (Azuhata *et al.* 2001; Blagoev *et al.* 2003; Temime-Smaali *et al.* 2008; Rousseau *et al.* 2010).

Another chromosome that shows instability in some ES cell lines is the X chromosome which has been shown to have multiple copies (Thomson *et al.* 1998; Sperger *et al.* 2003). Gains of an X chromosome can cause failure of X inactivation when the X (inactive)-specific transcript (XIST) gene is not expressed (Sperger *et al.* 2003).

Some female ES cell lines do not express the XIST gene and their undifferentiated progeny shows active X chromosomes. It appears as though X inactivation occurs through differentiation progression (Dhara and Benvenisty 2004). Oncogenes, such as members of the ETS (ELK1) oncogene family located in the Xp11.2 region and v-raf murine sarcoma 3611 viral oncogene homolog (ARAF) located in the Xp11.4 region, are cell signaling molecules present in X chromosomes and may be involved in cell growth and development (Wu *et al.* 1996; Yang *et al.* 2003; Allegrucci *et al.* 2007).

1.1.5 Epigenetic instability in embryonic stem cells

Epigenetic factors regulate gene expression without changing DNA sequences. Promoter methylation or chromatin assembly modifications are responsible for this modulation of gene expression during pluripotency and cell lineage commitment early during embryonic development. ES cell lines carry inherent differences in gene expression and epigenetic modifications, including changes in the DNA methylation patterns of genes required for pluripotency, self-renewal, and differentiation (Allegrucci *et al.* 2007). Under appropriate differentiation conditions, ES cells have the potential to become any and all cell types in the human body (Shiota *et al.* 2002; Jaenisch and Bird 2003). Transcriptome deregulation, DNA methylation losses or gains, histone acetylation, and chromatin remodeling modifications can be acquired over time in ES cell *in vitro* cultures and are examples of epigenetic alterations (Jaenisch and Bird 2003). Epigenetic deregulation leads to reactivation of imprinted genes that lead to a loss of pluripotency and promotes cell differentiation or cell transformation.

DNA methylation is an epigenetic modification that regulates gene expression during cell proliferation, differentiation, imprinting, nucleosome remodeling, and X

chromosome inactivation. OCT4, SOX2, and NANOG are unmethylated in ES cells and become repressed by *de novo* DNA methylation as a signal of lineage commitment during early cell differentiation. The methylation patterns are maintained across the subsequent cell divisions, ensuring the epigenome cellular program of that specific lineage commitment (Ahmed *et al.* 2010). Promotor hypermethylation of genes responsible for embryonic development and cell differentiation are a crucial epigenetic modification for ES cell maintenance *in vitro* (Allegrucci *et al.* 2007). This silencing of developmental genes is maintained between subsequent cell cycles (Jaenisch and Bird 2003). In addition, the regulation of gene expression can be mediated by methylation of promoter regions associated with CpG islands which are also referred to as differentially methylated regions (DMRs). Usually, these DMRs are tissue specific (Shiota *et al.* 2002). The culture environment induces chromosomal and genomic instability in DNA methylation patterns and confers adaptation of ES cells *in vitro* to maintain an undifferentiated state over extended periods of time through methylation changes at sensitive loci. In contrast, DNA hypomethylation is frequently seen in ES cells during *in vitro* expansion and *in vivo* cancer transformation. Establishment and maintenance of DNA methylation is important in ES cell development, expansion, and genomic stability (Kim *et al.* 2004).

Effects of genomic instability and DNA methylation on mutation rates are now an important research focus for improving the culture environment of ES cells intended for therapeutic uses. Changes in the DNA methylation of gene promoters in undifferentiated cell during long-term *in vitro* expansion have been observed for imprinted genes such as insulin like growth factor 2 (somatomedin A) (IGF2) involved in embryonic development, and X-inactivated specific transcript (XIST) in charge of X inactivation

(Allegrucci *et al.* 2007). ES cell lines are characterized by a set of criteria that change during cell line development and differentiation. These criteria include differences in gene expression and can be observed in alterations of allelic expression in imprinted genes such as XIST, which is a crucial gene for X-inactivation (Adewumi *et al.* 2007; Shen *et al.* 2008).

In vitro culture of ES cells contributes to changes in CpG methylation patterns and genomic instability in different cell lines established over the years (Tomkins *et al.* 2002). The initiation and maintenance of XIST is extremely important for embryogenesis and adult cell physiology (Shen *et al.* 2008). Demethylation of XIST promoter CpG islands have been related to increased levels of gene expression of X-linked genes such as plastin 3 isoform 1 (PLS3) located in the Xq23 region, retinoblastoma binding protein 7 (RBBP7) located in the Xp22.2 region, and SWI/SNF related matrix associated and actin regulator of chromatin subfamily A member 1 (SMARCA1) located in the Xq25 region which are responsible for chromatin remodeling (Ye *et al.* 2009; Wirt *et al.* 2010). ES cell databases showing differentiation-associated gene expression revealed that ES cell lines exhibit patterns of loss of methylation in genes that are normally up-regulated during cellular differentiation. These changes in methylation are also similar to those that occur during tumorigenesis (Smiraglia and Plass 2002; Baker *et al.* 2007). In comparison to cell transformation during tumorigenesis, hypomethylation can induce microsatellite instability and chromosomal instability (Eden *et al.* 2003). DNMT1 and MLH1 have binding sites for several genes involved in DNA replication (Umar *et al.* 1996; Guo *et al.* 2004; Athanasiadou *et al.* 2010; Sen *et al.* 2010). It has been reported that human MLH1 is silenced by a hypermethylated pattern in its promoter CpG islands in about 15-20% of colorectal cancers that exhibit MSI (Imai *et al.* 2008). This association of DNMT1 and

MLH1 in the same pathway of genomic instability confirms that down-regulation of gene expression is due to changes in the epigenetic patterns of DNA methylation (Lengauer *et al.* 1997; Guo *et al.* 2004).

1.1.6 Applications of embryonic stem cell

More than 100 companies in 300 countries are interested in regenerative medical applications of ES cells (Parson 2008**.** Improvements in the standardized protocols for ES cell *in vitro* culture maintenance, isolation in xeno-free conditions, specific differentiation in all different cell lineages of the body, and large scale production can only continue depending on the scientific discoveries detailing the specifications to which ES cells can be safely used in clinical applications (Holm *et al.* 2010). Complete and constant interaction is needed between scientists and clinicians for the selection of appropriate patients that could be candidates for ES cell transplants. Although ES cells are the source of restoring tissue function, they need the best available tissue environment to guarantee their complete *in vivo* stability and functionality.

Clinical studies have evaluated and validated protocols to direct cell lineage specificity into one of three different embryonic layers. An example is the potential use of ES cells for pancreatic tissue development. Researchers studied gene pattern and transcription factors involved in pancreatic differentiation and the determination of activin and fibroblast growth factor-2 (FGF2) that mediated pancreatic development *in vitro* (Burke *et al.* 2007). One goal is to drive these pancreatic cells into specific β-cells for insulin production (Dor *et al.* 2004; D'Amour *et al.* 2006). Another example is the potential use of ES cells for cardiac tissue regeneration; embryoid bodies show contractile cardiac muscle characteristics and are troponin positive. Experimentation in

rats has shown improvement of cardiac function after ES cell transplant (Min *et al.* 2003; Zhou *et al.* 2008). Yet another example is the potential use of ES cells to regenerate neural tissue. This has been studied in primate ES cell lines differentiated into dopamanergic cells for treatment of a primate Parkinson disease model (Ben-Hur *et al.* 2004). In mice, transplantion of oligodendrocyte progenitors increased myelination and locomotion (Keirstead *et al.* 2005). These are a few examples of the successes in transplanting ES cells for use as a source of cells in tissue regeneration for different pathologic approaches.

1.1.7 Cell transformation and tumorigenesis signaling

Cell transformation signals are similar to signals for cell specialization during early embryonic development. Several studies have reported that tumor cells expressed pluripotency genes allowed cell proliferation and tumor formation (Ince *et al.* 2007; Ratajczak *et al.* 2010; Lindgren *et al.* 2011). Cancer cells have been shown to have both genomic instability and hypermethylation of DNA repair and tumor suppressor mechanisms, aiding tumor initiation and progression. Evans, et al, reported the similarities of morphological characteristics between embryo carcinoma colonies and the blastocyst inner cell mass which are known for their variety of undifferentiated stem cells and differentiated cells from the three germinal layers (Evans and Kaufman 1981).

In normal cells, methylation patterns are maintained across cell divisions, only allowing gene expression of tissue-specific genes necessary for cellular functions. Cancer is induced by disruption of these methylation patterns established during differentiation or during *de novo* methylation early in embryonic development (Calvanese *et al.* 2008). Deregulation through hypermethylation of tumor suppressor genes during tumor

transformation have been reported in p53, BRCA1, RB1, INK4, APC, PTEN, and p21 (Melki *et al.* 1999) and hypomethylation of oncogenes such as RAS, BCR/ABL, CCND1, ERG1, MYC, EGFR, and FOS. Mutation noted in KRAS and p53 are examples of those originated by oxidative stress damage to the cells leading to missense mutations common in different cancer types such as glioma, liver, and bladder cancer (Rauch *et al.* 2008). Deregulated MMR mechanisms have also been induced by genomic instability that allows accumulation of mutations leading to tumor transformation (Rodríguez-Jiménez *et al.* 2008) ensuring cell proliferation and avoiding apoptosis signals.

The environment can affect genomic integrity and induce epigenetic changes responsible for losses in repressive chromatin in developmental genes. These genes can then become active and lead the cell to oncogenic transformation, contributing to expansion and migration of tumor cells in the body. Developmental genes showed particular histone patterns that ensure gene silencing in specialized tissue. However, modifications in histone during differentiation lineage commitment are responsible for mutations in somatic cells. These tumor stem cells then initiate tumor transformation (Gupta *et al.* 2005; Ince *et al.* 2007; Imai *et al.* 2008).

1.2 Significance of the research

Understanding critical pathways of pluripotency, self-renewal, and differentiation during early development is important for the evaluation of the therapeutic potential of ES cells because of their potential for tumor transformation due to genetic and epigenetic instability acquired during *in vitro* culture maintenance. ES cells are a perfect model in developmental biology studies due to their potential to differentiate *in vitro*. Cultured ES cells and embryoid bodies can be used as a model for determination of the earliest

embryonic developmental pathways of pluripotency and self-renewal that lead to cell lineage commitment *in vitro*. Refinement of culture systems will allow the differentiation of specific lineages that are a source of all types of cells for regenerative medicine. They provide an ideal population of lineage-specific cells that can be used as a model system to measure toxicity and pharmaceutical drug safety. Genetically modified ES cells with specific genotypes provide a model for understanding mechanisms of disease initiation, progression, and treatment. Determining genetic and epigenetic modifications, including single tandem repeat instability, gene expression changes, and chromatin modifications, are essential for determining potential biomarkers for diagnostic purposes that ensure ES cell stability and integrity needed for regenerative medicine.

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CHAPTER II

DEVELOPMENT OF SINGLE TANDEM REPEAT SEQUENCE MULTIPLEXES FOR HUMAN EMBRYONIC STEM CELL EVALUATIONS OF GENOMIC AND EPIGENOMIC INTEGRITY DURING *IN VITRO* CULTURE

2.1 Abstract

Twenty multiplexes with 64 single tandem repeat markers were standardized to determine genomic instability involved in cell differentiation of ES cells and cell transformation in ovarian tumor progression. These multiplexes included markers located near pluripotency, self-renewal, differentiation, and chromatin assembly genes. Each multiplex showed simultaneous amplification of 3 to 5 markers labeled with FAM or HEX fluorescent dyes. Standardization was performed in different conditions that included primer design avoiding overlapping, PCR product size in range of 100 to 400 base pairs (bp), PCR buffer, $MgCl_2$, primer, and Taq polymerase concentration. Additionally, DMSO and BSA reagents were tested at different concentrations as enhancers of the PCR and different steps on the amplification protocol were examined: annealing temperature, final extension time, and number of amplification cycles. PCR conditions optimized for evaluation of DNA integrity by detection of unstable repeat markers at the single genome equivalent level (25-50 pg/µl) of DNA are presented. These conditions ensure sensitivity to detect wild type and mutated alleles at their appropriate frequency of *in vitro* samples during and after culture passages, cell differentiation of ES cells, and cell transformation on ovarian tumor cells.

2.2 Introduction

Single tandem repeats (STR) of 2-6 nucleotide units are polymorphic genetic markers useful in multiple scientific areas such as disease predisposition, susceptibility, diagnostics and prognostics, human identification for forensic cases or paternity probes, population genetics, and gene mapping (Shuber *et al.* 1995; Jakupciak and Wells 1999; Berg *et al.* 2000; Butler *et al.* 2001; Collins *et al.* 2003; Krenke *et al.* 2005). Accurate standardization of genetic markers ensures the efficiency of diagnostic methods used in human diseases such as cancer, neurodegenerative, or fragile sites in chromosome related diseases.

STR multiplexing refers to simultaneous amplifications of many STRs in the same PCR reaction. Several reports have been published about multiplexing STR markers for forensic and cancer research (Berg *et al.* 2000; Butler *et al.* 2001; Mulero *et al.* 2006). However, no reports that validate specific STR markers located in close proximity to specific genes involved during embryonic development, differentiation, chromatin assembly, and genomic imprinting pathways exit.

STRs may be potential biomarkers to determine genomic stability during human embryonic developmental events. Instability detection in repetitive markers near these genes could be a signal of pluripotency or differentiation of ES cell lines maintained *in vitro*. Therefore, STR standardization methods are important for ES cell characterization and validation for safe application in regenerative medicine. This protocol allows for the selection and validation of specific STR markers in multiplex sets. Validation of STR multiplexes is a novel tool for evaluation ES cell genome integrity during and after culturing in long term *in vitro* passages.

2.3 Methodology

2.3.1 DNA samples

Genomic DNA was isolated from blood samples donated for this research by informed consent (IRB approval number 11-088) and ES cells H1-WA01 and H7-WA07 purchased from the National Stem Cell Bank – Wisconsin International Stem Cell Bank (Appendix B) with the Purelink™ genomic DNA mini-kit (Invitrogen Carlsbad, CA) following the manufacturer's protocol. All DNA samples were quantified using a NanoDrop™ ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Titrations of DNA were made at different concentrations from 500 pg/µl to 25 pg/µl. High DNA concentrations were used for amplification of both wild type alleles and low concentration (single genome equivalent DNA concentration) that allow detection of wild type and mutated alleles with accurate frequency.

2.3.2 Selection of tandem repeat sequences

We located 312 tandem repeats containing repeat motifs (mono- , di-, tri-, tetra-, penta-, and hexanucleotide repeats) located in or near promoter regions of specific genes involved in pluripotency, self-renewal, differentiation, chromatin assembly, and imprinting (Appendix A). To determine the presence of tandem repeat motifs near promoter regions, we analyzed gene sequences 1000 bp upstream and downstream of the promoter using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/) and the NCBI database (http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi). A total of 3,489 target genes involved in pluripotency and self-renewal were analyzed: 623 genes transcribed by OCT4 transcription factor, 1,587 genes transcribed by NANOG transcription factor, 1,279 genes transcribed by SOX2 transcription factor, and 353 genes transcribed by

OCT4, NANOG and SOX2 transcription factor. The target genes of OCT4, NANOG and SOX2 transcription factor database are available at www.wi.mit.edu/young/hESregulation/ (Boyer *et al.* 2005)

2.3.3 Primer design

2.3.3.1 Software designer

To select the ideal oligonucleotide to be standardized by high concentration DNA (500 pg/µl) and low concentration DNA (single cell DNA equivalent 50 pg/µl or 25 pg/µl), primers were designed to amplify identified single tandem repeats in promoter regions with oligo-perfect designer software (http://tools.invitrogen.com/content.cfm?pageid=9716) (Invitrogen, Carlsbad, CA). This software facilitates the design of oligonucleotide primers when target sequences are in FASTA format. These oligonucleotide sequences were validated in UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/) to confirm the chromosomal location and DNA sequence that contained the specific repeat sequence of interest.

2.3.3.2 Gene bank primer sequences

Other markers were identified in specific genes using the NCBI database (http://www.ncbi.nlm.nih.gov/unists/). The respective primer sequences identified in this database were validated using the UCSC Genome Browser. In Appendix A, 312 identified tandem repeat motifs located in promoter proximal regions of important pluripotency, self-renewal, differentiation, chromatin assembly, and imprinting genes are summarized.

2.3.4 Singleplex PCR optimization

Before standardization of primers, we collected the STR marker's reference allele size, motif, and primer concentration reported in NCBI database and previous public reports. We used BLAST with the designed primers to check for potentially negative primer interactions. Fluorescent primers were purchased from IDT (Coralville, IA) at 100 µM. The labeled dyes used were 6-FAM (blue), HEX (green), and NED (yellow). For initial testing, forward and reverse primers were combined at a final concentration of 25 µM each to create a singleplex primer stock.

Each STR primer pair was optimized to obtain amplified products with robust signal intensity and balanced peak heights from DNA samples in three concentrations 500, 100, and 50 pg/µl. Each locus was standardized in single PCR reactions to optimize the primer balance (concentration), specificity, and sensitivity of each amplified signal from each STR. The final concentration of primers was tested with two concentrations (0.8 and 1.5 µM). PCR amplification was carried out in a total reaction volume of 10 µl that contained: 1X of buffer D (800 mM Tris HCL, 200 mM $(NH_4)_2SO_4$, 0.2% w/v Tween 20) (US DNA, Fort Worth, TX), 2.5 mM of $MgCl_2$ (US DNA, Fort Worth, TX), 1.25 U of Hot-MultiTaq DNA polymerase (US DNA, Fort Worth, TX), 300 µM of dNTPs mix (Applied Biosystems, Foster City, CA). PCR was performed on a PE 9600 thermocycler (Perkin Elmer, Waltham, MA) using the following protocol: 1 cycle of 95°C for 11 minutes; 30 cycles (ramp 4 minutes to 94°C, hold for 10 seconds and ramp 50 seconds to 70°C, hold for 60 seconds); then, final extension of 60°C for 30 minutes, and hold at 4°C. Negative controls were included for each run to check for contamination and dye artifacts.

2.3.5 Multiplex PCR Optimization

After optimizing all STR markers with single marker PCR reactions, we began selection for multiplexing primers based on allele base pair (bp) size. The markers set per multiplex were organized between ranges of 100-400 bp. Determined space between markers was estimated, which is dependent upon the number of bases in the repeat motif (Idury and Cardon 1997). For example, shorter distances are needed between mononucleotides and dinucleotides, and larger distances are needed between tri-, tetra-, or pentanucleotides markers. Primers were multiplexed together according to similarities in primer concentration, repeat motifs (mononucleotides with mononucleotides, dinucleotides with dinucleotides, etc), and primers with the same fluorescent label (6-FAM, HEX, or NED). Two different concentrations of DNA were used, 100 pg/µl and 50 pg/ul, and the final concentration of primers that was determined for singleplex PCR. We tested different conditions and concentrations for PCR to ensure co-amplification of the primers in each multiplex designed. The following different PCR components and concentrations were tested: standard buffer with 800 mM Tris HCL, 200 mM $(NH_4)_2SO_4$, and 0.2% w/v Tween 20 (US DNA, Fort Worth, TX), two concentrations of $MgCl_2$ (2.0 and 2.5 mM) (US DNA, Fort Worth, TX), and three concentrations of Hot-MultiTaq (1.5, 2.0, and 4.0 U) (US DNA, Fort Worth, TX). Each component was tested individually as a series of titrations around a singleplex optimized condition. PCR was performed on a PE 9600 thermocycler (Perkin Elmer, Waltham, MA) using the following protocol: 1 cycle of 95°C for 11 minutes; 30 cycles (ramp 4 minutes to 94°C, hold for 10 seconds and ramp 50 seconds to 70°C, hold for 60 seconds); then, final extension of 60°C for 30 minutes, and hold at 4°C. Negative controls were included for each run to check for contamination and dye artifacts.

2.3.6 PCR enhancers

Amplification sensitivity was test with PCR enhancers by using two concentrations of dimethylsulfoxide (DMSO) (2 and 4%) and bovine serum albumin (BSA) (0.2 and 0.4 mg/µl). DMSO decreases hydrogen bond formation between DNA strands, and BSA helps stabilize and enhance DNA polymerase activity during PCR amplification (Sahdev *et al.* 2007; Eilert and Foran 2009) (Figure 2.1).

2.3.7 Final PCR standardized conditions

PCR amplifications were performed in a total reaction volume of 10 µl containing 1X of buffer D (800 mM Tris HCL, 200 mM $(NH_4)_2SO_4$, and 0.2% w/v Tween 20) (US DNA, Fort Worth, TX), 2.5 mM of $MgCl_2$ (US DNA, Fort Worth, TX), 1.25 U of Hot-MultiTaq DNA polymerase (US DNA, Fort Worth, TX), 4% of DMSO (Sigma Aldrich, Saint Louis, MO), 0.4 mg/ml of BSA (Thermo Scientific, Rockford, IL), 300 µM of dNTPs (Applied Biosystems, Foster City, CA), and 1X of Solution L (US DNA, Fort Worth, TX). Primer concentration, sequences, genebank information, and dye label for each of the 64 STR primers are shown on Table 2.1-2.2. PCR ramping protocol: 1 cycle of 95°C for 11 minutes; 1 cycle of 96°C for 1 minute; 10 cycles of [94°C for 30 seconds, ramp 68 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 25 cycles of [90°C for 30 seconds, ramp 60 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 1 cycle of 60°C for 30 minutes for final extension; and hold 4°C.

2.3.8 Detection and analysis of PCR products

Detection of PCR products did not vary throughout standardization of the process. A volume of 0.5 µl for each amplified product was mixed with 4.35 µl of Hi-Di™

Formamide and 0.15 µl GeneScan™ 500 LIZ Size Standard (35-500 bp) (Applied Biosystems, Foster City, CA) and denatured for 3 min at 95°C and detected on a Genetic Analyzer AB3130xl (Applied Biosystems, Foster City, CA). Data were analyzed with software GeneMapper version 4.0 (Applied Biosystems Foster City, CA). Quantification of the allele size in comparison with the internal lane size standard (Genescan 500 LIZ size standard) was scored for each sample replicate per marker.

2.4 Results

2.4.1 Single tandem repeats are located near embryonic developmental genes

Three hundred twelve STR markers were initially selected by location near embryonic developmental genes. Sixty-four STR markers were successfully standardized and organized in 20 different multiplexes. Chromosome location, PCR product length range, repeat motif, Genebank number, fluorescence dye used for labeling the primer, concentration, and sequences of each primer are reported for each STR marker characterized (Table 2.1 and 2.2). From these 64 markers, 11 were related to pluripotency genes, 33 were related to differentiation genes, 12 were related to chromatin assembly genes and 8 were related to imprinting genes (Table 2.3).

2.4.2 Size of repetitive markers

Repeat motifs were found to be important determinants for STR marker selection. STRs with a minimum six repeat units, including those with mononucleotides to hexanucleotides motifs were selected. Dinucleotides and tetranucleotides are more common across the genome than trinucleotides and pentanucleotides (Collins *et al.* 2003). Out of 64 markers that were standardized in section 3.1, 5 are mononucleotides, 45 dinucleotides, 1 trinucleotide, 11 tetranucleotide, and 2 pentanucleotides across the

genome (Table 2.1). Note that all these markers are located in intragenic, coding or un-translated regions either upstream or downstream of embryonic developmental gene promoters.

2.4.3 Optimization of PCR conditions in order to detect repetitive sequences

2.4.3.1 Primer concentration

Primers were standardized in singleplex reaction to determine optimal concentrations to ensure independent amplification of the STR fragment from the total DNA in the reaction. Primers showing similar conditions of amplification were organized in multiplexes of 3 to 5 STR markers in a size range of 100-400 bp to avoid overlapping of the allele peaks that could impede data interpretation. Simultaneously, amplified products of these multiplexes were analyzed and the concentration of the primer was empirically adjusted to achieve a homogeneous height if a multiplex primer balance displayed heterogeneous allele peak height or some markers failed for amplification. Several adjustments of primer concentrations were made until optimize simultaneous amplification of markers in each multiplex without twofold differences in peak heights were achieved. STR markers were removed if they did not show better or balanced amplified products in comparison with other STRs. For this reason, some multiplexes have 3, 4, or 5 STRs each from the total of 20 standardized multiplexes.

2.4.3.2 PCR standard components

A balanced amplification of the STR in multiplexes was obtained using selected concentrations of $MgCl_2$ at 2.5 mM. In contrast, higher concentrations of $MgCl_2$ displayed an increase of unspecific peaks that negatively affected the data interpretation.

Final concentration of Taq polymerase was 1.25 U as non-specific differences were observed with higher Taq polymerase concentrations.

2.4.3.3 PCR standard conditions

Annealing temperature was also tested at three different points: 58°C, 59°C, and 60°C. Protocols of forensic STR standardization have reported that higher melting temperatures improve STR amplification in multiplexed PCR (Butler *et al.* 2001). We found that 59°C was an optimal annealing temperature to our multiplexes observing that 58°C allowed poor amplification of some markers and 60°C increased non-specific amplifications.

Number of amplification cycles varied among 30, 35 and 40. The optimal cycle number was set at 35 due to the fact that all markers displayed an average peak height in a range of 1000 to 2000 relative fluorescent units (RFU) at single DNA genome equivalent concentration. After 40 cycles of amplification, non-specific peaks appeared and interfered with allele identification.

The final extension step of PCR protocol allows the addition of adenines to the 3' end of the double strand DNA during 30 minutes at 60°C. This step minimizes split peaks, ensuring that all amplified products are the same length and optimal shape. This facilitates differentiation of wild type and mutated alleles (expanded or contracted) (Brownstein *et al.* 1996).

2.4.4 PCR enhancers increased successful amplification of STR multiplexes

Some samples showed low amplification rates using initially standardized conditions. During the experimental process, reagents known as enhancers of PCR were added to increase the success of amplification of GC rich templates and difficult

templates in very low DNA concentration considered as single cell genome equivalents, or templates that could make secondary structures (Chakrabarti and Schutt 2001; Haqqi *et al.* 2002; Hubé *et al.* 2005). Dimethylsulfoxide (DMSO) and bovine serum albumin (BSA) enhancers were used in our multiplexes in order to improve the optimization for PCR amplification of these markers using single cell DNA equivalent concentration.

DMSO at 2% concentration did not show significant differences on the amplified products on the multiplexes in comparison with high concentration of 4% that show impact on the amplification balance between markers in the multiplex. Literature reported that DMSO decreases hydrogen bond formation between DNA strands giving a higher yield of PCR amplification products (Varadaraj and Skinner 1994; Sahdev *et al.* 2007).

In addition to enhanced multiplex amplification affected by the excess of residual dye from the multiple primers, we used BSA to help stabilize and enhance DNA polymerase activity during PCR amplification (Butler *et al.* 2001; Eilert and Foran 2009). In the absence of BSA, PCR amplification of a 4 marker multiplex displayed low peak heights in 3 markers (vWA, TPOX, FGA) and failure for the amplification of one marker (D8S1179). In contrast, when BSA was added into the PCR reaction at a concentration of 0.4mg/ml, BSA enhanced the simultaneous amplification of the same 4 heterozygous markers resulting in balanced peak heights (Figure 2.1). The results did not show differences when low concentration of BSA 0.2mg/µl was used.

2.4.5 Residual dye artifacts

After PCR amplification, some primers displayed dye artifacts due to residual dye impurities from manufacturing processes, improper primer storage that induces primer light degradation or continuous freeze/thaw conditions. Filtration of PCR amplified

products is recommended to remove free dye labels that can interfere with data interpretation and allele designations (Smith and Ballantyne 2007). Residual dyes from some markers were observed and they were identified as a background on the negative control products (Figure 2.2). To help prevent dye artifacts, primers were stored protected from light exposure and in small stock aliquots to ensure primer stability. Negative controls were exhaustively analyzed to differentiate real alleles and background dye signals on specific markers.

2.4.6 DNA concentration as a determinant factor on STR multiplexes validation

Large pool PCR amplification (500 and 75 pg/μl of DNA concentration) and single genome equivalent PCR amplification (50 and 25 pg/μl of DNA concentration) were optimized to ensure sensitivity and the efficiency of multiplexes in detection and discrimination of wild type and mutate alleles in their corresponding frequencies. Identification of the wild type allele was achieved by PCR amplification with large DNA concentration in comparison with low DNA concentration or single DNA genome equivalents that allow amplification of wild type or mutate allele in each sample replicate.

Using more than 500 pg/μl of DNA induced an amplified product with intense fluorescence that impacted the optimal wild-type and mutated alleles discrimination. This is in contrast to using lowest DNA concentration (below 25 pg/μl) that displayed poor amplification by low peak heights (below 100 RFUs) or complete absence of amplification on some STRs multiplexes this caused great difficulty in the analysis and interpretation of the data. Therefore, the final DNA concentration used to determinate

single tandem repeat instability between samples in these markers was between 25 to 50 pg/μl.

2.5 Discussion

STR markers have been cataloged as a useful tool in studies of disease detection and progression. The presence of expansion or contractions of STR markers may be a genetic signal responsible for losses of DNA integrity that induce cell transformation. Disruption of STRs has been involved as a signal that contributes to deregulation of gene expression in cells. Therefore, studies of genomic instability by STR markers have demonstrated the utility of those markers to predict susceptibility to disease mainly during tumorigenesis.

ES cells have been isolated and maintained *in vitro* during several passages. Some reports have shown that chromosomal aberrations accumulate after long term passages of ES cells (Amit *et al.* 2000; Maitra *et al.* 2005). The aim of this study was to determine if STR instability was present during ES cell *in vitro* passages. STR markers located near pluripotency and differentiation genes were identified and standardized by PCR. These STR markers optimized could constitute novel biomarkers that useful for determination of ES cell genomic instability. Characterization of STRs is an important tool for determining the status of DNA integrity during ES cells *in vitro* culture maintenance.

This study demonstrated that these multiplexes are robust and have efficient reagent concentrations and PCR conditions to amplify ES cell samples regardless of the DNA concentration amplified, large DNA concentration as well as single cell DNA equivalent concentration. Strict primer design and PCR conditions are key elements for creating successful multiplexes and ensuring cost-effective advantages by simultaneous

PCR amplification of different STRs of interest. The ability to determine product amplification and separation characteristics, such as stutter (artifact peaks preceding the true allele peak) or unspecific amplification artifacts, allowed us to determine the specific range of normal and abnormal allele shifts per STR marker. This was an important observation when determining accurate mutation frequencies for unstable repeat motifs in independently tested sample replicates.

The reported multiplexes will facilitate the genetic integrity evaluation of ES cell samples at different times of *in vitro* culture. In addition, the present molecular technique will open new doors to discovery and validation of new and informative STR markers that could be used to determine predisposition, susceptibility, diagnostics, and prognostics of abnormalities occurring during early embryonic development.

These PCR combinations significantly impact the sensitivity for detection of alleles in their accuracy frequency. Standardization of STR multiplexes is a combination of careful primer design, optimization, and evaluations of PCR reagents and conditions needed for robust and balanced STR peaks amplification independent of the DNA concentration. Additional validations are needed to predict STRs informativity and which of them could give the major sensitivity in ES cell genomic integrity evaluation.

Application of STR analysis for detection of genomic instability losses allows the identification of target repeat elements on the genome. Instability in STRs involved during improper ES cell signals *in vitro* may have the potential to increase the knowledge about ES cells pluripotency maintenance or cell fate initiation early in embryonic development. In conclusion, specific STR markers could be identified as informative markers allowing the characterization of ES cells as a safe source of cells for regenerative applications.

Table 2.1 Characteristics of single tandem repeats analyzed

n.	Marker name	Chromosome location	Size range (bp)	Repeat motif	Genbank number
1	NANOG	12p13	159-163	(AAAG)12	(-)
2	D16S3034	16q12	268-274	(CA)11	Z52393
3	D12S1719	12q21	221-234	(CA)18	Z51889
4	D3S1541	3q22	273-277	(GAAA)16	L18687
5	D1S1656	1q23.3	148-168	(GATA)16	G07820
6	D1S551	1q23.3	176-184	(GATA)10	G07849
7	D4S2623	4q25	195-211	(GATA)8	G08380
8	D12S1682	12p12.2	135-143	(CA)24	Z53902
9	D11S4090	11q23.1	178-188	(CA)23	Z52338
10	D2S134	2p23	200-210	(CA)9 (CG)11	4211
11	D1S2630	1q22.23	260-270	(CA)15 (TA)5	Z52190
12	D11S1331	11p15	192-196	(CA)16	Z23980
13	D6S2384	6p22	208-221	(CA)13	G31311
14	D7S488	7p29.6	130-154	(CA)27	Z16593
15	D6S1001	6p	187-193	(TG)16(GA)6	GDB:363825
16	D4S1625	4q31	178-202	(GATA)11	G08328
17	HISTH4A	6p21.3	114-130	(A)11	(-)
18	HISTHB2	6p22.1	104-112	(T)	(-)
19	D10S529	10q22	134-152	(CA)7(CA)20	Z23410
20	D22S447	22q11.2	148-152	(GT)14	BV079566
21	D1S430	1p22	176-186	(CA)21	Z23588
22	D2S290	2p14	205-212	(CA)12	Z23527
23	D6S416	6q21	254-260	(CA)18	Z23615
24	D2S2327	2q33.1	161-170	(CA)25	Z53995
25	D8S11268	8	198-210	(CA)18	BV096928
26	kLF4-1	9q31	100	(T)40	(-)
27	NANOG	12p13.31	159	(CAAA)9	(-)
28	D3S1583	3p24	146-152	(CA)12	Z23961
29	DXS458	Xq21.3	182-190	(CA)16 (TA)5	1730
30	D22S941	22q11.2	248-254	(TA)6 (CA)11	7290
31	D9S1840	9q33	108-116	(CA)19	Z53489
32	D7S638	7p21.1	158-164	(CA)22	Z23645

Table 2.1 continued

n.	Marker name	Chromosome location	Size range (bp)	Repeat motif	Genbank number
33	D21S1909	21q22.1	178-200	(CA)21	Z51272
34	D6S1698	6p12	236-242	(CA)17	Z51754
35	D2S144	2p23	134-142	(CA)19	Z16833
36	GRB10PROM	7p12-p11.2	183-191	(T)15	(-)
37	D10S1653	10p14-p12	202-210	(CA)20	Z52361
38	G60405	10q11.23	264-272	(CA)13	G60405
39	D11S909	11p15	145-153	(CA)17	Z16685
40	D6S2252	6p21	108-118	(CA)21	85235
41	D5S2021	5p13.1-p12	110-122	(CA)12	Z53326
42	D20S821	20q476.4	128-136	(CT)3	G07497
43	IGF2R	6q26	164-172	(TG)	(-)
44	D18S63	18p11.3	270-278	(CA)15	Z16909
45	DIRAS3PROM	1p31	104-114	(T)8(TTTTA)34	(-)
46	D4S1542	4q21.3	190-198	(CA)11	Z23454
47	DXS981	Xq11.2	216-228	(ATCT)12	ID:57760
48	D14S588	14q22.1	124-136	(GGAA)9	G07885
49	D3S2459	3q12.3	194-206	(GATA)12	G08268
50	D3S1611	3p21.3	256-264	(CA)15	Z24553
51	D17S2180	17q21.3	92-110	(TTG)5(TTA)6	G10326
52	EGFR	7p12	248-256	(CA)16	(-)
53	PEG10PROM	7q21	98-108	(CCCCT)3	(-)
54	SNURF10PROM	15q12	250-258	(CT)13	(-)
55	D16S3091	16q24.2-q24	117-125	(CA)22	Z53416
56	D1S468	1q36.3	162-186	(CA)15	Z23994
57	TNFa3	6p12	96-112	(CA)11	(-)
58	DNMT3	19	136-144	(CTTT)5	(-)
59	IGF2PROM	12	108-122	(T)20	(-)
60	D15S983	15q23	164-172	(CA)12	Z52727
61	IGF	12	189-197	(CA)20	(-)
62	DXS1208	Xp11.23	244-252	(CA)15	Z23944
63	D5S426	5p13.3	190-198	(CA)23	Z17066
64	D11S2179	11q22-q23	131	(CA)17	472

Table 2.2 List of single tandem repeat primers sequences

n.	Marker name	Label dye	Primer volumen (µl)	Primers sequence	
1	NANOG	FAM	0.4	GAAAGAAAGAAAAGAAAGAAAAAGAAA	TGACTTCATCCTAATCAACAGCA
2	D16S3034	HEX	1	TAATCTAGTTAAAGATGCAACTGCC	GCTCAGAAGTTTGATGCC
3	D12S1719	FAM	1	TCCTCCAGTTTCAGTAATGTTT	GGTGGTTGATGCCTGTAA
4	D3S1541	FAM	1	TATGGACTGTAAGAAATGCCA	TGTGGGGTGGATAGAAAGAG
5	D1S1656	FAM	1	GTGTTGCTCAAGGGTCAACT	GAGAAATAGAATCACTAGGGAACC
6	D1S551	FAM	1	CTGCCAGAGAATAGGGTGAA	TTGTAATTCTTGGTCCTGCC
7	D4S2623	FAM	1	AACTAGGCTGCTTCCCAGAT	GCCAGATACATGGCTAAGGA
8	D12S1682	FAM	0.8	GGGACAAGAGTGAGACTTGG	CCTTTATTGAAGTAAACTGTGAAGC
9	D11S4090	FAM	0.8	GAGAGTGGGTCAGGTCG	GCTGCAGTTTCGGGAA
10	D2S134	FAM	1.6	AACGTCTGCTCGTCAGAGTC	CGACTACGTGCTGGCTACTT
11	D1S2630	FAM	0.8	CCCAGAAGGTTGAGAGTGC	CAGTAATCCCATAGACAGTAAATCG
12	D11S1331	FAM	0.8	GCTGCTTCCATGAGAGGATACTG	GCAGAGCCCTTTGCAGTCTT
13	D6S2384	HEX	1	ATGTCTCCTGCGAAGTAG	GAAGTCTGAAAAAGTCTGATTG
14	D7S488	HEX	0.7	ACCTCTCCCTGACCTCATTA	AAAAAATAAGCCAGCAAGGA
15	D6S1001	HEX	1.5	gtttcttTCTGGGATTCCTGTCCAATG	CCTGACATATAGTAGGCACTC
16	D4S1625	FAM	1	GACTCCAAATCACATGAGCC	GTCTCTGCATTTGCTGGTTT
17	HISTH4A	HEX	1	GCTCACGCCTGTAGTCACTG	TGCACCCAGTGTGTAGGTTT
18	HISTHB2	HEX	0.6	AAGTTTGCTTTCGGTTTTCG	CGGCACTGCACTTCATCCT
19	D10S529	HEX	1	AGCAGGCGCTAGACTGTGAC	AGTGATGCCTTGCAGATGCT
20	D22S447	FAM	0.6	AGCACAGGAAGGAAGCTGTT	GTTGGCAGATGCTTCAGGA
21	D1S430	FAM	1.7	TCCAGATTTAGTGTCATTTCCC	CACTTACAGTAACAAGCCCCAG

Table 2.2 continued

n.	Marker name	Label dye	Primer volumen (µl)	Primers sequence	
22	D2S290	FAM	0.8	CGACTCTGGTGAATTGCTTG	CGACTCTGGTGAATTGCTTG
23	D6S416	FAM	0.9	GGCCCCACTTCCAGTAAGG	GGCCCAGGATAAAATGGTTG
24	D2S2327	HEX	0.7	CAACTGAATTTTTCAGACTTGTC	AATTAGAGCCAGATTTTAAAGGA
25	D8S11268	HEX	1.1	GACATTTCACCGGATTTGAG	TCTCTCTCCCTTTTCCCTTG
26	kLF4-1	HEX	0.9	CAACCTTGGGAGAATGGAGA	GCCTGGGCAATAGAGTGAGA
27	NANOG	HEX	1.2	GAGGCGGAGGTTACAGTGAG	GGGGCTTTTCATCCAAAAA
28	D3S1583	HEX	1	AGCTTGTAAATAGGTCCTAACAGAG	TGGTTTAATAGGCACCGTTT
29	DXS458	HEX	1	GATAAAACTGCATAGAAATGCG	CAACTGGGATATTGACATTG
30	D22S941	HEX	1	CAGGTTACAAAGTACATTAACTT	CAAGAAATGGTTGGAGCTGGT
31	D9S1840	HEX	1	ACCAATCAGAAACCTTGCC	TTAAGAACAGAAGCGCATAGGAG
32	D7S638	HEX	0.6	GCCAAAGGAAGGTTAAGTGT	CCACGCATATATGTACAGCA
33	D21S1909	HEX	1	CTGTGATTGTGTTTTCCATTTAGCA	TTCCACACTGAGTCAAGAGCAGG
34	D6S1698	HEX	1.2	TGCAGGTAATTTGACTACCC	ACACCCCTCATATATACTTGAGTGT
35	D2S144	FAM	1.3	TCTCCCTGACAGACTCTGCG	GCTGCATAGGCCGTACTGAG
36	GRB10PROM	FAM	0.6	ACAGCATTATGGCTGCAAAA	TTGGCTTTGTGTCACATTCG
37	D10S1653	FAM	1.3	CCTTTGGATAAAGCCTCCT	TATCATTGTCTCATCCGGG
38	G60405	FAM	1	CTTAGAGTCTCATGGGAAAAACAGAC	AAAATTTCACACGTTGTTTCCTTG
39	D11S909	HEX	0.8	GATATAACACCAAAAGCGCG	GGTATTCTTACAGCACAAAAGTTCT
40	D6S2252	HEX	0.8	CTAATCTCCAAATGCCTAAG	GATTTAGAAATGTAGGCCAG
41	D5S2021	FAM	1.2	TTCT1.2ACGGATTCCAATCAC	CAAAAGCAACTTAACCACG
42	D20S821	FAM	0.8	ACAGGAAATAAACTAGGCATGAGG	CAACTCGATGAAACTAAGATTTCAAC

Table 2.2 continued

n.	Marker name	Label dye	Primer volumen (µl)	Primers sequence	
43	IGF2R	FAM	1.2	TTCTACGGATTCCAATCAC	GTATCATGAGAACCTGAAGAG
44	D18S63	FAM	1	AGCTCATGTTGGATGTATCA	GTCAGACTACGCGCCTT
45	DIRAS3PROM	FAM	0.8	TCTCTTCACATCTGGAAACTTCA	GCCTGGGTAACATAGGGAGA
46	D4S1542	FAM	1	CTTTTCAAAGATCGACTCCAGTG	ATTCTCCCAGATAGCAGGGC
47	DXS981	FAM	2	TCAGAGGAAAAGAAGTAGACATACT	TTCTCTCCACTTTTCAGAGTCA
48	D14S588	FAM	1	GCCGAAAGAAAGAAAAAAGG	CGAATGCATACTTGCTGTTG
49	D3S2459	FAM	1	CTGGTTTGGGTCTGTTATGG	AGGGACTTAGAAAGATAGCAGG
50	D3S1611	FAM	1	CCCCAAGGCTGCACTT	AGCTGAGACTACAGGCATTTG
51	D17S2180	HEX	1	GCGTCGAGTTTTCACATCTT	TAGTCTTGTCTTAGCTCTGGACG
52	EGFR	HEX	1	GTTTGAAGAATTTGAGCCAACC	TTCTTCTGCACACTTGGCAC
53	PEG10PROM	HEX	1	GGGCAATTGCATTCTTGG	GGATGCTGATGCTGAACTGG
54	SNURF10PROM	HEX	1	ATTGCACCATTGCACTCCAG	TCTAATTTGGGAACATGACTTCC
55	D16S3091	FAM	1	GGGAGATAGCCTTAAACTTTCTTAC	TGTTGCTAATAACACTAGGCCA
56	D1S468	FAM	1	AATTAACCGTTTTGGTCCT	GCGACACACACTTCCC
57	TNFa3	FAM	1	CCTCTCTCCCCTGCAACACACA	GCCTCTAGATTTCATCCAGCCACA
58	DNMT3	FAM	1	AACCCAGGTAGCCAGAGACC	CCTGTCATCCTGCTTTGGA
59	IGF2PROM	FAM	1	CGGGAGATTATCGGGTTTG	GCGCCGCCTTCCACATTAGA
60	D15S983	FAM	1	TCTGAAACGATGGGCTG	AAGGTGATTCCGTCCCTG
61	IGF	HEX	1	GCTAGCCAGCTGGTGTTATT	ACCACTCTGGGAGAAGGGTA
62	DXS1208	HEX	1	CGGCACGTAAGGACAG	GTTAAAGGATTTGGGAGGC
63	D5S426	HEX	1	AAATTCTTGCTTTCATAGCCA	AGACTAAATAAAATCACTGCCG
64	D11S2179	HEX	1	TAGGCAATACAGCAAGACCCTG	GCACTGGAATACGATTCTAGCAC

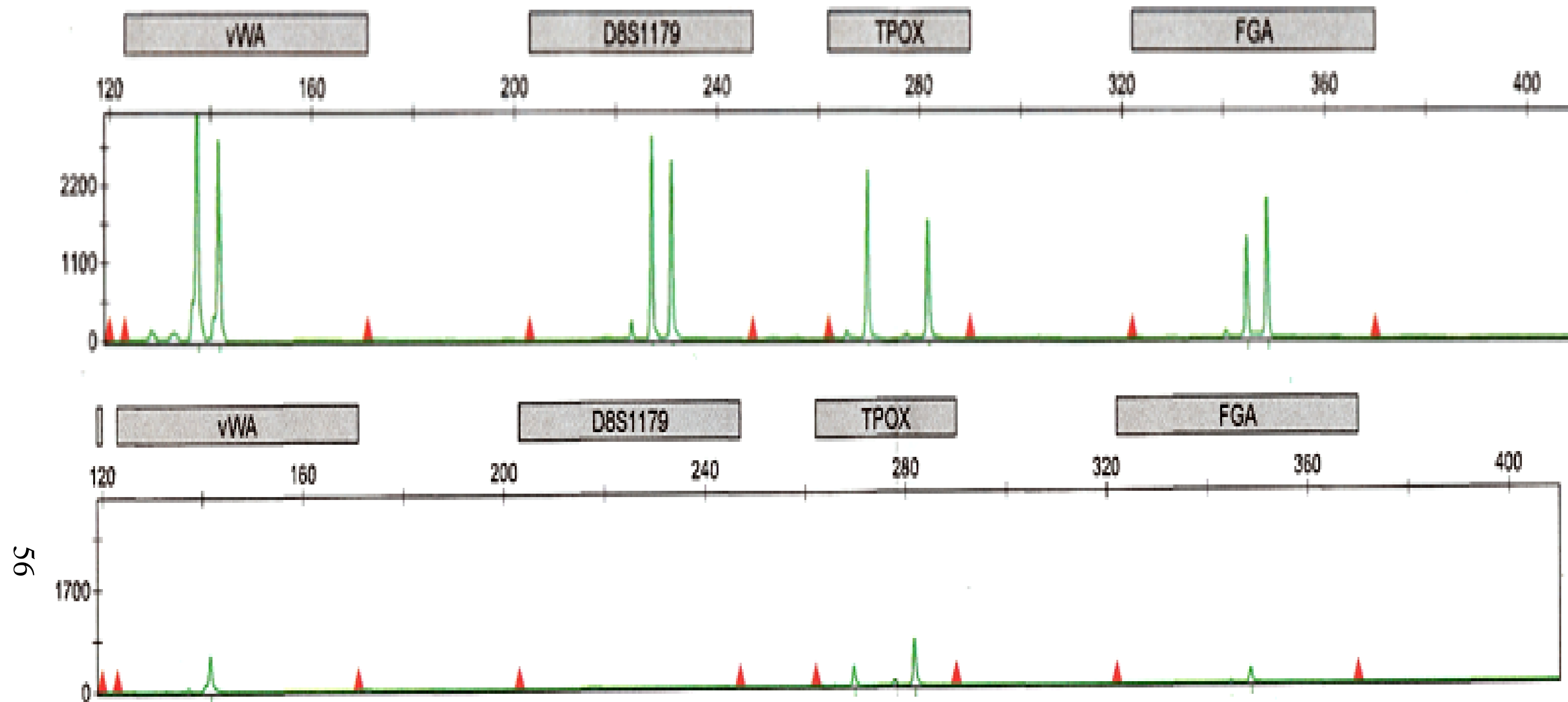


Figure 2.1 Examples of electropherograms.

Notes: Top panel shows PCR amplification results in the absence of bovine serum albumin (BSA) that clearly indicates a failure for the amplification of marker D8S1179 and the other markers in this panel display low peak heights. The bottom panel shows PCR amplification results with addition of 0.4mg/ml of BSA and displays how BSA enhanced the simultaneous amplification of the same 4 heterozygous markers. Both panels are amplifications from the same DNA sample and PCR conditions, with the exception of addition / absence of BSA.

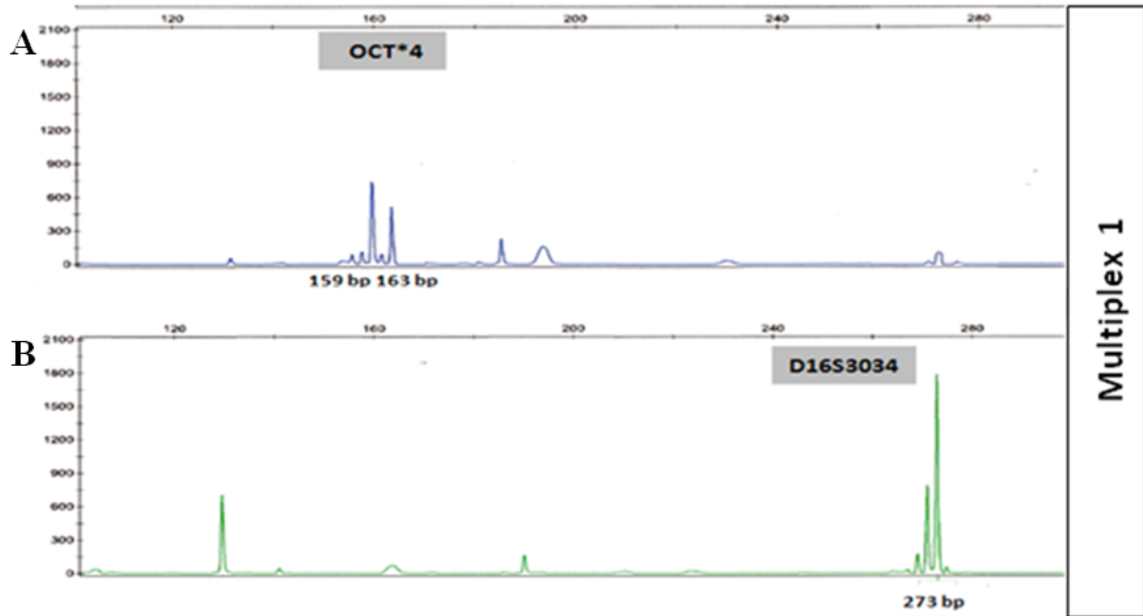


Figure 2.2 Examples of Electropherograms

Notes: Twenty fluorescent multiplexes of 62 single tandem repeats markers standardized are shown in these panels. Names for each marker are shown above the corresponding peak. Alleles are distinguished by different colored peaks. Each marker is either 6-FAM (blue) or HEX (green) labeled. The base pairs (bp) size of the alleles is shown below each corresponding peak. Panel (A and B) Multiplex 1 (OCT*4 and D16S3034 markers). Panel (C) Multiplex 2 (D2S1719 and D3S1541 markers). Panel (D) Multiplex 3 (D12S1682, D11S4090, D2S134, and D1S2630 markers). Panel (E) Multiplex 4 (D1S1656, D1S551, and D4S2623 markers). Panel (F and G) Multiplex 5 (D11S1331 and D6S2384 markers). Panel (H and I) Multiplex 6 (HISTH4A, D4S1625, D3S1583, DXS458, and D22S941 markers). Panel (J and K) Multiplex 7 (HISTHB2, D10S529 D22S447, D1S430, D2S290, and D6S416 markers). Panel (L) Multiplex 8 (D2S2327, and D8S11268 markers). Panel (M) Multiplex 9 (D9S1840, D7S638, D21S1909, and D6S1698 markers). Panel (N) Multiplex 10 (D2S144, GRB10-PROM, D10S1653, and G60405 markers). Panel (O) Multiplex 11 (D6S2252, D11S909, D2S2333, and D5S2115 markers). Panel (P) Multiplex 12 (DIRAS3-PROM, D4S1542, and DXS981 markers). Panel (Q) Multiplex 13 (D5S2021, D20S821, IGF2R, and D18S63 markers). Panel (R) Multiplex 14 (D14S588, D3S2459, and D3S1611 markers). Panel (S and T) Multiplex 15 (D17S2180, EGFR, D16S3091, and D1S468 markers). Panel (U) Multiplex 16 (PEG10-PROM and SNURF2-PROM markers). Panel (V) Multiplex 17 (IGF2-PROM and D15S983 markers). Panel (W) Multiplex 18 (D7S488 and D6S1001 markers). Panel (X) Multiplex 19 (TNFa3 and DNMT3 markers). Panel (Y) Multiplex 20 (IGF and DXS1208 markers).

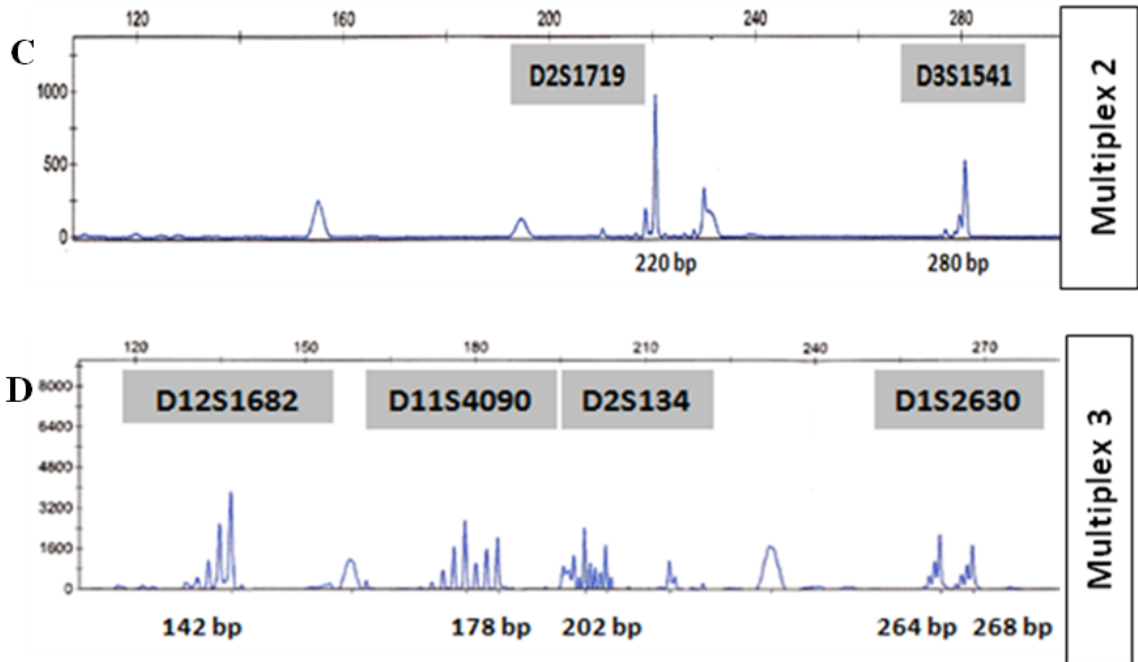


Figure 2.2 continued

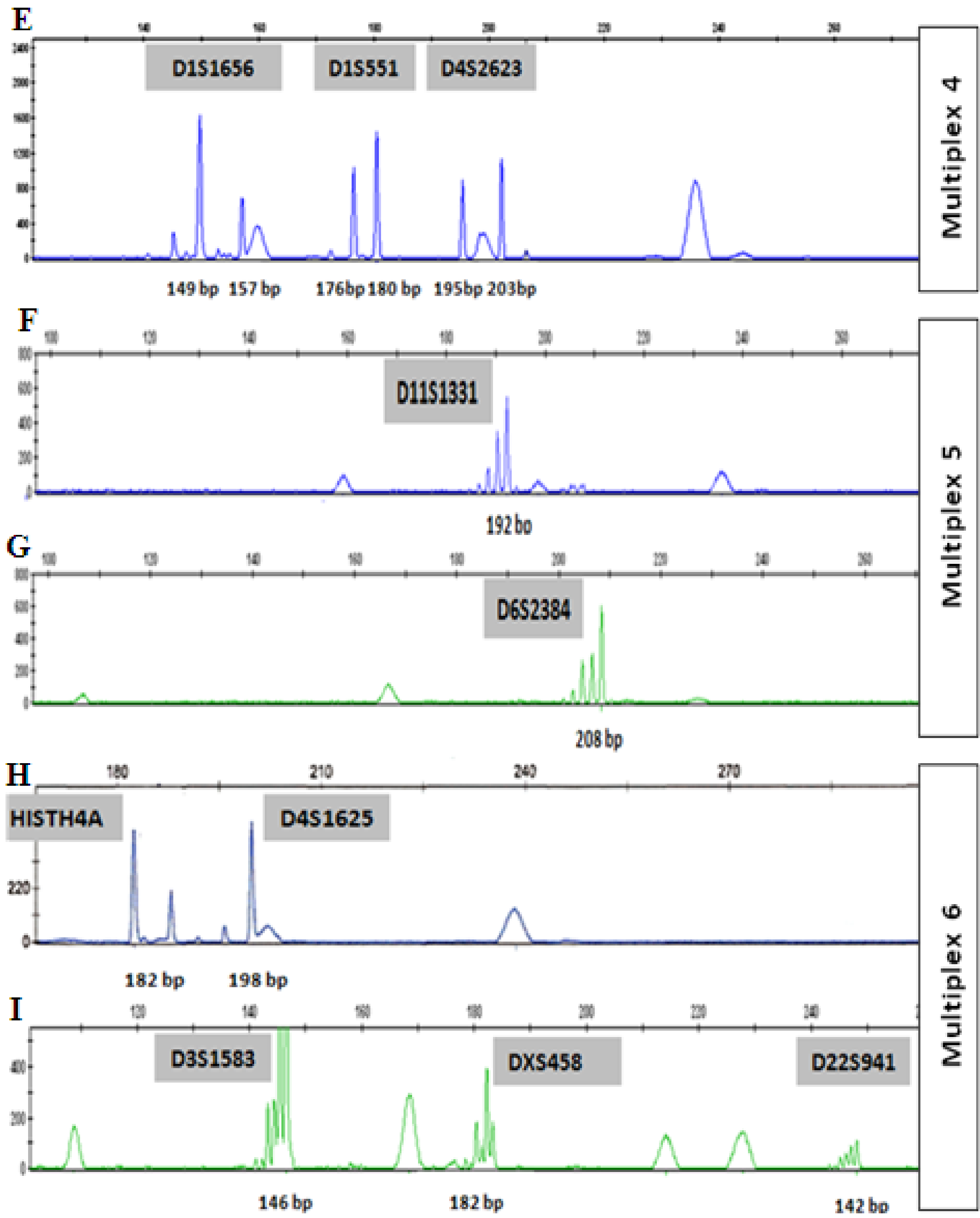


Figure 2.2 continued

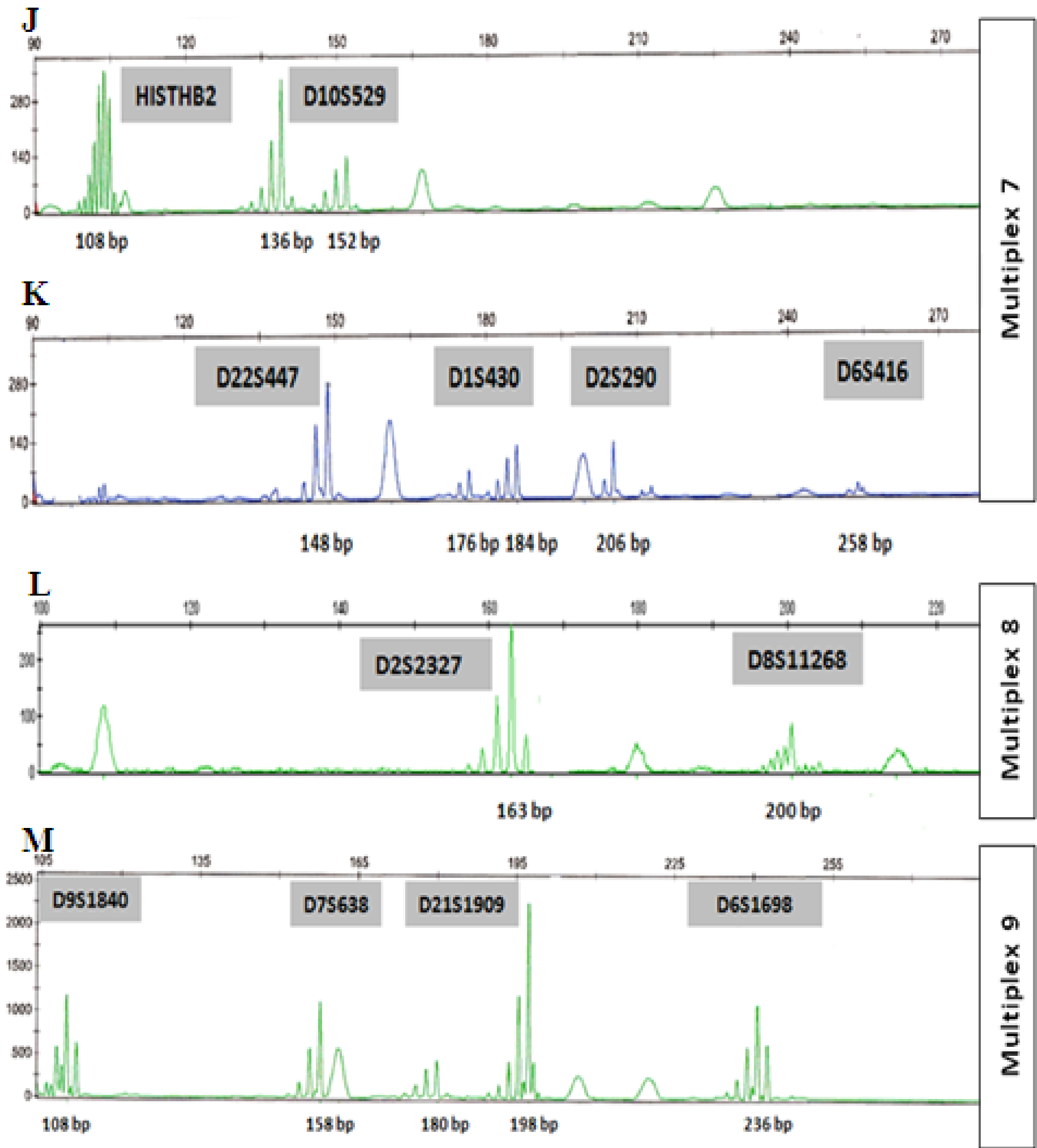
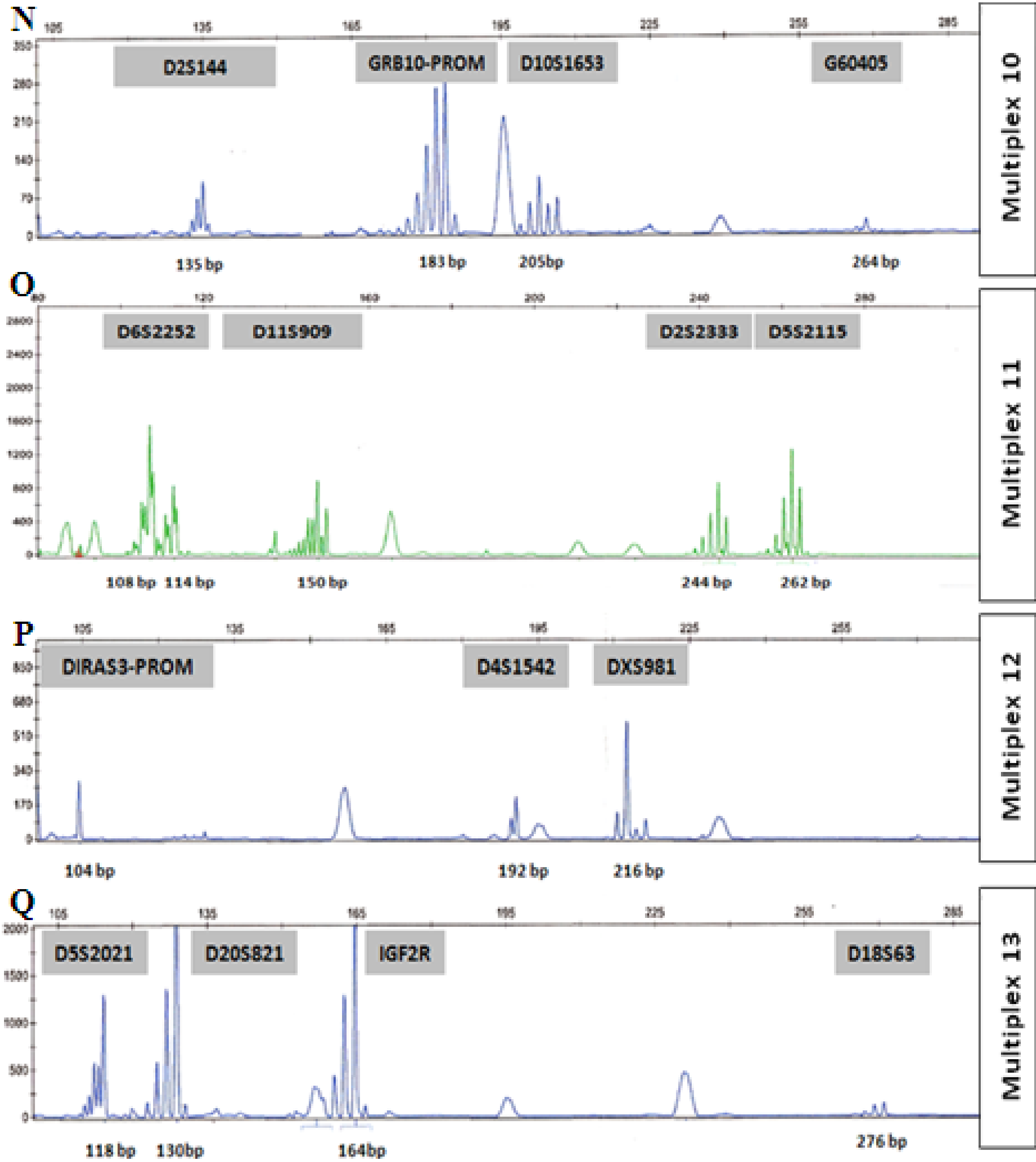


Figure 2.2 continued



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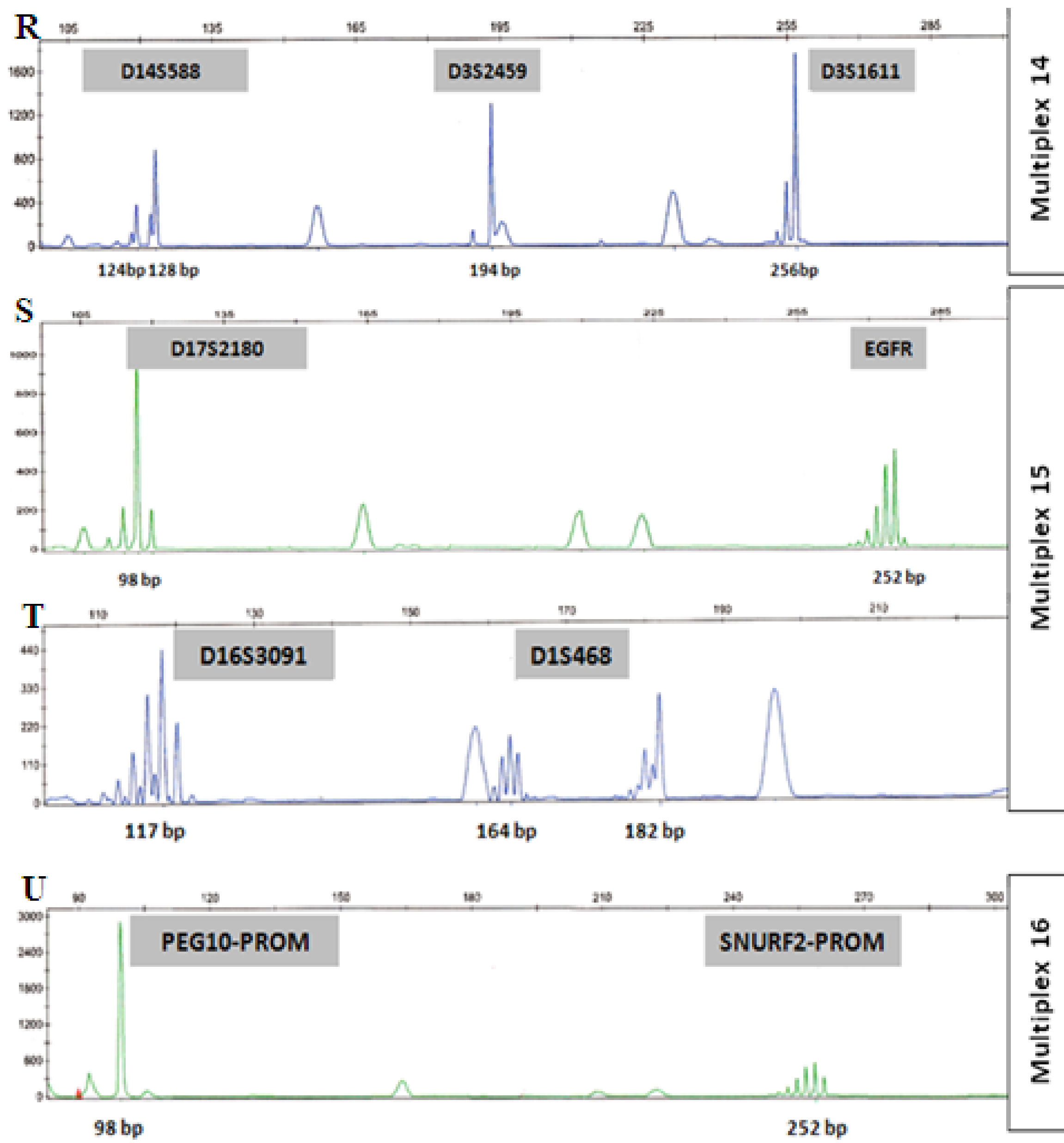

Figure 2.2 continued

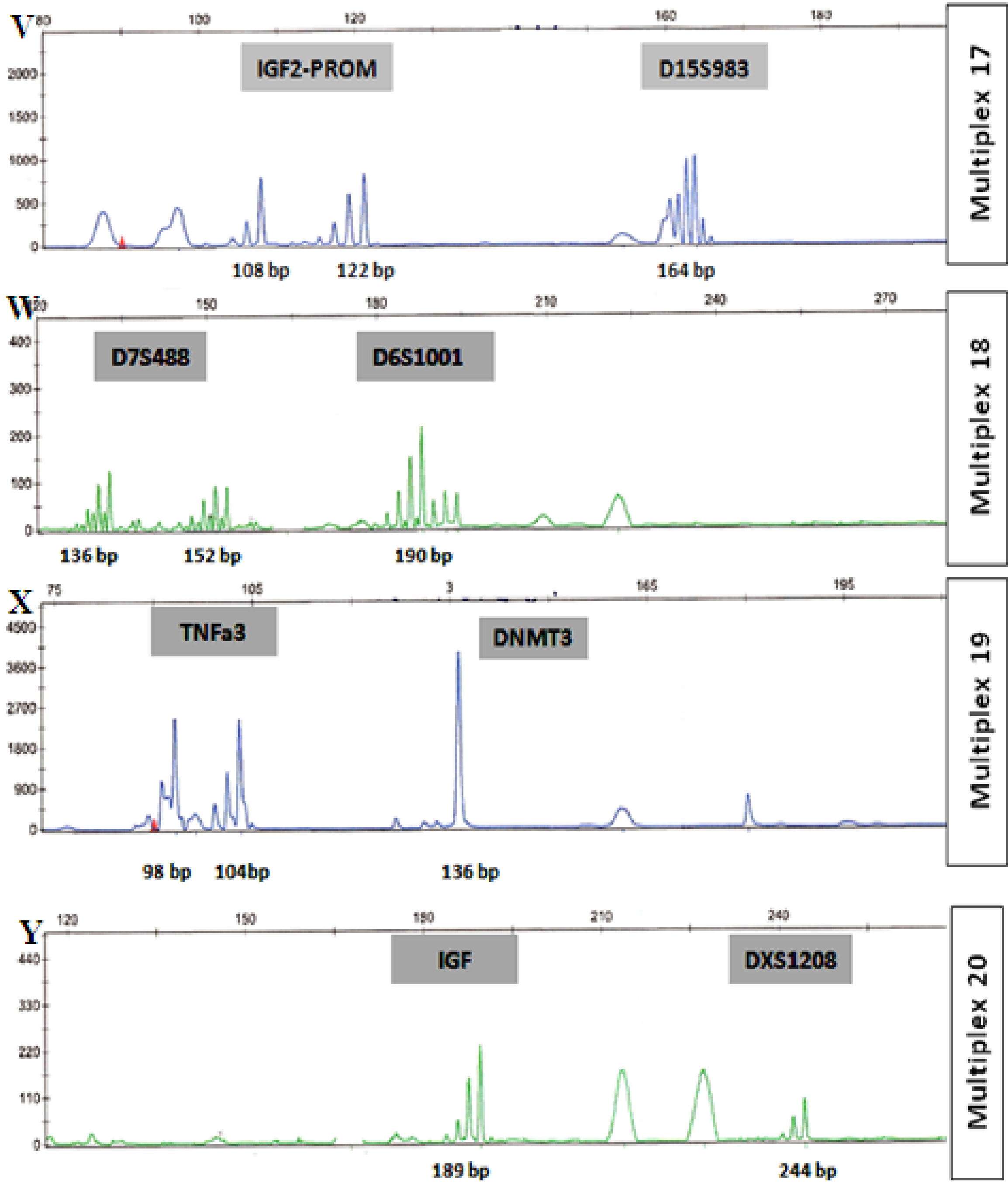

Figure 2.2 continued

2.6 Acknowledgements

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CHAPTER III

EMBRYONIC STEM CELL GENOMIC INSTABILITY RESULTING FROM CULTURE PASSAGES MAY BE A MECHANISM OF ADAPTATION AND PLURIPOTENCY MAINTENANCE

3.1 Abstract

Embryonic stem (ES) cells have the ability to maintain pluripotency and self-renewal during *in vitro* maintenance, which is a key to their clinical applications. ES cell quality has been widely evaluated through determination of the specific genetic and epigenetic profiles. The hypothesis of this study was that genetic stability in repetitive sequences located near key genes involved in pluripotency, self-renewal, differentiation, chromatin assembly, and imprinting could be a signal for adaptation of the ES cell *in vitro*. Instability in specific repetitive sequences is present and increases during ES cell passages. ES cells displayed significant mean frequencies of instability in twelve markers out of 64 related to pluripotency (OCT4, D1S551), early differentiation (G60405, D18S63, and D1S468), chromatin assembly (D22S447, D6S2252, D10S529, and HISTB2), and imprinting (GRB10-prom, D2S144, and IGF2-prom). Interestingly, instability was distinct between H1 and H7 ES cell lines. In summary, these results suggest that instability in tandem repeat sequences located near early embryonic developmental genes is associated with failure of ES cell pluripotency and self-renewal maintenance over consecutive culture passages. These results suggest that instability determination is a potential indicator of gene deregulation and epigenetic modification

that involves chromatin modification and imprinting establishment during ES cell cultures. Finally, instability in specific genes could be a signal that contributes to adaptation of ES cells to *in vitro* culture or could be the switch that initiates early cell specialization *in vitro*.

3.2 Introduction

Since the first human embryonic stem (ES) cells were isolated two decades ago, this field of research has generated uncountable advances and knowledge about early embryonic development and cell fate differentiation (Evans and Kaufman 1981; Thomson *et al.* 1998; Brimble *et al.* 2004; Enver *et al.* 2005). ES cell pluripotency and self-renewal led to significant discoveries and clinical applications as the source of all cell types from the three embryonic germinal layers. However, continued maintenance *in vitro* leads to cellular, genetic, and epigenetic changes in the ES cells, which creates many questions about their real therapeutic potential. The accepted culture conditions used for ES cell maintenance around the world are limited. ES cell research continues to face doubts about their clinical applications because of a wide range of variability in the maintenance of homogeneous and undifferentiated ES cells over time during culture passages (Toyooka *et al.* 2008; Ying *et al.* 2008).

Several studies have reported changes in ES cell gene expression profiles that occur during long term cultures (Abeyta *et al.* 2004; Brimble *et al.* 2004; Xu *et al.* 2005). Also, the presence of chromosomal abnormalities in late passage cultures of ES cells has been reported (Amit *et al.* 2000; Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005; Ogawa *et al.* 2006). Furthermore, the signals or initial steps that lead to gene expression and epigenetic changes remain unknown. A simple screening method to select

the best ES cells would be of great use in the field. This study focuses on determining the role of instability in repetitive DNA sequences as a signal of ES cell adaptation or differentiation, and the identification of possible biomarkers useful for screening and determining the quality of ES cells to be used for regenerative therapies.

Instability in flanking regions of developmental genes could affect enhancer or repressor elements that regulate transcriptional patterns of ES cells during *in vitro* maintenance. In order to understand how genomic instability affects pluripotency of ES cells, self-renewal, and differentiation, we have tested a key characterization method to evaluate the safety of the ES cell treatments. As a first step to investigate the instability effects of repetitive sequences on ES cells over time, we have determined the mean frequency of instability in different markers located in close proximity to sequences of important genes responsible for ES cell pluripotency, self-renewal, cell differentiation, chromatin assembly, and imprinting. We analyzed H1 and H7 ES cell lines during early, middle, and late passages to compare the genomic instability across passages. By determining the mean frequencies of instability for each marker, we identified sensitive repetitive markers that showed significant instability in ES cell cultures over time. In addition, specific genes that were identified as related to the unstable marker were evaluated. This study has established that instability in these specific regions could modulate gene expression and epigenetic signals that determine ES cell adaptation or differentiation stages.

3.3 Materials and Methods

3.3.1 Embryonic stem cell maintenance

Frozen aliquots from human ES cells H1-WA01 passage 27 and H7-WA07 passage 26 were purchased from the National Stem Cell Bank – Wisconsin International Stem Cell Bank (Appendix B). H1 and H7 ES cells were seeded onto a mouse embryo fibroblast-CF1 (MEF) feeder layer previously inactivated with mitomycin C. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) knockout medium (Invitrogen, Carlsbad, CA) supplemented with 10% knockout serum replacement (Invitrogen, Carlsbad, CA), 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA), 100 μM β-mercaptoethanol (Sigma Aldrich Saint Louis, MO), 20 ng/ml basic fibroblast growth factor (b-FGF) (Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Invitrogen Carlsbad, CA), 2 nM L-glutamine (Invitrogen, Carlsbad, CA), and 20 ng/ml of leukemia inhibitor factor (LIF) (Chemicon/Millipore Billerica, MA). ES cells were maintained in a humidified atmosphere at 37°C in 5% CO_2. Medium was changed daily.

3.3.2 Mouse embryo fibroblast CF1 feeder layer

The mouse embryo fibroblast (MEF-CF1) feeder layer cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) (Appendix B). MEF feeder layer cells were cultured in a T-25 flask (Falcon, Becton Dickinson Labware, NJ). The culture medium consisted of DMEM high glucose medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). MEF cells were mitotically inactivated for 2 hours with 10 mg/ml mitomycin C (Sigma Aldrich, Saint Louis, MO), seeded at densities of 130,000 cells/ml in gelatin coated one-well dishes (Falcon, Becton Dickinson

Labware, NJ) and cultured 24 to 48 hours before ES cells were seeded onto the feeder layer. These cells were maintained in a humidified atmosphere at 37°C in 5% CO_2.

3.3.3 Embryonic stem cell passages

ES cell colonies with undifferentiated morphologies were mechanically dissected into small pieces under a stereomicroscope and seeded onto a fresh MEF feeder layer during 20 passages (5 months). Cells were passaged every 4-6 days (Figure 3.1). Periodically, ES cells were tested for the presence of alkaline phosphatase activity, which is an indicator of the undifferentiated state. We used the alkaline phosphatase detection kit following the manufacturer's recommended protocol (Millipore, Chemicon, Billeria, MA). Sqamples of ES cell colonies were dissected for isolation of DNA and RNA early in the culture time (passage 27-28) and during the middle of the culture time (passage 40-42) in both ES cell lines.

3.3.4 Immunohistochemical analysis

ES cell colonies were fixed with 4% paraformaldehyde (Sigma Aldrich, Saint Louis, MO) for 15 minutes at room temperature, washed in PBS, and immunostained. The primary antibodies used were rabbit anti-OCT4 polyclonal antibody, mouse anti-SOX2 monoclonal antibody, and mouse anti-SSEA-1 alexa fluor 488 (Chemicon/Millipore, Billerica, MA). Secondary antibodies included goat-anti-rabbit IgG rhodamine and C5Y-conjugated antibody (Chemicon/Millipore, Billerica, MA). Each antibody was diluted 1:200 in PBS, 0.1% Triton X-100, and 3% BSA. Nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI) staining (Vysis Abbott Laboratories, Abbott Park, IL). Staining without primary antibody served as a negative control. Images were captured using a fluorescence microscope Axiovert 135 (Carl Zeiss

International) with FITC and rhodamine filter set. Fluorescence intensities were measured with image software developed at the National Institute of Health (Bethesda, MD) downloaded from http://rsb.info.nih.gov/ij/index.html. Accumulation was calculated by averaging the fluorescent ratio between exposed and non-exposed areas in the nucleus.

3.3.5 DNA isolation

DNA was prepared from each sample of ES cells in early passage (27-28) and middle passage (40-42). DNA from late passage (78-82) was provided by the Michigan Center for human ES Cell Research (Ann Arbor, MI). DNA was isolated with the Purelink genomic DNA mini kit (Invitrogen Carlsbad, CA) following the manufacturer's protocol. All DNA samples were quantified using a NanoDrop™ ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

3.3.6 Single tandem repeat markers selection and standardization

Single tandem repeats (STRs) are located in or near promoter regions of specific genes responsible for embryonic stem cell pluripotency and self-renewal. We identified DNA sequences that were approximately 1000 bp upstream or downstream of the promoter using UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/) gene sorter and uni-STS-NCBI database (http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi). A total of 64 STR were selected and classified according to ES genetic network regulation database available at (http://www.wi.mit.edu/young/hESregulation/). Eleven markers were related to pluripotency genes, 33 were related to differentiation genes, 12 were related to chromatin modification genes, and 8 were related to imprinting genes (Table 3.1). Each STR was optimized to obtain amplified products with robust signal intensity and

balanced peak heights from ES cell samples in early passage (27-28), middle passage (40-42), and late passage (78-82).

Samples were analyzed with differing amounts of genomic DNA: large DNA concentration (DNA concentration of 0.1 to 1 ng/µl) and single cell DNA concentration (single genome equivalent between DNA concentrations 12.5 to 50 pg/µl). The average for amplifiable DNA (λ) was calculated by Poisson distribution: $\lambda = -\ln$ (number of replicates with non-amplification / total number of replicates) (Zhang *et al.* 2002). A $\lambda < 2$ means that single genome equivalent of DNA was present in the amplification.

Each locus was standardized in separate PCR reactions to optimize and ensure specificity and sensitivity of the system. Labeled primers with either 6-FAM or HEX dye were used to allow automatic detection. Primers were tested at concentrations of 0.8-1.5 µM in standard PCR conditions and reagents.

3.3.7 Genomic instability determination by single cell PCR

Single cell PCR was performed on 64 STRs (Table 3.1). Less than a single diploid genome-equivalent of DNA (25-50 pg/µl), was used to perform single cell PCR analysis in 48 replicates for each marker. These concentrations of DNA ensure sensitivity of the PCR to detect wild type and mutated alleles at their appropriate frequency (Coolbaugh-Murphy *et al.* 2004). Total reaction volume of 10 µl containing 1X of buffer D (800 mM Tris HCL, 200 mM $(NH_4)_2SO_4$, 0.2% w/v Tween 20) (US DNA, Fort Worth, TX), 2.5 mM of $MgCl_2$ (US DNA, Fort Worth, TX), 1.25 U of Hot-MultiTaq DNA polymerase 5 U (US DNA, Fort Worth, TX), 4% of DMSO (Sigma Aldrich, Saint Louis, MO), 0.4 mg/ml of BSA (Thermo Scientific, Rockford, IL), 300 µM of dNTPs mix (Applied Biosystems, Foster City, CA), and 1X of Solution L 5X (enhancer solution for

amplification of difficult templates) (US DNA, Fort Worth, TX). The primer concentration for each primer is shown on Table 2.2 in CHAPTER II.

PCR was performed on a PE 9600 thermocycler using a ramping protocol: 1 cycle of 95°C for 11 minutes; 1 cycle of 96°C for 1 minute; 10 cycles of [94°C for 30 seconds, ramp 68 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 25 cycles of [90°C for 30 seconds, ramp 60 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 1 cycle of 60°C for 30 minutes for final extension; and hold 4°C. Negative controls per run were included to check for contamination.

Amplified products were mixed with Hi-Di™ formamide and GeneScan™ 500 LIZ Size Standard (35-500 bp) (Applied Biosystems, Foster City, CA) and denatured for 3 min at 95°C to be separated and detected by fragment analysis on a Genetic Analyzer AB3130xl (Applied Biosystems, Foster City, CA). Data were analyzed with the software, GeneMapper version 4.0 (Applied Biosystems Foster City, CA). Quantification of the allele size in comparison with the internal lane size standard was scored in each single cell replicate. An average of 48 replicates per sample plus negative controls were amplified and scored for both ES cell lines.

STR makers are classified according to their repeat motif (number of nucleotides): mononucleotides (1 nucleotide motif), dinucleotide (2 nucleotide motif), trinucleotide (3 nucleotide motif), tetranucleotide (4 nucleotide motif), and pentanucleotide (5 nucleotide motif). Wild type alleles were determined for each microsatellite. Repeat motif shifts from the wild type allele size were considered a mutant allele. Mutant alleles for mononucleotides (e.g. GRB10-PROM, IGF2-PROM, and HISTBH2) were determined by a repeat shift greater than 3 repeats or less than 3 repeats.

For dinucleotides (e.g. D18S63, D6S2252, and D10S529), mutants were determined by a repeat shift greater than 2 repeats or less than 3 repeats. For trinucleotides (e.g. D17S2180), tetranucleotides (e.g. OCT4, and D1S551) and pentanucleotides (e.g. DIRAS3-PROM), mutants were determined by a repeat shift greater than 1 repeat or less than 2 repeats (Figure 3.2) (Boland *et al.* 1998; Suraweera *et al.* 2002; Coolbaugh-Murphy *et al.* 2004; Coolbaugh-Murphy *et al.* 2005; Goel *et al.* 2010).

3.3.8 Statistical analysis of genomic instability

Mutation frequencies (total number of wild type alleles related to the mutant alleles in each marker) were determined for each ES cell line and passage number by SP-PCR software version 2.0 (M.D. Anderson Cancer Center Houston, TX) (Appendix C). Differences in mutation frequencies were calculated with a two tailed t-test using raw mutation frequencies using a package SAS/win 9.2 (SAS Institute, Cary, NC). Mutation frequencies of informative markers were considered statistically significant when a p-value was ≤ 0.05, and were considered marginally significant if the p value was ≤ 0.10.

3.4 Results

3.4.1 Embryonic stem cell culture maintenance

The ES cells were continuously cultured for 20 passages to explore the potential role of genomic instability during ES cell maintenance *in vitro* under standard conditions with MEF and growth factors, such as b-FGF and LIF. ES cells from both cell lines (H1 and H7) retained their growth and morphological characteristics: ES cell showed homogenous round and compact colonies, ES cells showed a prominent nucleus and high nucleus: cytoplasm ratio, and ES cells showed positive alkaline phosphatase activity as well as expression of the specific pluripotency markers OCT4 and SSEA-1(Figure 3.3).

3.4.2 Embryonic stem cells displayed morphological changes across passages

ES cell cultures, in general, could display less than 20% of the colonies with heterogeneous morphology corresponding to differentiation. These heterogeneous colonies were then removed with a pipette under a stereomicroscope before the next subsequent passage (Adewumi *et al.* 2007; Veraitch *et al.* 2008; Kent 2009). H7 ES cells were subcultured/passaged more than 20 times continuously for more than 5 months. During that time, they exhibited round and compact colony morphologies. In contrast, H1 ES cells were cultured under the same conditions and time, yet they exhibited an increased number of irregular shapes of colonies with some differentiated cells at the periphery (Figure 3.4). To explore the ES cell morphological characteristics over passages, we compared differences in the shape of the colonies between H1 and H7 ES cells; we quantified the number of regular and irregularly shaped colonies from passages 28-42 in H1 ES cells and 27-42 in H7 ES cells. We found that H1 ES cells showed a significant increase in the colonies that exhibited signs of cell differentiation across passages in comparison to the H7 ES cell line (p=0.04). H1 ES cells in passage 40 showed a higher percentage (37%) of irregular colonies when compared to passage 27 (14%) (p=0.047) (Figure 3.5). H7 cell line did not show any significant difference across passages. Taken together, these results indicate that H1 ES cells failed to promote complete self-renewal of the ES cells across passages.

3.4.3 Genomic instability in single tandem repeat markers mediated embryonic stem cell culture adaptation

Because embryonic stem cells in culture maintain pluripotency and self-renewal via genetic rearrangements (Amit *et al.* 2000; Brimble *et al.* 2004; Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005; Ogawa *et al.* 2006), we asked whether ES cell

cultures are genetically stable in long term cultures. The efficiency of ES cells to maintain genomic stability was evaluated by analyzing single tandem repeat markers found close to specific genes involved in ES cell pluripotency and self-renewal (Table 3.1). Samples of DNA from H1 and H7 ES cells at three different times (early, middle, and late passages) were analyzed to determine genomic instability in specific markers. There was significant genomic instability in 21 out of 64 single tandem repeat markers evaluated. Both ES cell lines were unstable over passages in these markers. However, H1 ES cells became much more unstable than H7 ES cells. H1 ES cells showed significant instability differences between early to middle (p=0.002) and between early to late passages (p=0.025) but differences were not significant between middle to late passage. In contrast, H7 ES cells show a significant difference only between early to middle passage (p=0.057) (Figure 3.6). These results indicate genomic instability was present during long term ES cell cultures and suggest these could be a signal of cell adaptation.

3.4.4 Genomic instability could be a signal of embryonic stem cell pluripotency and self-renewal loss during long term cell culture

Increasing evidence suggests that culture passages of ES cells lead to significant changes in gene expression (Abeyta *et al.* 2004; Brimble *et al.* 2004; Xu *et al.* 2005; Gu *et al.* 2010). Our results have shown that during long term culture and subsequent passages, ES cells accumulated instability in single tandem repeats. These markers are located near important genes involved in pluripotency and differentiation. H1 ES cells were unstable in three markers related to pluripotency genes (OCT4, D1S551, and D1S2630) that were completely stable in H7 ES cells over passages. In addition, H1 ES cell showed instability in eight markers related to genes expressed during early differentiation (D2S134, D3S1583, G60405, D11S909, D18S63, DXS981, D17S2180,

and DXS1208). In contrast, H7 ES cells showed instability in three different markers related to differentiation (D16S3091, D1S468, and D12S1682). Both ES cell lines showed instability in the differentiation marker DXS1208, but the difference did not reach significance. Statistically significant differences were observed in two pluripotency related markers (OCT4 and D1S551) and three differentiation related markers (G60405, D18S63, and D1S468). D1S551, D18S63, and D1S468 markers showed higher mean values of mutation frequencies at a significant level ($p<0.05$) compared with the other unstable markers analyzed (Figure 3.7 and Table 3.2). We suggest that the presence of genomic instability in these specific pluripotency or differentiation genes could be a signal of gene expression changes that induce adaptation or differentiation of the ES cell during long term cultures and multiple passages.

3.4.5 Epigenetic changes that occur during embryonic stem cell *in vitro* culture could result from genomic instability

Imprinting, chromatin assembly, and methylation are essential epigenetic mechanisms that modulate ES cell maintenance (Bibikova *et al.* 2006; Collas 2009; Ahmed *et al.* 2010). We found significant differences in ES cell genomic instability following passages. H1 ES cells showed instability in three markers (D22S447, D6S2252, and D10S529) and H7 ES cells in two markers (D10S529 and HISTHB2) that were related to chromatin assembly (Figure 3.8 and Table 3.3). All four chromatin assembly markers were significantly unstable. D22S447 and D6S2252 showed higher mean values of mutation frequencies at significant levels ($p<0.05$). Instability of the HISTHB2 marker was highly statistically significant in the H7 ES cells ($p <0.001$). H1 and H7 ES cells showed significant instability differences in the D10S529 marker ($p<0.03$) (Figure 3.8). Additionally, unstable markers for imprinting genes were

determined. A single tandem repeat in the promoter of GRB10 imprinting gene was found to be unstable in both H1 and H7 ES cells, with a significant difference between them (p=0.026) (Figure 3.9) (Table 3.4). H7 ES cells also showed high instability in two additional markers (D2S144 and IGF2-PROM), whereas H1 ES cells were stable for these markers. D2S144 was significantly unstable compared with the IGF2-promoter marker that showed less significance (p=0.04 and p=0.08 respectively) (Figure 3.9 and Table 3.4). These findings related to instability of markers located near genes that participate in epigenetic modifications support the idea that genomic instability could be essential to generating epigenetic modifications during ES cell maintenance *in vitro*.

3.5 Discussion

Embryonic stem cells have the capacity for unlimited stem cell proliferation and the ability to differentiate into all cell lineages from the three germinal layers. Questions about the molecular signals of pluripotency and self-renewal maintenance *in vitro* are still unsolved and are the key to clinical ES cell applications. We evaluated early developmental molecular markers responsible for pluripotency and cell differentiation characteristics of ES cells to determine the genomic stability

Accumulation of DNA damage is observed during cellular stress responses. ES cells in long term cultures have shown genomic instability in the form of chromosomal abnormalities after more than 100 passages in response to environmental changes during *in vitro* maintenance (Amit *et al.* 2000; Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005). Genomic instability in single tandem repeats create frame-shift mutations, enhancer, or repressor modifications that originate gene expression changes affecting cellular processes, which has been explored widely in tumorigenesis studies (Cahill *et al.*

1998; Roelofs *et al.* 2000; Smiraglia and Plass 2002; Kremenskoy *et al.* 2003; Gorringe *et al.* 2005).

ES cells and tumors have common molecular pathways that maintain their cellular characteristics and functions (Summersgill *et al.* 2001; Sperger *et al.* 2003; Wang *et al.* 2004; Andrews *et al.* 2006; Baker *et al.* 2007; Barber *et al.* 2008). Instability analysis of a single tandem repeat located downstream or upstream of specific pluripotency and self-renewal genes is a reliable tool to characterize the genomic stability during ES cell *in vitro*. It can be a potential biomarker to predict and evaluate pluripotency losses and uncontrolled cell differentiation processes during ES cell maintenance.

Our data suggest that instability in pluripotency and differentiation markers is a signal of balance between culture adaptation of ES cells and the differentiation process that is observed by morphological characteristics and genetic stability. H1 colonies became more irregular than H7 colonies through culture passages. Colony irregularities are morphological signs of differentiation during cell culture and could be related to the DNA instability found in specific markers located near essential genes responsible for optimal ES cell functions. ES cells show low instability during early passages when compared to the mean frequencies of instability during middle and late passages. Several reports suggest that late passages significantly increase the frequency of chromosomal instability due to environmental signals from the *in vitro* system used to maintain ES cell lines in culture (Amit *et al.* 2000; Draper *et al.* 2004; Inzunza *et al.* 2004; Maitra *et al.* 2005). Our results support the idea that ES cell lines exhibit different adaptation processes involved in genomic instability in early and middle passages as a part of cell adaptation *in vitro*. However, during later passages, chromosomal instability occurs in some stem cell lines that enable maintenance of ES cell pluripotency. Some studies report

that the H1 ES cell line showed trisomy in chromosomes 12 and 17 at 144 passages (Draper *et al.* 2004; Maitra *et al.* 2005; Baker *et al.* 2007). In contrast, H7 ES cell line showed trisomy in chromosome 20 and translocation between chromosome 6 and 17 at passage 209 (Draper *et al.* 2004; Maitra *et al.* 2005; Baker *et al.* 2007). Apparently, chromosomal instability and single tandem repeat instability occur by independent processes that happen during long term ES cell culture. H1 and H7 ES cell lines showed high rates of single tandem repeat instability during passages 27-28 and 42, but instability frequencies decreased at late passages (78-82 respectively) (Figure 3.6).

Important key findings emerged from our data: failure to maintain pluripotency, tendency to differentiate, and epigenetic changes over ES cell passages. We identified twelve unstable markers localized near pluripotency, differentiation, chromatin assembly, and imprinting genes that play important roles during early embryogenesis. These genes are involved in specific cell signals that determine genetic and epigenetic modifications relevant to the ES cell: DNA transcription, cell cycle, cell differentiation, tissue specification, apoptosis, and DNA repair.

First, ES cell genes for pluripotency and self-renewal are actively expressed and are responsible for maintaining all characteristics of the ES cell. When genomic instability occurs around these specific genes, it could lead to loss of pluripotency and self-renewal in the ES cells. We found two unstable pluripotency markers in H1 ES cells; OCT4 and D1S551. OCT4 (POU class 5 homeobox 1) is a transcription factor that plays a role in embryonic development and has been identified as an important gene for ES cell pluripotency (Ying *et al.* 2003; Boyer *et al.* 2005; Masui *et al.* 2007). OCT4 is part of the ES cell gene network that regulates pluripotency by transcription regulation. OCT4, NANOG, and SOX2 are transcription factors that regulate themselves and bind

common target developmental genes important for ES cell maintenance and embryonic development (Boyer *et al.* 2005; Babaie *et al.* 2007; Chen *et al.* 2008; Fernandez-Tresguerres *et al.* 2010). D1S551 is located near a regulator of G protein signaling gene. G protein is involved in many cell signaling pathways (Strubing *et al.* 1997; Neves *et al.* 2002; Charlesworth *et al.* 2006; Ebert *et al.* 2006). In mouse ES cells, G protein signaling is present during early neurogenesis and provides control of neuronal differentiation. Studies in mice and rat demonstrated that G-protein is a modulator of calcium channels, neurotransmitter gamma-aminobutyric acid (GABA), and opioid receptor (Strubing *et al.* 1997; Rusin and Moises 1998).

Second, several reports have shown how gene expression changes occur during ES cell culture passages, but the exact mechanism is not clear (Abeyta *et al.* 2004; Gu *et al.* 2010). Accumulation of DNA damage creates changes in gene expression that induce cell function decline and loss of the cell's integrity over time (Abeyta *et al.* 2004; Brimble *et al.* 2004; Xu *et al.* 2005; Gu *et al.* 2010). Long term cultures and passages generate ROS that are a source of DNA damage, apoptosis, and cell cycle defects (Wu *et al.* 1996; Lengauer *et al.* 1997; Eden *et al.* 2003; Allegrucci *et al.* 2007). For example, mouse ES cells, after exposure to ionizing radiation, show DNA damage that induces fibroblast cell differentiation (Saretzki *et al.* 2004; Maynard *et al.* 2008). From our results, we believe genomic instability could be a signal of gene expression deregulation. Early embryonic differentiation genes show genomic instability in H1 ES cells over multiple passages. H1 ES cells cannot completely maintain pluripotency, whereas H7 ES cells can. Differentiation markers that show instability in H1 ES cells were D2S134, D11S909, D18S63, and DXS981. Interestingly, these are specific markers located next to genes expressed during early embryonic neuroectoderm specialization (Tamagaki *et al.*

2000; Pazmany and Tomasi 2006; Wang *et al.* 2008; Hamid and Brandt 2009; Mojsin and Stevanovic 2009; Göhring *et al.* 2010; Xiang *et al.* 2010). D17S2180 and DXS1208 are related to endoderm and mesoderm specialization genes, respectively (Fu *et al.* 2003; Wu *et al.* 2007; Lui *et al.* 2008; Kumarapeli *et al.* 2010; Schwarz *et al.* 2010) (Table 3.5). In comparison to H7 ES cell unstable markers, D16S3091 is related to early mesoderm gene differentiation, D1S468 is a gene that promotes apoptosis, and D12S1682 is both an endoderm and mesoderm differentiation gene (Wechsler *et al.* 2002; Kim *et al.* 2007; Sayan *et al.* 2010) (Table 3.5).

Third, genomic instability is a multistep process that involves genetic and epigenetic modifications that induce opposite effects on the status of ES cell pluripotency. Epigenetic changes such as chromatin assembly, imprinting, and methylation are responsible for determining transcriptional patterns dependent upon the cell stage. Imprinting is a switch for gene transcription that ensures cell proliferation, development, and tissue specific functions (Kamakaka and Thomas 1990; Jaenisch and Bird 2003; Dhara and Benvenisty 2004; Allegrucci *et al.* 2007; Kim *et al.* 2007; Shen *et al.* 2008). Developmental genes for ES cells have a specific pattern of histone modifications that determine the status of activation of specific genes involved in embryonic development and cell fate during differentiation by *de novo* methylation. For example, the OCT4 gene is unmethylated during pluripotency by bivalent histone modifications to ensure cell proliferation and development. However, OCT4 is completely repressed when cell differentiation occurs (Loh *et al.* 2006; Mikkelsen *et al.* 2007; Chamberlain *et al.* 2008). ES cell lines *in vitro* fail to maintain a specific epigenetic pattern, inducing changes in the cellular status that leads to loss of ES cell pluripotency over time (Bibikova *et al.* 2006; Allegrucci *et al.* 2007; Yu *et al.* 2007; Ahmed *et al.*

2010). In our results, H1 and H7 ES cells showed significant differences of instability in markers that were located next to chromatin assembly and imprinting genes across time. Genomic instability was observed in markers such as D22447, D6S2252, HISTHB2, and D10 S529, all of which were located close to genes that code for basic nuclear histone proteins. Histones are proteins responsible for the octameric structure of the nucleosome; they are formed by two molecules of each histone H2A, H2B, H3, and H4. The D6S2252 marker is located next to HIST1H2AH (linker histone H1), which interacts with the DNA between nucleosomes and is responsible for chromatin compaction (Wang *et al.* 2004; Zhang *et al.* 2005; Petty *et al.* 2009). D10S529 is a marker for a variant histone, H2AFY2 that contributes to the inactivation of X chromosome (Chadwick and Willard 2001; Buschbeck *et al.* 2009; Gamble *et al.* 2010). In zebra fish embryos, it has been observed that H2AFY2 is involved in the activation of neuronal differentiation genes such as the homeobox A1 gene (HOXA), which encodes a DNA-binding transcription factor to control gene expression during embryonic development and cell differentiation (Buschbeck *et al.* 2009). D22S447 is a histone cell cycle regulator A (HIRA) that is a homolog of *Saccharomyces cerevisiae* histone. HIRA is responsible for controlling cell growth by regulation of cell cycle related genes (Ahmad *et al.* 2005). Taken together, our results suggest that instability in these markers could be the signals that induce X chromosome inactivation, ES cell growth, and differentiation through gene expression changes in developmental and differentiation genes over multiple passages. Additionally, imprinting markers also showed instability and are involved in the embryonic methylation process. D2S144 is a marker for the DNA (cytosine-5-)-methyltransferase 3 alpha gene (DNMT3A) that is responsible for epigenetic modification of *de novo* DNA methylation important for embryonic development, differentiation, imprinting, and X-

chromosome inactivation (Chen *et al.* 2002; Wienholz *et al.* 2010). Other unstable markers are located next to the promoter region of imprinting genes, such as GRB10 and IGF2, which are imprinted in a tissue specific manner. These results confirm that H1 and H7 ES cells have a constant and actively regulated process across passages that control genetic and epigenetic outcomes to ensure ES cell growth, maintenance of cell feature characteristics or cell differentiation *in vitro.*

In conclusion, our findings indicate that maintenance of ES cell genetic and epigenetic characteristics is compromised by the loss of DNA integrity in tandem repeat sequences that flank specific genes that are responsible for the pluripotency and self-renewal of ES cells maintenance, cell fate during differentiation, chromatin assembly, imprinting, and methylation. From our data, we can support the idea that genomic instability could be responsible for genetic and epigenetic imbalances originating in long term ES cell cultures. The exact signals that coordinate this process are complex and not completely known. Even so, our data support our hypothesis that instability in repetitive sequences located close to specific genes could be the signal for adaptation or differentiation of ES cells in culture passages over time.

Furthermore, our results give rise to the identification of biomarkers that could be part of an ES cell characterization process that evaluates genomic integrity through *in vitro* maintenance procedures. Understanding the role of genomic instability in ES cell maintenance could lead to the origin of an accurate approach for the safety needed in regenerative medical applications of human ES cells.

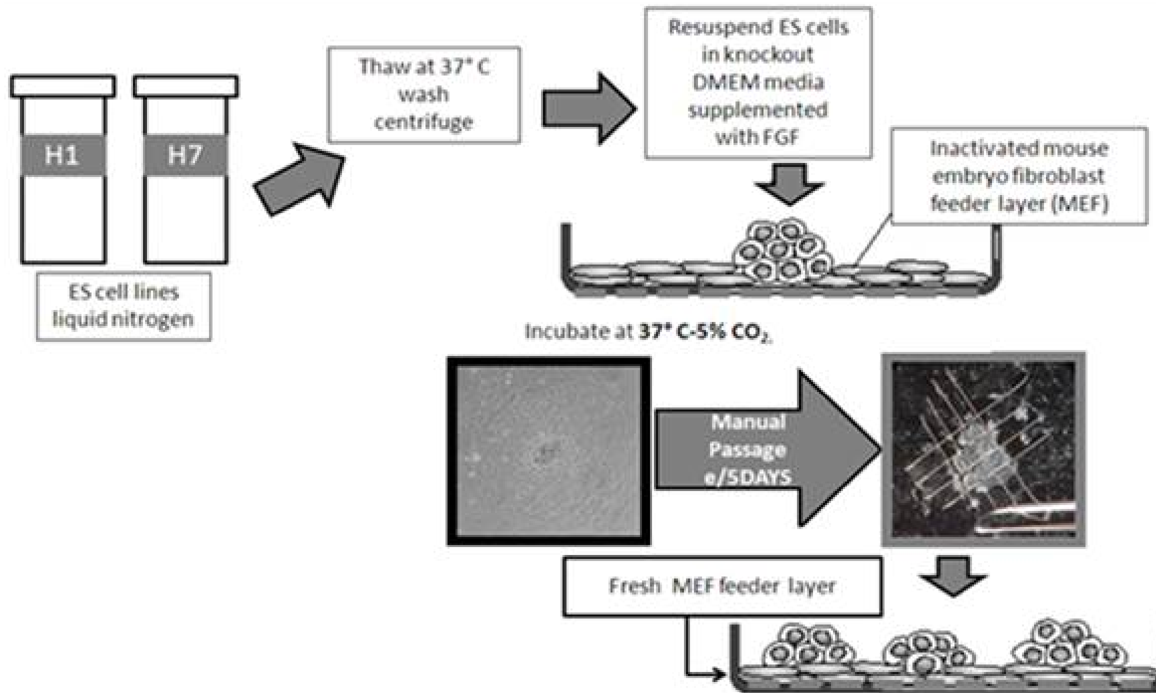

Figure 3.1 Flowchart of H1 and H7 ES cell line maintenance in culture

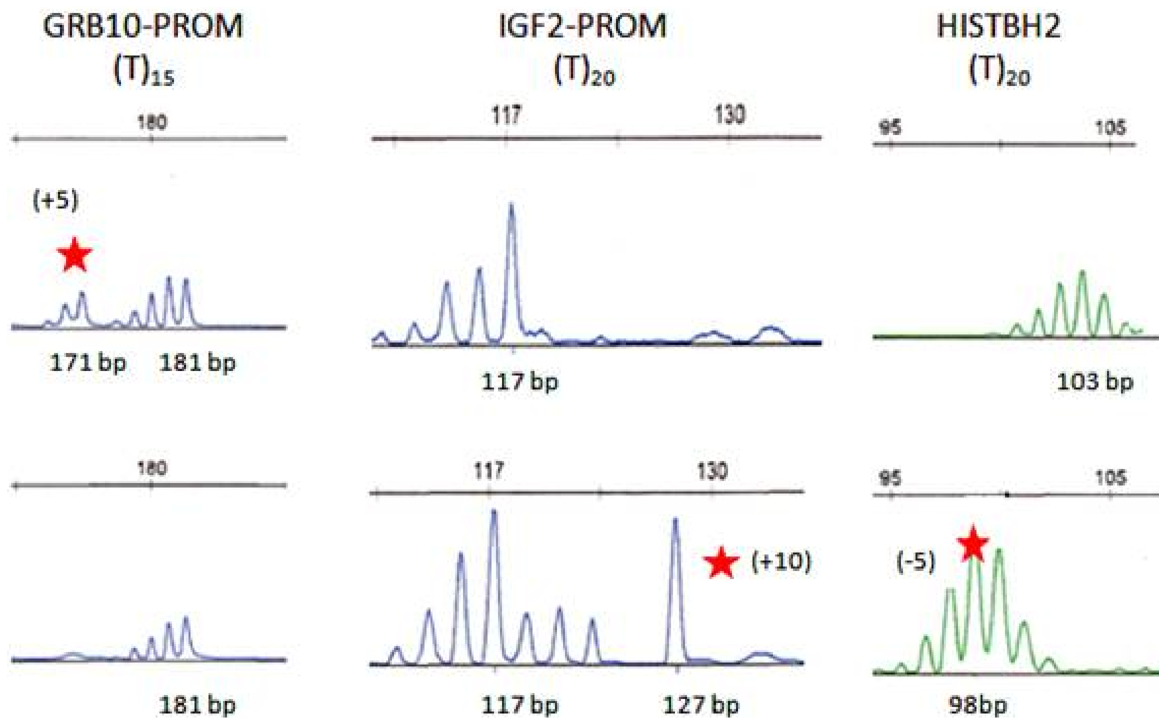

Figure 3.2 Examples of electropherograms

Notes: Examples of normal and mutated alleles of mononucleotide markers (GRB10-PROM, IGF2-PROM, and HISTBH2) showing the corresponding normal allele as well as the mutated allele that was shifted greater than 3 repeats or less than 3 repeats. Mutated alleles are shown with a red star and the number of repeat shifts in parentheses. (-) means a loss of repeat units, while (+) means a gain of repeat units. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of the allele in base pair (bp). Markers are labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

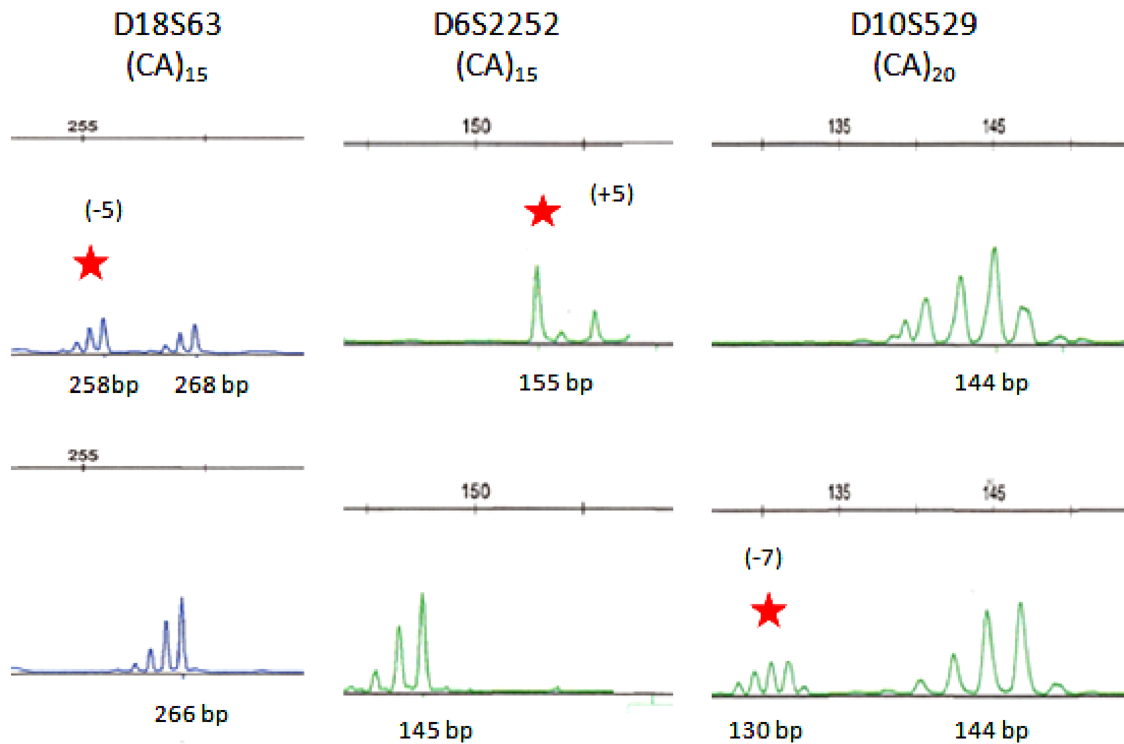

Figure 3.2 Continued

Notes: Examples of normal and mutated alleles of dinucleotide markers (D18S63, D6S2252, and D10S529) showing the corresponding normal allele as well as mutated allele that was shifted greater than 2 repeats or less than 3 repeats. Mutated alleles are shown with a red star and the number of repeat shifts in parentheses. (-) means a loss of repeat units, while (+) means a gain of repeat units. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of the allele in base pair (bp). Markers are labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

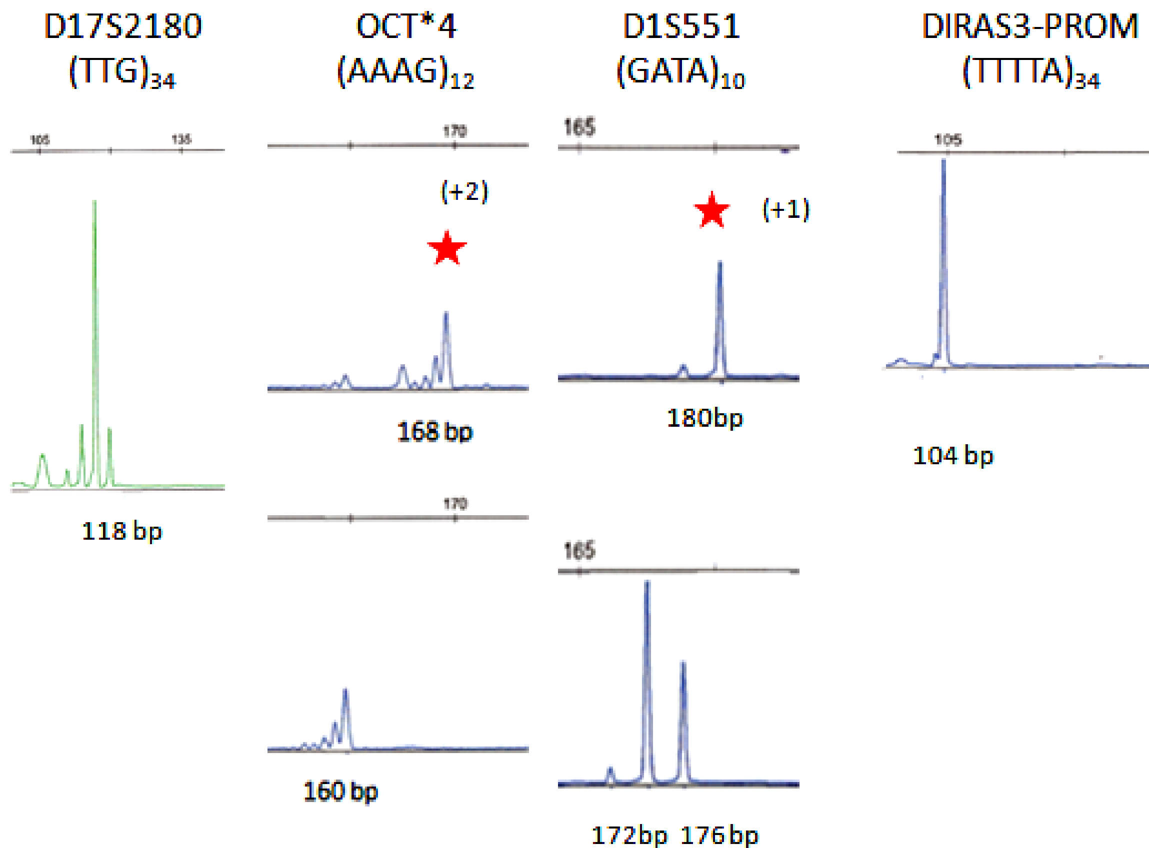

Figure 3.2 Continued

Notes: Examples of normal and mutated alleles of trinucleotide marker (D17S2180), tetranucleotide markers (OCT4, and D1S551), and pentanucleotide marker (DIRAS3-PROM) showing the corresponding normal allele as well as the mutated allele that were shifted greater than 1 repeat or less than 2 repeats. Mutated alleles are shown with a red star and the number of repeat shifts in parentheses. (-) means a loss of repeat units, while (+) means a gain of repeat units. The markers D17S2180 and DIRAS-PROM only show the normal allele because these markers were stable for the samples analyzed. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the base pair (bp) size of the allele in base pair (bp). Markers are labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

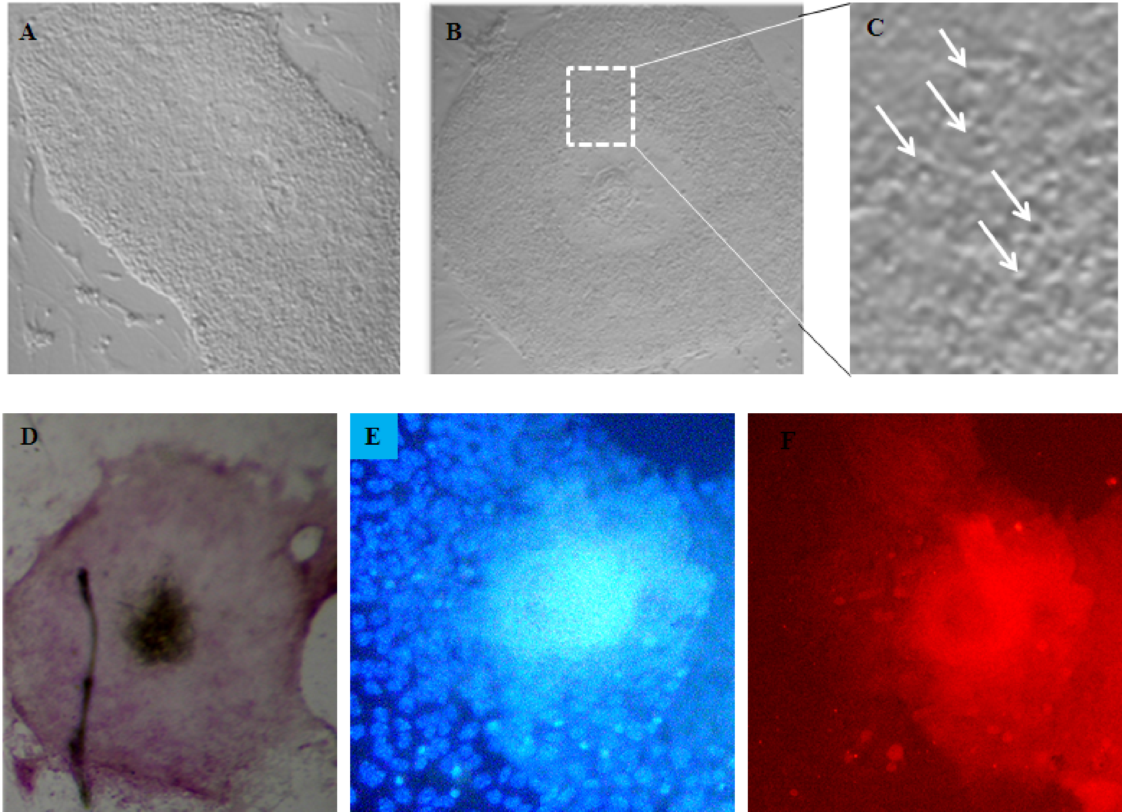

Figure 3.3 ES cell characterization

Notes: (A and B) H1 and H7 undifferentiated ES cells colonies, respectively. Colonies show a condensed and multilayer pattern of growth over the mouse embryonic fibroblast feeder layer (MEF) (Phase contrast photomicrographs with magnification 10X). (C) Boxed region from B, shows in 60X magnification of multilayer colony and displays typical H1 ES cells morphology in culture. The white arrows indicate ES cells with a high nucleus to cytoplasm ratio. (D) Alkaline phosphatase positive H7 ES cells colony. (E) H7 ES cells showing SSEA-1 positive expression (green). (F) ES cells showing OCT4 positive expression (red). Nuclei were visualized with DAPI stain (blue). Fluorescence photomicrographs are show with magnification of 60X.

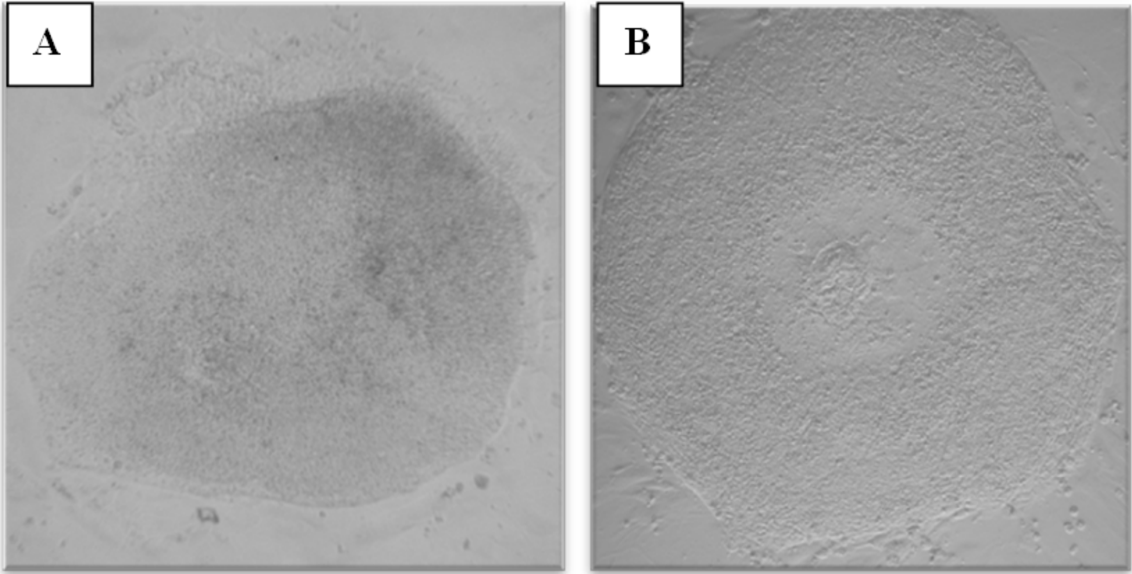

Figure 3.4 Morphologies of H1 ES cell colonies

Notes: (A) Phase contrast image shows an undifferentiated homogeneous colony. (B) Phase contrast image shows heterogeneous colony morphology with differentiation at the periphery of colony (white arrows). Phase contrast photomicrographs have a magnification of 10X.

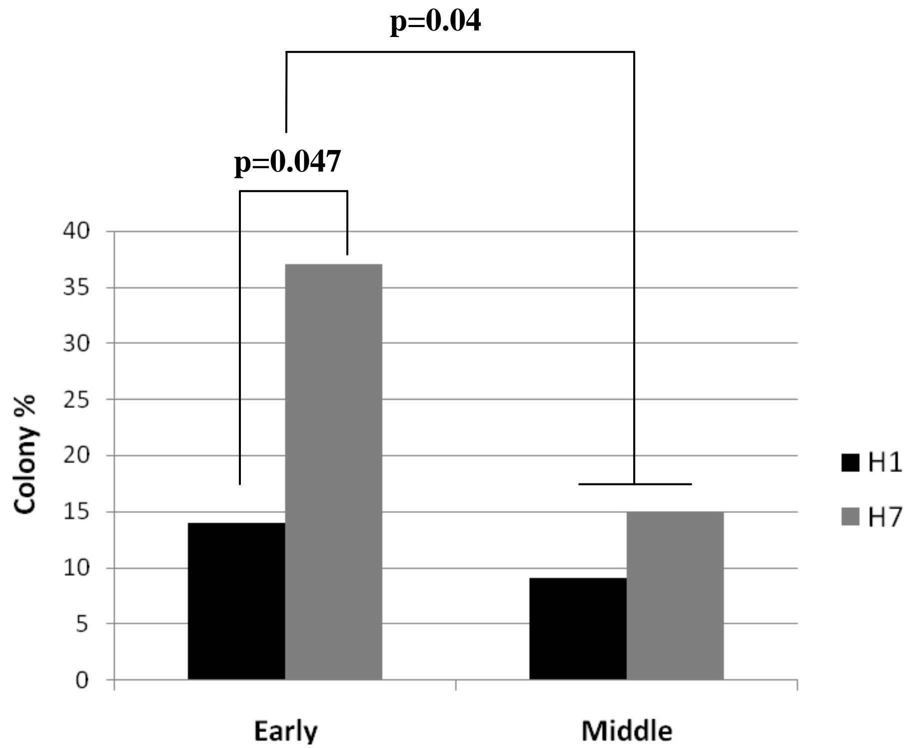

Figure 3.5 Percentage of H1 and H7 ES colony morphology changes *vs* culture passages

Notes: ES cells were subcultured/passaged approximately 20 times over 4 months by mechanical dissection of the colonies. Throughout, ES cells failed to retain their normal morphology. Values are the percentage of colonies with irregular morphology across passages. The differences in morphology for colonies of ES cell lines were statistically significant between H1 and H7 ES cell lines when compared to early (passages 27-28) and middle (passages 40-42). Error bars, SD, $p<0.05$ (n=4).

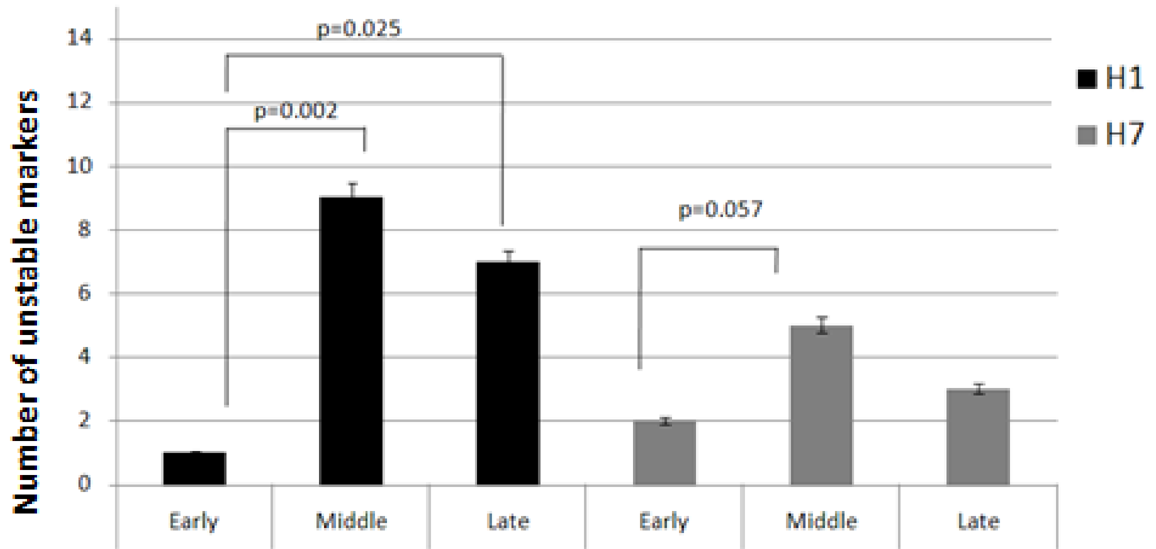

Figure 3.6 Number of unstable markers across culture passages

Notes: H1 cells show statistically significant differences for frequencies of unstable markers across passages in comparison to H7 cells ($p<0.05$). Values represent the number of markers that show instability through the passages in each ES cell line. Early (passages 27-28), middle (passage 42), and late (passage 78-82).

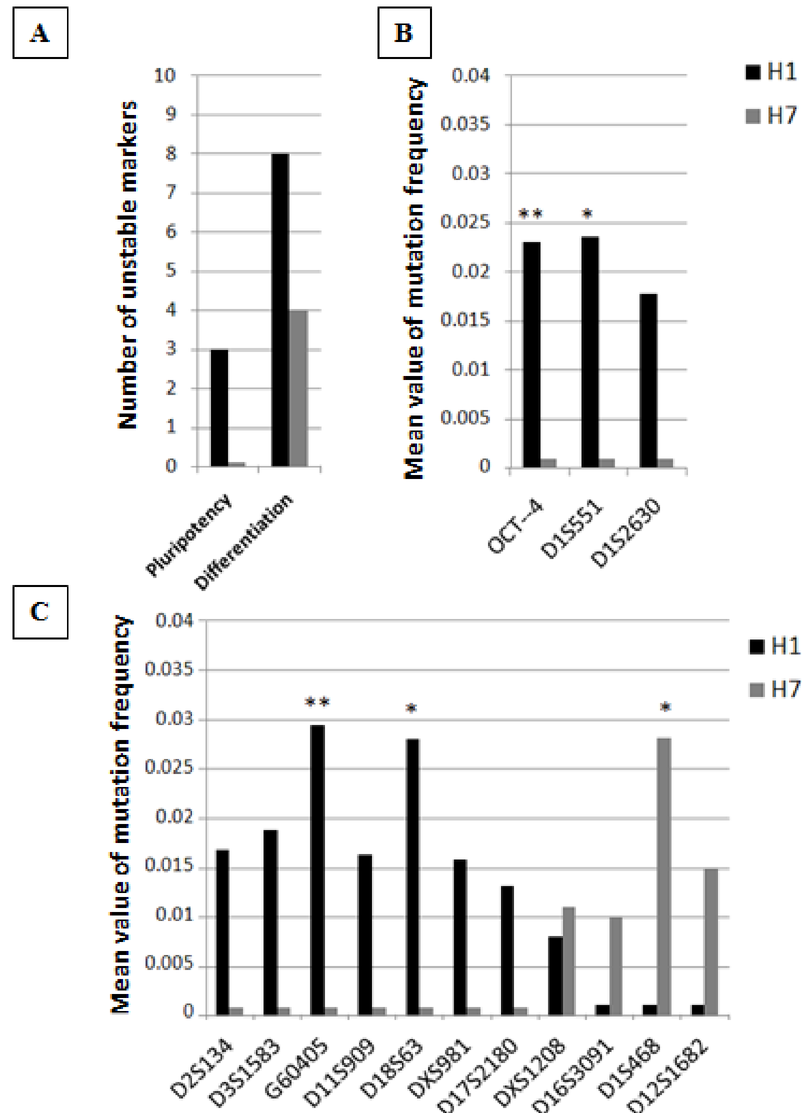

Figure 3.7 Unstable markers related to genes involved in pluripotency and early differentiation

Notes: Differences in the number of unstable markers and mean mutation frequencies were observed between H1 and H7 ES cell lines. (A) Shows the number of unstable markers per ES cell line, and the cellular status of either pluripotency or differentiation. (B) Mean values of mutation frequencies of unstable markers related to pluripotency genes. (C) Mean values of mutation frequencies of unstable markers related to differentiation genes. Values represent the mean value of mutation frequency of sample replicates (n=48) per marker that was calculated with SP-PCR software (MD Anderson Cancer Houston, TX). Statistically significance differences *p≤0.05, marginally significance p≤0.10.

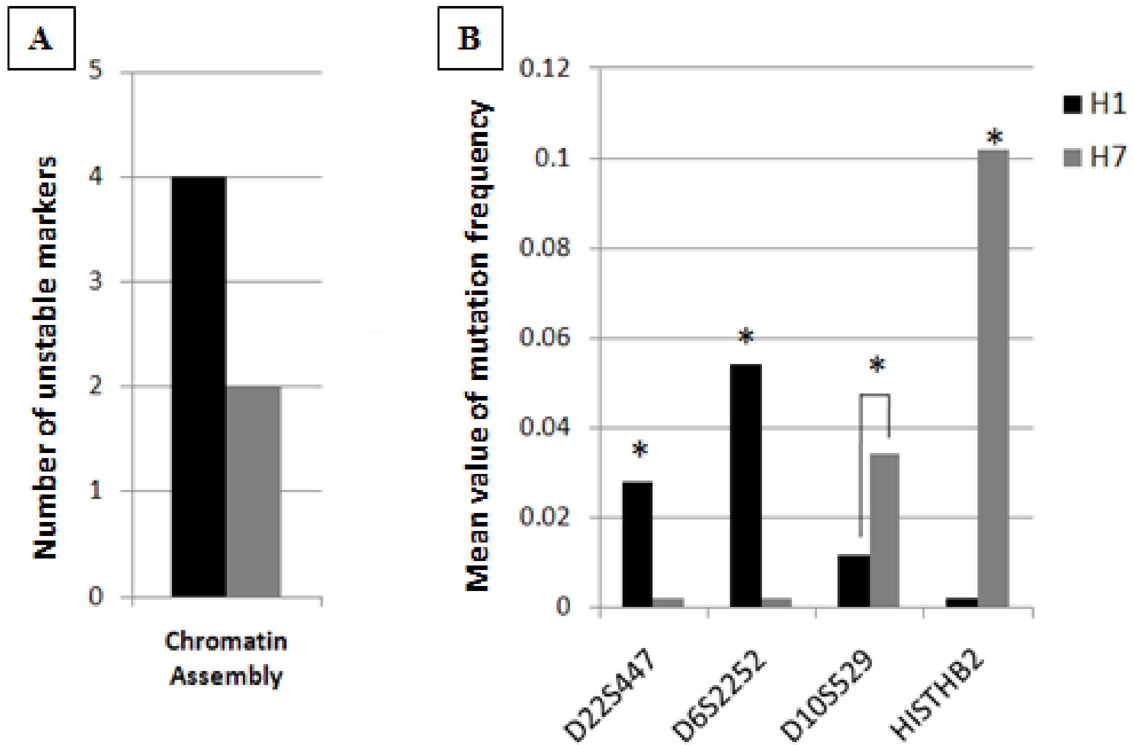

Figure 3.8 Unstable markers related to chromatin assembly genes

Notes: Differences in the number of unstable markers and mean mutation frequencies were observed between H1 and H7 ES cell lines. (A) Number of unstable markers per ES cell line. (B) Mean values for mutation frequencies of unstable markers related to chromatin assembly genes. Values represent the mean mutation frequency of sample replicates (n=48) per marker calculated with SP-PCR software (MD Anderson Cancer Houston, TX). HISTHB2 shows highly significant differences in mean mutation frequencies of H7 ES cells ($p < 0.001$). D10S529 shows instability in both ES cells lines, but H7 ES cells show a significantly higher mutation frequency compared to H1 ES cells ($p = 0.03$). Statistically significance *$p<0.05$.

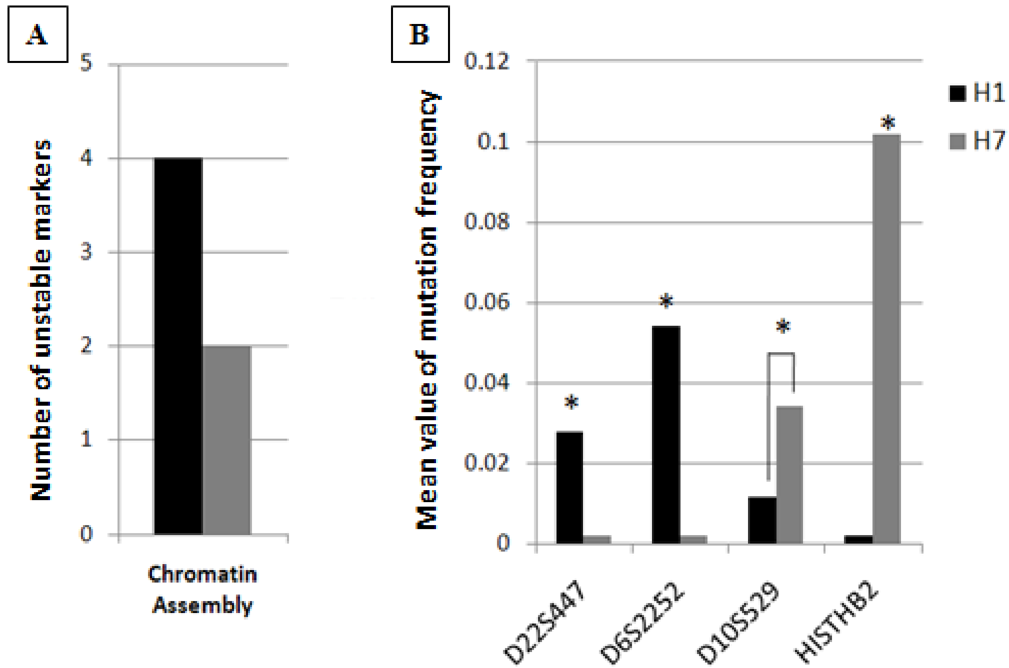

Figure 3.9 Unstable markers related to imprinting genes

Notes: Differences in the number of unstable markers and mean mutation frequencies were observed between H1 and H7 ES cell lines. (A) Number of unstable markers per ES cell line. (B) Mean values for mutation frequencies of unstable markers related to imprinting genes. Values represent the mean mutation frequency of sample replicates (n=48) per marker calculated with SP-PCR software (MD Anderson Cancer Houston, TX). GRB10-PROM shows instability for both ES cell lines, but H1 ES cells show a significantly higher mutation frequency compared to H7 ES cells ($p = 0.026$). Statistically significance $^*p<0.05$. Marginally significant differences $^{**}p <0.10$.

Table 3.1 List of single tandem repeat markers

Pluripotency	Differentiation		Chromatin Structure	Imprinting
OCT4	D16S3034	D4S1542	D7S488	GRB10PROM
D1S1656	D12S1719	DXS981	D6S1001	D20S821
D1S551	D4S2623	D14S588	HISTH4A	IGF2R
D12S1682	D2S134	D3S2459	HISTHB2	DIRAS3PROM
D1S2630	D11S1331	D17S2180	D10S529	PEG10PROM
D6S2384	D4S1625	EGFR	D22S447	SNURF10PROM
D6S416	D1S430	D16S3091	D8S11268	IGF2PROM
D2S2327	D2S290	D1S468	D22S941	IGF
kLF4-1	D3S1583	TNFa3	D7S638	
NANOG	DXS458	D15S983	D6S2252	
D9S1840	D21S1909	DXS1208	D2S144	
	D6S1698	D5S426	DNMT3	
	D10S1653	D3S1541		
	D11S909	G60405		
	D5S2021	D3S1611		
	D18S63	D11S2179		

Notes: Eleven markers were related to pluripotency genes, thirty-three were related to differentiation genes, twelve were related to chromatin structure genes, and eight were related to imprinting genes.

Table 3.2 Mutation frequencies of five single tandem repeat markers located near genes related to pluripotency and differentiation

ES cells	Passage Number	OCT4			D1S551			G60405			D18S63			D1S468		
		n	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*
H1	28	46	2	0.031	54	0	0.000	33	0	0.000	37	0	0.000	45	0	0.000
	42	37	0	0.000	56	2	0.024	48	2	0.029	37	2	0.028	29	0	0.000
	82	35	1	0.016	63	0	0.000	32	0	0.000	41	0	0.000	33	0	0.000
H7	27	33	0	0.000	48	0	0.000	33	0	0.000	63	0	0.000	55	0	0.000
	42	27	0	0.000	74	0	0.000	25	0	0.000	34	0	0.000	64	3	0.028
	78	75	0	0.000	74	0	0.000	35	0	0.000	42	0	0.000	61	0	0.000
	p-value			**0.060**			**0.046**			*0.077*			**0.036**			**0.050**

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated by SP-PCR software with SP-PCR software (MD Anderson Cancer Houston, TX). p-values ≤ 0.05 are in **bold,** p-value ≤ 0.10 in *italic*

Table 3.3 Mutation frequencies of three single tandem repeat markers located near genes related to chromatin assembly

ES cells	Passage Number	Chromatin Assembly Markers											
		D6S2252			**HISTHB2**			**D10S529**			**D22S447**		
		n	***m***	***f***	***n***	***m***	***f***	***n***	***m***	***f***	***n***	***m***	***f***
H1	28	67	0	0.000	47	0	0.000	47	0	0.000	46	0	0.000
	42	41	3	0.054	38	0	0.000	39	0	0.000	37	2	0.028
	82	49	0	0.000	40	0	0.000	40	1	0.012	45	0	0.000
H7	27	33	0	0.000	35	7	0.102	40	3	0.034	53	0	0.000
	42	42	0	0.000	34	0	0.000	40	0	0.000	38	0	0.000
	78	63	0	0.000	37	0	0.000	36	0	0.000	68	0	0.000
	p-value			**0.018**			**< 0.001**			**0.030**			**0.050**

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated by SP-PCR software with SP-PCR software (MD Anderson Cancer Houston, TX). p-value ≤ 0.05 are in bold

Table 3.4 Mutation frequencies of three single tandem repeat markers located near genes related to imprinting genes

		Imprinting Markers								
ES cells	**Passage Number**	**GRB10-PROM**			**D2S144**			**IGF2-PROM**		
		n	***m***	***f***	***n***	***m***	***f***	***n***	***m***	***f***
H1	28	44	0	0.000	45	0	0.000	66	0	0.000
	42	45	1	0.016	28	0	0.000	54	0	0.000
	82	40	0	0.000	34	0	0.000	65	0	0.000
H7	27	35	0	0.000	37	0	0.000	53	0	0.000
	42	71	1	0.009	41	1	0.020	58	2	0.022
	78	63	0	0.000	64	0	0.000	45	0	0.000
	p-value			**0.026**			**0.040**			*0.080*

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated with SP-PCR software (MD Anderson Cancer Houston, TX). p-values ≤ 0.05 are in bold, p-value ≤ 0.10 in *italic*

Table 3.5 List of unstable markers

Name Marker	Gene Symbol	Gene Name	Gene Function	References
OCT4	POU2 F1	POU class 2 homeobox 1	Developmental transcription factor	Chambers I, 2003 Loh YH, 2006
D1S551	RGS4	Regulator of G-protein signaling 4	Signal transduction regulator	Strübing C, 1997 Neves S, 2002
D1S2630	POU2 F1	POU class 2 homeobox 1	Developmental transcription factor	Masui S, 2007 Chen X, 2008 Fernandez T,2010
D2S134	MEIS1	Meis homeobox 1	Embryo development	Xiang P, 2010 Mojsin M, 2009
D3S1583	RARB	Retinoic acid receptor, bet	Embryo development	Elizalde C, 2011 Sheng N, 2010
G60405	ERCC6	Excision repair cross-complementing group 6	DNA repair	Pastoriza-G, 2007 Sabatino M, 2010
D11S909	ST5	suppression of tumorigenicity 5	Tumor suppressor	Göhring I, 2009
D18S63	TGIF1	TGFB-induced factor homeobox 1	Growth factor activity	Hamid R, 2009 Pazmany T, 2006
DXS981	SPG16	spastic paraplegia 16	Developmental gene	Tamagaki A, 2000
D17S2180	HOXB5	homeobox B5	Developmental transcription factor	Fu M, 2003 Lui VC, 2008
DXS1208	HSPB1	Heat shock thermic protein	Developmental transcription factor	Asangi RK, 2010 Schwarz L, 2010
D16S3091	CDH13	cadherin 13, H-cadherin (heart)	Growth factor activity	Li L, 2010

Notes: Summary of characteristics of genes located in close proximity to unstable markers involved in embryonic development. Twelve markers showed statistically significant instability frequencies and are identified in bold ($p<0.05$).

Table 3.5 continued

Name Marker	Gene Symbol	Gene Name	Gene Function	References
D1S468	TP73	tumor protein p73	Developmental transcription factor	Berna S, 2010 Kim KP, 2007
D12S1682	PDE3A	phosphodiesterase 3A, cGMP-inhibited	Tissue remodeling	Jeremy W, 2002
D22S447	HIRA	HIR histone cell cycle regulation defective homolog A	Nucleosome assembly	Ahyar A, 2005 Zhang R, 2005
D6S2252	HIST1 H2AH	histone cluster 1, H2ah	Nucleosome assembly	Hengbin W, 2004 Zhang R, 2005
D10S529	H2A FY2	H2A histone family, member Y2	Nucleosome assembly	Matthew J, 2010 Marcus B, 2009
HISTHB2	HISTH B2	H2A histone family, member Y2	Nucleosome assembly	Lee MG, 2007
GRB10	GRB10	growth factor receptor-bound protein 10	Growth factor activity	Norio T, 2007 Monk D, 2009
D2S144	DNMT3 A	DNA (cytosine-5-)-methyltransferase 3 alpha	DNA methylation	Taiping C, 2002 Bethany L, 2010
IGF2	IGF2	insulin-like growth factor 2	Growth factor activity	Tabano S, 2010 Demars J, 2010

3.6 Acknowledgements

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CHAPTER IV

GENOMIC INSTABILITY DURING EARLY DIFFERENTIATION OF EMBRYONIC STEM CELLS

4.1 Abstract

Understanding how genomic instability could be involved in the regulation and establishment of cell lineage commitment during embryonic stem (ES) cell differentiation into an embryoid body (EB) would provide crucial knowledge of stem cell biology. Therefore, defining the signaling pathway that controls early cell fate decisions is an important focus of research. Here, we determine the degree of instability in single tandem repeat markers located near embryonic developmental genes responsible for pluripotency, differentiation, and imprinting of the ES cells. We determined that the mean values of instability frequencies in EB from H1 and H7 ES cell lines showed significant differences across time between ES cell lines. Markers that became unstable during spontaneous differentiation showed higher instability frequencies associated with pluripotency (D1S551), differentiation (D16S3034, D16S3090, D14S588, D11S4090, D3S1583, D1S468, DXS1208, D4S2623, and D18S63), and imprinting (IGF2PROM, GRB10PROM, HISTHB2, D6S2252, D2S144, D3S1611, D7S488, and D10S529). Genomic instability influences the loss of pluripotency and the gain of cell lineage specialization. Interestingly, the differentiation potential of EBs from the two stem cell lines varied. EBs from H1 were prone to neuroectoderm differentiation in comparison to EBs from H7, which showed functional differentiation into mesoderm in the form of

contractile cardiac muscle. We suggest that genomic instability in repetitive regions could be a signal for cell fate decision during differentiation among ES cell lines. Our results indicate correlation of instability in specific markers located near developmental genes and epigenetic modulators in EB that underwent spontaneous differentiation *in vitro*. The significance of elucidating possible molecular mechanisms of genomic instability and validation of novel biomarkers could potentially lead to use of ES cell derivatives are safe source for cell tissue replacement in clinical applications.

4.2 Introduction

ES cells are derived from the inner cell mass of a blastocyst after the first differentiation stage during embryonic development. During this stage, the embryo loses totipotency and displays pluripotent characteristics that ensure its potential for differentiation into the three germinal cell layers (Thomson *et al.* 1998; Chen *et al.* 2008). ES cells can differentiate into all cell types of the embryo by spontaneous differentiation *in vitro* into EBs. Colonies of ES cells can be cultured in suspension with conventional ES cell culture techniques in a medium supplemented with serum containing many undefined growth factors that induce differentiation into EB. Differentiation into EB is spontaneous process that always displays a heterogeneous mix of cell populations and is a technique to demonstrate the pluripotency capacity of the ES cells to differentiate into all three germinal layers (Thomson *et al.* 1998; Enver *et al.* 2005). However, these protocols are inappropriate for obtaining large numbers of homogeneous and pure cell type populations that are needed for cell regeneration treatments of human diseases.

Conditions for culture establishment and maintenance are a constant scientific challenge to improve the methodology used for this therapeutic aim. Determination and

validation of precise growth factors that direction of ES cell specialization towards a homogeneous population of a selected germinal layer such as ectoderm for neurons, endoderm for pancreatic cells, or mesoderm for cardiac muscle cells still is a work in progress. Several approaches have been reported for supplementing media with specific exogenous growth factors that direct ES cell differentiation to induce mature specialized cell types, such as noggin factor that induces neural lineage commitment, activin A that induces definitive endoderm, and bone morphogenic protein (BMP) plus activin A that induce mesoderm differentiation into early cardiac muscle cells (Ben-Hur *et al.* 2004; D'Amour *et al.* 2006; Yao *et al.* 2006; Jiang *et al.* 2007; Yang *et al.* 2008; Zhou *et al.* 2008).

Differentiation is a constant process of gene modification and chromatin regulation that is responsible for the specific signals that induce morphological and functional changes in early cell progenitor derivatives during embryonic development (Niwa *et al.* 2005). Differentiation reflects the alteration of balance between ES cell pluripotency and self-renewal. ES cells that differentiate lose pluripotency and gain the lineage-specific signature that displays specific cell tissue identity through gene and chromatin modification in the promoter regions of developmental genes responsible for pluripotency and early cell differentiation (Mohn *et al.* 2008). Identification of the molecular switches that regulate differentiation of early cell progenitors could be used as a tool for target ES cell pluripotency and achieve differentiation homeostasis.

Pluripotency, self-renewal, and differentiation signals in ES cells naturally occur as a result of extracellular environmental stimuli. Regulation of these specific cellular signals during ES cell maintenance *in vitro* contributes to correct cell fate decisions (Niwa 2007). Genetic and environmental changes influence the phenotype of the ES cell

lines. It is important to fully characterize ES cell lines for comparative analyses in order to completely define their identity. Precise and well standardized biomarkers are needed to characterize ES cells at molecular and functional levels to ensure their quality and efficiency for cellular transplant and organ regeneration applications. In addition, this will be a crucial tool for basic embryonic development, drug testing, toxicology, and tumorigenesis research.

Several studies reported successful EB formation from ES cell lines through spontaneous differentiation into the three germinal layers. Gene expression and epigenetic pattern characteristics that underline differentiation of ES cell lines *in vitro* have been widely reported (Brimble *et al.* 2004; Ware *et al.* 2006; Adewumi *et al.* 2007; Allegrucci *et al.* 2007; Osafune *et al.* 2008). However, the precise molecular signals that coordinate ES cell differentiation are not understood. Identification of unstable repetitive sequences of the DNA is a sensitive molecular technique to evaluate DNA integrity of ES cells. ES cells in culture acquire different genetic and epigenetic modifications in order to maintain pluripotency or induce ES cell differentiation into the functional phenotype of lineage specific neuroectoderm, mesoderm, or endoderm cell layers (Enver *et al.* 2005; Allegrucci *et al.* 2007; Baker *et al.* 2007).

We determined genomic instability during spontaneous differentiation of EB from H1 and H7 ES cell lines. EB samples were obtained at three time points of EB progression at 7, 14, and 30 days after differentiation induction. The frequency of genomic instability in 63 single tandem repeat markers located near pluripotency, differentiation, and imprinting genes was determinate by calculating the instability frequency of each sample per marker. The aim of this study was to determine novel molecular biomarkers for monitoring ES cell signals that govern differentiation *in vitro.*

We report that genomic instability could be the signal that leads to ES cell differentiation through modulation of gene expression and epigenetic modifications during cell lineage and tissue derivation of cell populations in EBs from H1 and H7 ES cell lines. Finally, determination of reported single tandem repeat stability offers a mechanism for characterization as well as defining new protocols for directing ES cell differentiation *in vitro* towards particular cell lineages that are needed for clinical applications

4.3 Materials and Methods

4.3.1 Embryonic stem cell culture conditions

Frozen aliquots from human ES cells H1-WA01 passage 27 and H7-WA07 passage 26 were purchased from the National Stem Cell Bank – Wisconsin International Stem Cell Bank (Appendix B). H1 and H7 ES cells were seeded onto a mouse embryo fibroblast-CF1 (MEF) feeder layer previously inactivated with mitomicyn C. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) knockout medium (Invitrogen, Carlsbad, CA) supplemented with 10% knockout serum replacement (Invitrogen, Carlsbad, CA), 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA), 100 μM β-mercaptoethanol (Sigma Aldrich Saint Louis, MO), 20 ng/ml basic fibroblast growth factor (b-FGF) (Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Invitrogen Carlsbad, CA), 2 nM L-glutamine (Invitrogen, Carlsbad, CA), and 20 ng/ml of Leukemia Inhibitor Factor (LIF) (Chemicon/Millipore Billerica, MA). ES cells were maintained in a humidified atmosphere at 37°C in 5% CO_2. The medium was changed daily.

4.3.2 Embryoid body formation

For *in vitro* differentiation of ES cells through embryoid body formation, undifferentiated ES cell colonies at 3-5 days post-passage, maintained on a mouse embryo fibroblast (MEF) feeder layer during 15 passages for both ES cell lines, were mechanically dissociated and transferred into a low attachment petri dish containing embryoid body (EB) medium. EB medium consisted of Dulbecco's Modified Eagle Medium (DMEM) knockout medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen, Carlsbad, CA), 1% non essential amino acids (NEAA) (Invitrogen, Carlsbad, CA), and 0.1 mM β-mercaptoethanol (Sigma, Aldrich Saint Louis, MO). EBs were cultured in suspension for 5 days with medium changes every other day. Then, EBs were transferred to 60 mm center-well culture dishes (Corning Incorporated, Corning, NY) previously coated with 0.1% gelatin (Sigma, Aldrich Saint Louis, MO) and cultured for 30 days with EB-medium in a humidified atmosphere at 37°C and 5% CO_2. During the 30 day culture period, medium was renewed every 2 days, but less frequent medium changes were made depending on EB culture density. Samples of EBs in culture were collected at 7, 14, and 30 days for histopathology, immunohistochemical, and molecular analysis.

4.3.3 Histopathology

EBs were harvested and fixed with formalin (Sigma-Aldrich, Saint Louis, MO) overnight at room temperature, washed in 1X PBS, dehydrated with an ethanol wash, and embedded in paraffin. The five micrometer thick sections from the paraffin embedded EBs were placed on slides. After deparaffinization of the slide by xylene, alcohol, and water washes, slides were stained with hematoxylin-eosin for routine histological examination under microscope.

4.3.4 Immunohistochemical analysis

EB samples attached to gelatin were fixed with 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO) for 15 minutes at room temperature, washed in 1X PBS, and immunostained (Miltenyi Biotec Inc, Auburn, CA). The primary antibodies used were rabbit anti-GATA4 polyclonal antibody (endoderm), anti-myosin heavy chain monoclonal antibody (mesoderm), and anti- β-III Tubulin clone AA2 alexa fluor 488 conjugated mouse monoclonal antibody (ectoderm) (Chemicon/Millipore Billerica, MA). Antigen detection was performed with secondary antibodies; goat-anti-rabbit IgG rhodamine and C5Y conjugated secondary antibody (Chemicon/Millipore Billerica, MA). Each antibody was diluted to 1:200 in 1X PBS with 0.1% Triton X-100 and 3% BSA. Nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI) staining (Vysis Abbott Laboratories, Abbott Park, IL). EBs stained without the primary antibody served as a negative control. Images were captured using a fluorescence microscope, Axiovert 135, (Carl Zeiss International), with a FITC and Rhodamine filter set. Fluorescent intensities were measured with a semi quantitative method using image software developed at the National Institute of Health (Bethesda, MD, USA). Software was downloaded from http://rsb.info.nih.gov/ij/index.html. Accumulation of fluorescence was calculated by averaging the fluorescent ratio between exposed and unexposed areas in the nuclei.

4.3.5 Germinal layer separation from embryoid bodies

After 30 days in differentiation culture, EBs were harvested using 0.5 mg/ml collagenase D (Sigma Aldrich, Saint Louis, MO) in DMEM for 5 minutes at 37°C and pipetting to obtain a single cell suspension. EBs were washed twice with 1X PBS. Suspensions of cells were separated by positive selection with the Mini-MAC system cell

separation into three different types of cell populations (Miltenyi Biotec Inc, Auburn,CA). Ectoderm cells were magnetically labeled with Anti-PSA-NCAM microbeads, mesoderm cells with CD56 microbeads, and endoderm cells with CD326 (EpCAM) microbeads (Miltenyi Biotec Inc, Auburn, CA). Cells from each specific separation (ectoderm, mesoderm, and endoderm) were collected for DNA isolation and molecular analysis.

4.3.6 DNA isolation

DNA was prepared from each sample of EBs (7, 14, 30 days of differentiation induction) that were previously collected in 1X PBS by mechanical disruption in pieces under a stereomicroscope. Samples from EBs and cell suspensions were isolated with the Purelink™ genomic DNA mini-kit (Invitrogen Carlsbad, CA) following the manufacturer's protocol. All DNA samples were quantified using a NanoDrop™ ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

4.3.7 Determination of genomic instability with single cell PCR

Analysis of genomic instability of all DNA samples (listed previously) was carried out using 63 STR markers. Eleven markers were located near promoters of pluripotency genes, thirty-two markers were related to differentiation genes, and twenty markers were related to imprinting genes (Table 4.1). Total reaction volume of fluorescent multiplex PCR reactions was 10µl containing 1X of 10X buffer D, 2.5 mM of $MgCl_2$, 1.25 U of Hot-MultiTaq DNA polymerase, 1X of Solution L (these four reagents are from US DNA, Fort Worth, TX), 4% of DMSO (Sigma Aldrich, Saint Louis, MO), 0.4 mg/ml of BSA (Thermo Scientific, Rockford, IL), 300 µM of dNTPs (Applied Biosystems, Foster City, CA), and primer sets at a final concentration ranging between

0.2-2 µM (Table 2.2 in CHAPTER II). Each replicate contained 9 µl of master mix with 1µl of DNA at a concentration of less than a single diploid genome-equivalent (25-50 pg/µl). This DNA concentration allowed detection of wild type and mutant alleles in the same replicate (Coolbaugh-Murphy *et al.* 2004). Forty eight replicates for each marker and each sample with appropriate negative controls were amplified. PCR was performed on a PE 9600 thermocycler using a ramping protocol: 1 cycle of 95°C for 11 minutes; 1 cycle of 96°C for 1 minute; 10 cycles of [94°C for 30 seconds, ramp 68 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 25 cycles of [90°C for 30 seconds, ramp 60 seconds to 58°C (hold for 30 seconds), ramp 50 seconds to 70°C (hold for 60 seconds)]; 1 cycle of 60°C for 30 minutes for final extension for adenine addition; and hold 4°C. Negative controls without DNA were included to check for contamination.

Fluorescent PCR products (0.5 µl) were denatured in formamide (4.35 µl) (Applied Biosystemss, Foster City, CA) and size standard Genescan 500-LIZ (0.15 µl) (Applied Biosystemss, Foster City, CA), to be detected by capillary electrophoresis on an AB3130xl Genetic Analyzer (Applied Biosystemss, Foster City, CA). Fragment size of alleles was estimated by GeneMapper software version 4.0 (Applied Biosystemss Foster City, CA). Wild type and mutated alleles of each marker were quantified according to standardization explained in the Materials and Methods section 3.3.7 in CHAPTER III. Examples of normal and mutated alleles per markers are shown in Figure 4.1.

4.3.8 Statistical analysis of genomic instability

Mutation frequencies were determined using SP-PCR software version 1.0 (M.D. Anderson Cancer Center Houston, TX) (Appendix C). Differences in mutation

frequencies were calculated with a two tailed t-test using raw mutation frequencies in the statistical package SAS/win 9.2 (SAS Institute, Cary, NC) to determine statistically significant differences (p-value ≤ 0.05) in the mean mutation frequencies of informative markers. Marginally significant differences were considered if the p value was ≤ 0.10.

4.4 Results

4.4.1 ES cells spontaneously differentiate into embryoid bodies *in vitro*

ES cell pluripotency is evaluated by the efficiency with which they form EBs and drive ES cell differentiation into the three germinal layers symmetrically and spontaneously upon removal of leukemia inhibitor factor (LIF) and the MEF feeder layer, and supplementing of the medium with 20% fetal calf-serum (Chambers *et al.* 2003; D'Amour *et al.* 2006). Initially, our EBs developed compact and tri-dimensional cell aggregates in suspension during the first 7 days. Once they attached, EB aggregates began to spread and display an irregular shape distribution. This was indicative of differentiation into a heterogeneous mix of cell populations derived from the three germinal layers from day 7 to day 30 after induction of EB differentiation. Microscopic morphology of EBs was determined with stained paraffin-embedded sections. The EB morphology showed stratified keratinizing epithelium (characteristic of ectoderm), cardiac muscle (characteristic of mesoderm), and pseudostratified columnar epithelium (characteristic of endoderm) (Figure 4.2). The morphology, coupled with the expression of specific immunofluorescence markers (β-III tubulin-ectoderm, Myosin-mesoderm, and GATA4-endoderm), confirmed that H1 and H7 ES cell lines maintained the capacity for spontaneous differentiation into EBs that display a mixed population of cells from the three germinal layers during *in vitro* culturing (Figure 4.3).

4.4.2 Genomic instability is present during ES cells differentiation *in vitro*

During early determination of cell fate, genes responsible for cell differentiation are activated and pluripotency genes are turned off. The molecular mechanisms that induce these gene expression changes are unclear (Boyer *et al.* 2005). The aim of this study was to determine if genomic instability was present at this time and could possibly be a signal of ES cells' spontaneous differentiation. ES cells that efficiently differentiate into EBs were evaluated for genomic instability with 63 single tandem repeat markers located near genes involved in pluripotency, differentiation, and imprinting (Table 4.1). DNA from H1 and H7 EBs at three time points (7, 14, and 30 days) after culture establishment were analyzed. Significant mean frequencies of instability in 18 out of 63 markers were detected. Markers located near differentiation and imprinting genes displayed higher instability frequencies compared to markers located near pluripotency genes. Only one marker located near a pluripotency gene showed significant instability (D1S551). In comparison, nine markers located near differentiation genes were significantly unstable (D16S3034, D16S3090, D14S588, D11S4090, D3S1583, D1S468, DXS1208, D4S2623, and D18S63), and eight markers related to imprinting were significantly unstable (IGF2PROM, GRB10PROM, HISTHB2, D6S2252, D2S144, D3S1611, D7S488, and D10S529). These results show that genomic instability was detected in specific single tandem repeat markers during EB differentiation and could be determinate signals for ES cell fate specialization.

4.4.3 Single tandem repeat instability increased during EB formation over time

ES cell differentiation occurs in a spontaneous multistep manner, which results in cell specialization. We found significant differences in genomic instability among EBs from H1 and H7 ES cell lines over time. At 30 days post differentiation, EB samples

were significantly unstable in comparison to EBs from 7 and 14 days. Instability frequencies at day 30 were increased two-fold in comparison with the earlier frequencies (day 7). EBs from H1 showed an instability mutation frequency of 0.018 at 7 days and 0.039 at 30 days, whereas EBs from the H7 showed an instability frequency of 0.016 at 7 days and 0.036 at 30 days ($p<0.05$) (Figure 4.4). EBs from H1 show an increased number of unstable markers (n=18) time in comparison to EBs from H7 (n=14). HISTHB2, IGF2PROM, and D3S1583 were unstable markers at 7 days of EB culture. In contrast, markers that were unstable at 14 days included D18S63, D3S1611, D6S2252, HISTHB2, D16S3034, and D3S1583. At 30 days unstable markers: D1S468, DXS1208, D2S144, GRB10PROM, D7S488, D4S2623, D10S529, D16S3090, IGF2PROM, D11S4090, and D14S588 (Figure 4.4). These observations confirmed that spontaneous differentiation occurred at the same time that genomic instability increased during EB formation. Therefore, genomic instability could potentially drive differentiation progression *in vitro.*

4.4.4 Embryoid bodies from H1 and H7 ES cell lines show different unstable marker profiles

During the last decade, complete gene expression profiles have been reported for ES cells. Gene expression modifications are due to ES cell culture maintenance *in vitro* (Boyer *et al.* 2005; Loh *et al.* 2006). The aim of this study was to determining whether EBs from H1 and H7 ES cell lines would show instability in the same markers. Our study reports that eight markers showed increased instability in markers for EBs of both ES cell lines (D16S3034, D10S529, D14S588, D16S3091, IGF2PROM, D11S4090, D3S1583, and D1S551) (Figure 4.5 and Table 4.2). EBs from H1 showed the highest significant mean frequencies of instability in seven additional markers (D1S468, DXS1208, D2S144, GRB10PROM, D18S563, D3S1611, and D7S488) ($p\leq0.05$) (Figure 4.6 and

Table 4.3) whereas, EBs from H7 showed instability in only three additional markers (HISTHB2, D6S2252, and D4S2623) ($p \leq 0.05$) (Figure 4.6 and Table 4.4). Notably, unstable markers in EB from H1 were located near genes involved in early neuroectodermal differentiation. In contrast, EBs from H7 displayed instability in markers located near genes involved in mesodermal and endodermal differentiation. EBs from both H1 and H7 ES cell lines showed instability in markers related to imprinting genes. Taken together these results confirm that ES cells showed differential profiles of unstable markers during EB differentiation *in vitro.*

4.4.5 Instability in repetitive regions related to differentiation genes coordinate cell fate decisions

Upon progression of differentiation, gene modifications act as signals that facilitate cell fate decisions (Smith 2005; Feldman *et al.* 2006; Galán *et al.* 2010). We searched for possible associations between genomic instability of specific markers and the differentiation preferences of individual ES cell lines. We characterized our ES cell lines by morphologic and genomic instability patterns throughout EB differentiation *in vitro*. EBs from H1 and H7 ES cell lines showed mixed populations of cells after 30 days of culturing in EB media supplemented with fetal calf serum. However, we observed that multiple neural rosette like neuroectoderm structures were more common in EB cultures from H1 when compared to EBs from H7 (Figure 4.7). This is positively correlated with instances of instability that showed increased mean frequencies for markers specific to the neuroectoderm layer (D11S4090, D3S1583, D1S468, DXS1208, and D18S63). In contrast, EBs from H7 showed instability in markers related to mesoderm (D14S588 and D16S3091) (Boie *et al.* 1995; Li *et al.* 2010), and endoderm differentiation (D4S2623) (Chu and Shen 2010) (Table 3.5). In addition, EBs from H7 showed morphological and

functional spontaneous differentiation into contracting EBs, increased cell confluency, as well as increased contraction rates in the developing EBs (Figure 4.8). Our results show that instability in repetitive regions near genes responsible for early cell differentiation of neuroectoderm and mesoderm were not equally regulated between differentiation of H1 and H7 ES cell lines *in vitro.*

4.4.6 Epigenetic modification during spontaneous EB differentiation a result from genomic instability

Early embryonic differentiation signals are regulated by epigenetic changes such as imprinting, chromatin changes, and methylation (Lee *et al.* 2006; Pasini *et al.* 2007; Christophersen and Helin 2010). We found that specific markers related to imprinting showed increased frequencies of instability during EB differentiation from both ES cell lines. Markers associated with tissue specific imprinting genes (IGF2, and GRB10), histone genes (HISTHB2, D6S2252, D7S488, and D10S529), *de novo* methylation genes (D2S144), and DNA repair genes (D3S1611) showed significant differences in instability in EB from H1 and H7 ES cell lines. Markers located in the promoter regions of the genes IGF2 and GRB10 displayed highly significant instability ($p<0.001$). The marker near IGF2 was unstable in EBs from both the H1 and H7, with the highest instability frequency found in EBs from the H7. The marker in GRB10 however, was exclusively unstable in EBs from the H1. Additionally, D2S144 was significantly, and exclusively, unstable in EBs from H1 ($p=0.0081$) compared with EBs from the H7 that were stable. D10S529 was unstable in EBs from both ES cell lines but was highly significantly unstable in EBs from the H1 compared with EB from H7 ($p=0.0308$). Together, these findings demonstrate that instability as a molecular signaling pathway might control the

epigenetic changes necessary to induce gene expression changes in ES cells that are undergoing early progenitor differentiation.

4.5 Discussion

Understanding the molecular signals that regulate the decisions determining ES cell fates *in vitro* during early progenitor differentiation can help identify reliable genetic markers that will be useful for characterizing the mix of cell populations obtained from the three germinal layers. Careful characterization of ES cells and their cell-type specific outcomes serve to validate them for prospective clinical applications that would require specific ES cell progenies isolated from spontaneous differentiation protocols *in vitro*. We evaluated single tandem repeat markers located near embryonic developmental genes related to pluripotency, differentiation, and imprinting of ES cells, to determine their stability during spontaneous differentiation of EBs *in vitro.*

Normally, ES cells can differentiate into a heterogeneous mixed population of EB cell types from the three germinal layers *in vitro*. EBs can be differentiated into a wide variety of cell types that are functionally equivalent to *in vivo* tissues (Chambers *et al.* 2003; D'Amour *et al.* 2006). We hypothesized that instability in repetitive sequences located near important genes responsible for cell differentiation could control the subsequent cell fate decisions during the progression of differentiation.

Differentiation is a process where pluripotency of the ES cells is lost through embryogenesis. ES cells differentiate progressively until they achieve complete cell specialization and functional cell-tissue capacities (Feldman *et al.* 2006; Galán *et al.* 2010). Our results show that EBs undergoing differentiation accumulates instability in different markers located near possible target genes that are responsible for early cell

differentiation and imprinting. This is consistent with our results that just one pluripotency marker was unstable in comparison with nine unstable markers related to differentiation, and eight unstable markers related to imprinting during EB culturing. This is in contrast, our findings that. Our data suggest that some pluripotency genes still act upon progression of differentiation until complete cell lineage commitment is achieved. In addition, the observation of progressive instability in markers located near differentiation and imprinting genes could be the signals of specific cell fate decisions that are required for each ES cell line.

First, ES cells undergoing differentiation need intracellular and extracellular signals that vary over time that regulate the transcriptional factor machinery. This induces cell type specific changes through completion of lineage commitment (Snykers *et al.* 2007; Chowdhury *et al.* 2010). Extracellular signals, such as stress from the culture environment and continual passaging, might affect the stability of ES cells undergoing differentiation. The efficiency to further direct homogeneous differentiation is also reduced because of subsequent losses in genomic integrity and changes in gene expression (Vallier *et al.* 2005). Additionally, differentiation efficiency depends on the level of cell confluency (Snykers *et al.* 2007). During differentiation induction *in vitro,* our EBs showed increased cell density after 20 days in culture. We observed high instability frequencies in EB samples at 30 days of culture. At that time, maximum efficiency in cell to cell interaction and cell differentiation was observed, and the confluence of EBs from H1 and H7 ES cell lines was almost 100%. Our data support the affirmation that differentiation is a dynamic process where interaction between cells and addition of chemical supplements to the culture medium can drive ES cells toward

differentiation in addition to playing an important role in inducing molecular signals needed to obtain specialized cell types.

Second, developmental genes are responsible for controlling differentiation in ES cells. Molecular markers located near specific genes could be good candidates for the evaluation of cell fate progression during embryogenesis. We found significant instability frequencies in 18 single tandem repeat markers localized near developmental genes. Our results support the idea that these markers are possible target sequences responsible for the molecular signals of pluripotency, differentiation, and imprinting during ES cell commitment specialization of lineage *in vitro* (Table 4.5). We found important correlations between genes in close proximity to the unstable markers analyzed in this study. For example, POU family transcription factor (POUF) is a regulator of pluripotency that prevents ES cell differentiation. Repression of this gene induces ES cell differentiation into primitive endoderm (Niwa *et al.* 2005; Feldman *et al.* 2006). D1S551 is a marker located near the POUF gene and was significantly unstable later in the EB formation process (30 day sample), indicating that instability acts as a signal to silence this pluripotency gene, and allows progression of differentiation of the ES cells. On the other hand, genomic instability in repetitive regions could be required for selective preference of differentiation into the three germinal layers during EB progression in H1 and H7 ES cells. In our study, signaling pathways that control spontaneous EB differentiation in H1 and H7 *in vitro* reveal differences in the capacity to achieve homogeneous cell populations at the end of differentiation. EBs from H1 and H7 did not differentiate equally well into the three germinal layers. EBs from H1 efficiently promoted more neuroectoderm structures in comparison to EBs from H7 that efficiently promoted more mesoderm structures as a functional cardiac muscle cells. Our

observations suggest that H1 and H7 ES cell lines show specific embryonic differentiation patterns. Interestingly, EBs from H1 and H7 show different molecular patterns of instability. Significant differences within unstable markers were observed and could be the source of differences in the noted cell morphological and functional characteristics. EBs from H1 were unstable for markers related to early neuroectoderm differentiation. For example, D1S468 is located near tumor protein p53 (TP73) gene, which is involved in the cellular stress response and development. Deletion of this gene is involved in neuroblastoma (Berna S, 2010; Kim KP, 2007). DXS1208 is located near the heat shock 27 kDa protein β1 (HSP25/27) gene implicated in astrocytic and cortical degeneration (Schwarz *et al.* 2010; Kirbach and Golenhofen 2011). D18S63 is located near the TGFβ-induced factor homeobox 1 (TGIF1), a transcription regulator during development, and is associated with structural brain abnormalities (Pazmany and Tomasi 2006; Wang *et al.* 2008; Hamid and Brandt 2009). D11S4090 is located near the gene neural cell adhesion molecule 1 (NCAM) that is necessary for the induction of synaptic plasticity in the hippocampus (Kleene *et al.* 2010). D3S1583 is located near the retinoic acid receptor beta (RARB) gene, which is a developmental gene responsible for cell growth and differentiation (Sheng *et al.* 2010; Elizalde *et al.* 2011). Additionally, EBs from H1 and H7 ES cell lines showed instability in markers involved in mesoderm differentiation. For example, D16S3034 is located near the chromodomain helicase DNA binding protein 9 (CHD9) gene that is involved in early osteogenic cell differentiation (Shur *et al.* 2006). D16S3091 is located near cadherin 13 H-cadherin (CDH13) gene that is a mediator of cell-cell interaction in the heart and negative regulator of neural cell growth (Li *et al.* 2010).

Third, epigenetic modifications are necessary to induce gene expression changes in ES cells undergoing differentiation into early progenitor cell types of the three germinal layers (ectoderm, endoderm, and mesoderm). Chromatin modification is a mechanism that potentially drives cell fate decisions, cell renewal, and lineage specialization. Improper silencing or activation of specific genes induces chromatin remodeling modifications (Boyer *et al.* 2005; Enver *et al.* 2005; Feldman *et al.* 2006; Baker *et al.* 2007). Changes in chromatin structure can regulate commitment specialization of ES cell lineage by modulating gene expression through two ways: first, by modification of histones and second, by methylation of promoters regulating specific developmental genes. These modifications ensure the expression or repression of target genes during cell differentiation. However, it is not fully understood how these steps are coordinated. Previous studies have confirmed that histone modifications are associated with transcriptionally active regions in the genome that regulate spontaneous differentiation of ES cells *in vitro* (Boyer *et al.* 2005; Azuara 2006). ES cells that failed to keep their repressive chromatin and lost the capacity to differentiate into the three germinal layers (Enver *et al.* 2005; Pasini *et al.* 2007). Consequently, histone H2A ubiquitination reduction and histone H3 and H4 acetylation enrichement modifications allow gene transcription to maintain ES cell pluripotency (de Napoles *et al.* 2004; Lee *et al.* 2006). Our results suggest that instability in repetitive regions near histone specific genes could be a signal for histone modification that generates repressive or active chromatin to modulate gene expression during cell lineage commitment. From our results, four significant unstable markers that showed high instability are related to the histone genes HISTHB2 and D6S2252 (HIST1H2AH), both of which are linker histones responsible for chromatin compaction (Wang *et al.* 2004; Zhang *et al.* 2005; Petty *et al.*

2009). Another histone gene, D10S529 (H2AFY2), is involved in inactivation of the X chromosome (Buschbeck *et al.* 2009; Gamble *et al.* 2010). All three markers were especially significant in the instability detected in EBs from H7 ES cell line when compared to EBs from H1. Studies have reported changes in X inactivation in female ES cells, which was congruent with our results because the H7 ES cell line is female (Chadwick and Willard 2001; Buschbeck *et al.* 2009; Gamble *et al.* 2010). Marker D7S488 located near the histone deacetylase 9 isoform 3 (HDAC) gene is responsible for tissue-specific gene expression during cell differentiation (Karamboulas *et al.* 2006). We found that this marker was exclusively unstable in EBs from H1. Taken together, our results suggest that instability in these sequences, which are near specific histone genes, could be a signal for chromatin modifications that repress expression of pluripotency genes during spontaneous differentiation.

In addition to chromatin modification by histones, methylation of gene promoter regions is responsible for establishing the epigenetic changes that allow for the pluripotency or differentiation status of ES cells. DNA (cytosine-5)-methyltransferases (DNMT) catalyze the addition of methyl groups to the cytosines in CpG islands that are located in promoter regions of genes, and they are responsible for controlling access of transcription factors to the genome (Jaenisch and Bird 2003). Changes in methylation during differentiation have been widely reported (Lagarkova *et al.* 2006; Meissner *et al.* 2008), supporting the idea that methylation is a key gene regulator of the pathway leading to ES cell fate decisions. D2S144 is located near the DNA (cytosine-5)-methyltransferase 3 alpha gene (DNMT3A) responsible for *de novo* DNA methylation during embryonic development, and displays significant instability (Chen *et al.* 2002; Wienholz *et al.* 2010). DNMT3A *de novo* methylation in ES cell lines induces silencing in pluripotency

and self-renewal genes in the differentiated cells and prevents de-differentiation or reactivation of pluripotency in somatic adult tissues (Lagarkova *et al.* 2006). Methylation is the mechanism for gene imprinting during early embryonic development. We also observed significant unstable markers located in promoter regions of two genes that are imprinted in tissue specific manner. Growth factor receptor-bound protein 10 (GRB10) gene is imprinted in the paternal allele in the brain and is responsible for modulation of tyrosine kinase activity. GRB10 overexpression results in suppression of embryonic growth (Tezuka *et al.* 2007; Monk *et al.* 2009). Insulin-like growth factor 2 (IGF2) gene, expressed only from the paternal allele, is involved in embryonic development and growth (Demars *et al.* 2010; Tabano *et al.* 2010). Therefore, epigenetic modifications have two essential functions, regulating cell fate decisions during stages of differentiation and preserving the cell specialization status throughout the cell's life.

Finally, identification of specific target sequences that are predominantly unstable during spontaneous differentiation might provide clues to deciphering molecular mechanisms used to express and/or repress genes during embryogenesis and cell lineage commitment. In addition, our results reveal a novel molecular tool for characterizing cell populations according to their genomic integrity through analysis of unstable markers located near important genes responsible for early cell differentiation. This novel tool has potential significance and practical applications for use in regenerative medicine. To our knowledge, this study is the first to identify potentially useful biomarkers that can determine the stability of specialized cell populations differentiated *in vitro* from ES cells. Further evaluation of these markers will enable more precise characterizations of ES cells and cell populations during development, so their applications could be fully assessed.

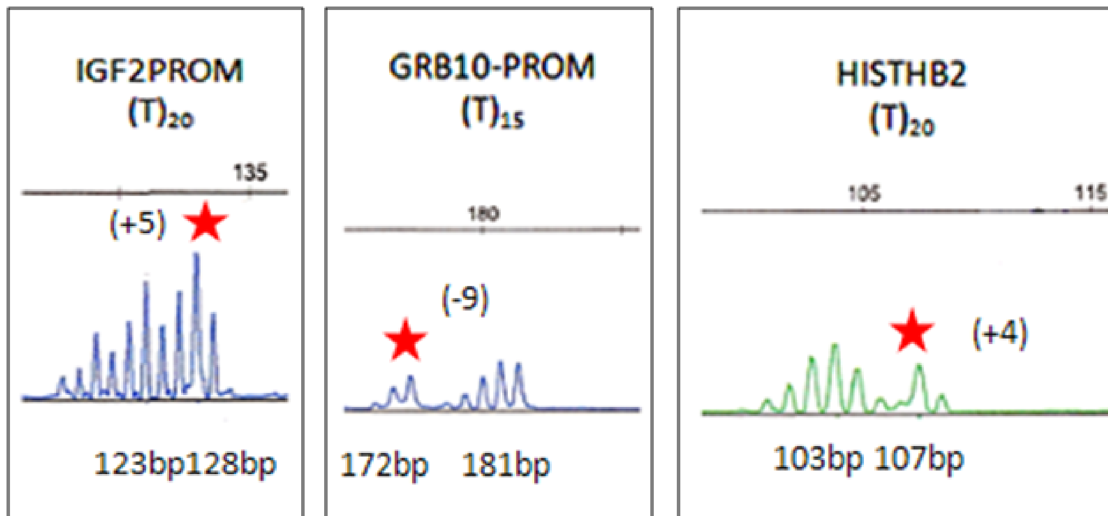

Figure 4.1 Examples of electropherograms from mononucleotide unstable markers.

Notes: Normal and mutated alleles are identified according to repeat motif shifts. Mononucleotide markers (IGF2-PROM, GRB10-PROM, and HISTBH2) show the corresponding normal allele, as well as the mutated allele that was shifted greater than 3 or less than 3 repeats motifs. Mutated alleles are indicated with a red star and the number of repeat motif shifts is in parentheses. (-) indicated a loss of repeat units, while (+) indicated a gain of repeat units. Normal alleles are shown next to their mutated alleles. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of each allele in base pair (bp). Markers were labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

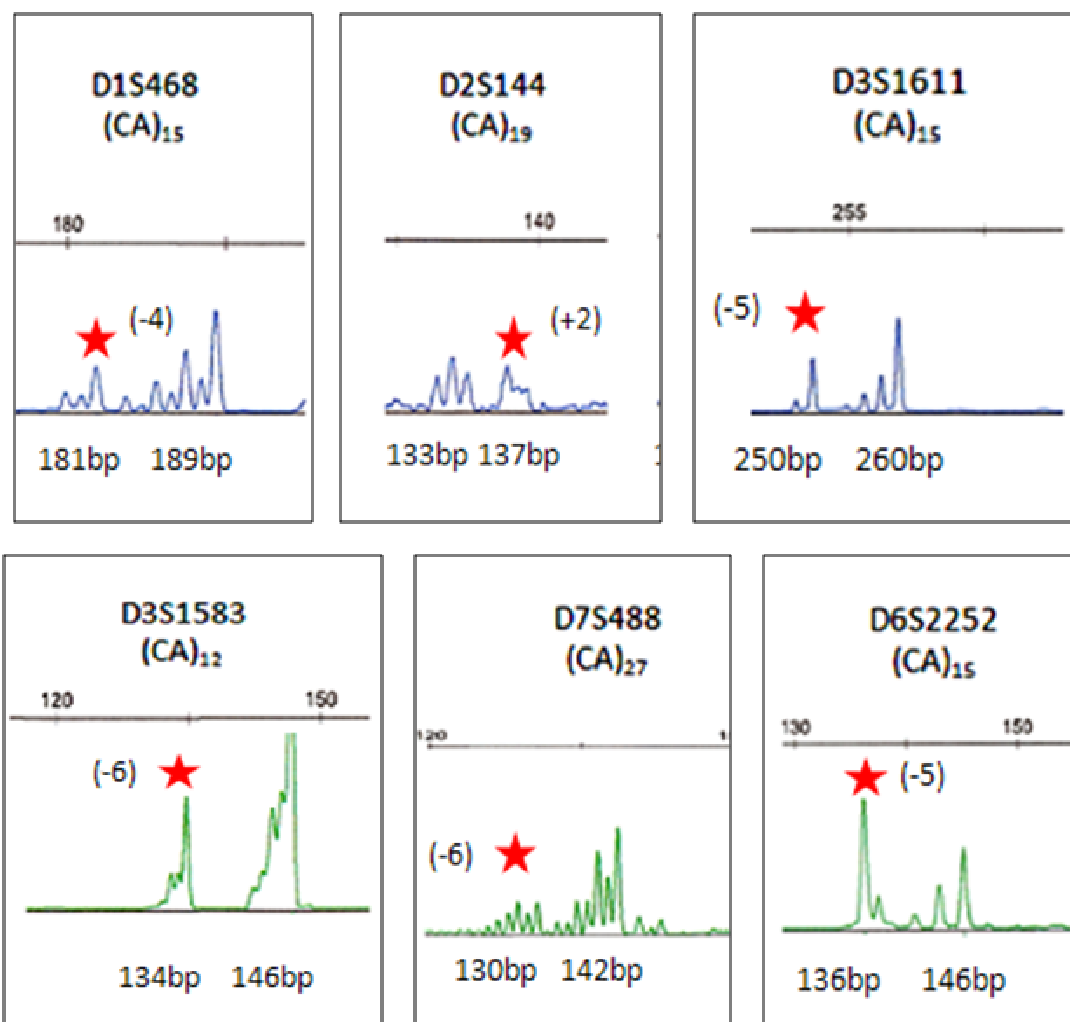

Figure 4.1 Continued.

Notes: Normal and mutated alleles are identified according to repeat motif shifts. Dinucleotide markers (D1S468, D2S144, D3S1611, D13S1583, D6S2252, and D7S488) show the corresponding normal allele, as well as the mutated allele that was shifted greater than 2 or less than 3 repeat motifs. Mutated alleles are indicated with a red star and the number of repeat motif shifts is in parentheses. (-) indicated a loss of repeat units, while (+) indicated a gain of repeat units. Normal alleles are shown next to their mutated alleles. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of each allele in base pair (bp). Markers were labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

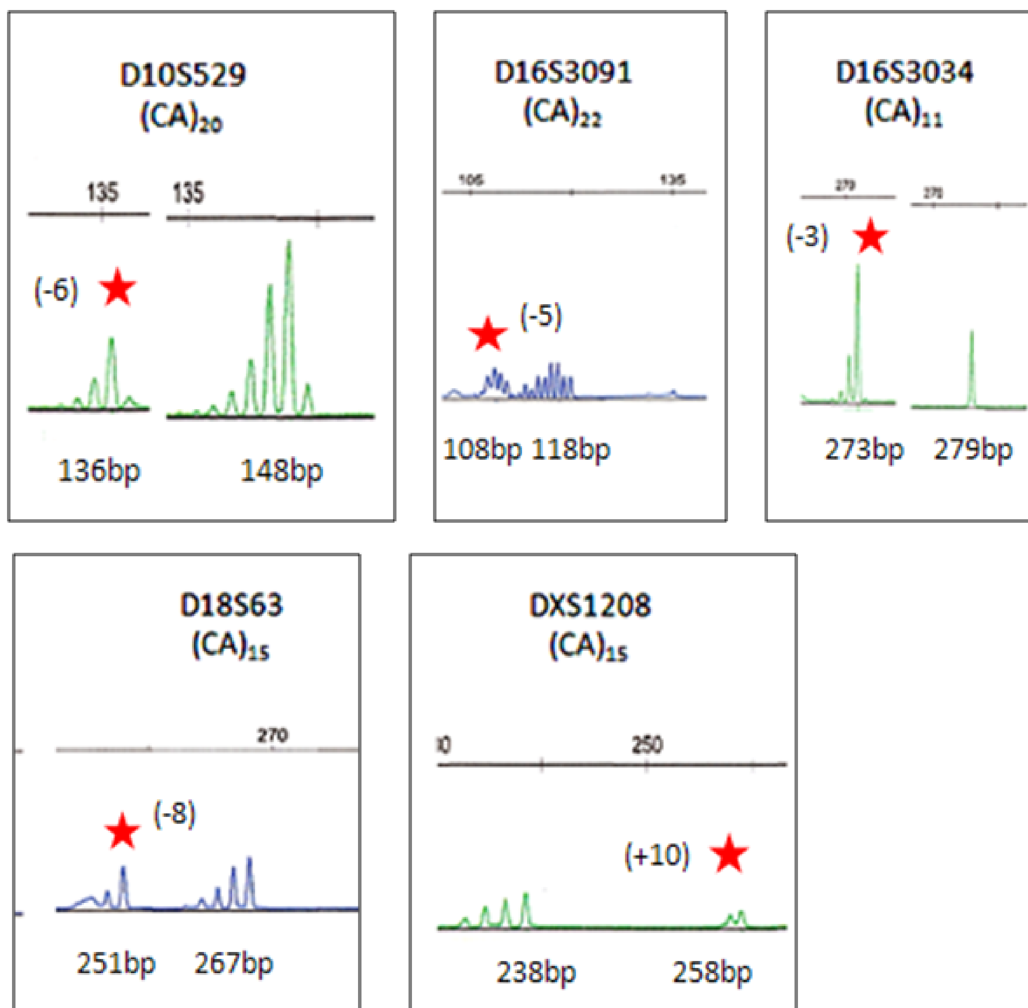

Figure 4.1 Continued.

Notes: Normal and mutated alleles are identified according to repeat motif shifts. Dinucleotide markers (D10S529, D16S3034, D16S3091, D18S63, and DXS1208) show the corresponding normal allele, as well as the mutated allele that was shifted greater than 2 or less than 3 repeat motifs. Mutated alleles are indicated with a red star and the number of repeat motif shifts is in parentheses. (-) indicated a loss of repeat units, while (+) indicated a gain of repeat units. Normal alleles are shown next to their mutated alleles. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of each allele in base pair (bp). Markers were labeled with either 6-FAM (blue) or HEX (green) fluorescent dyes.

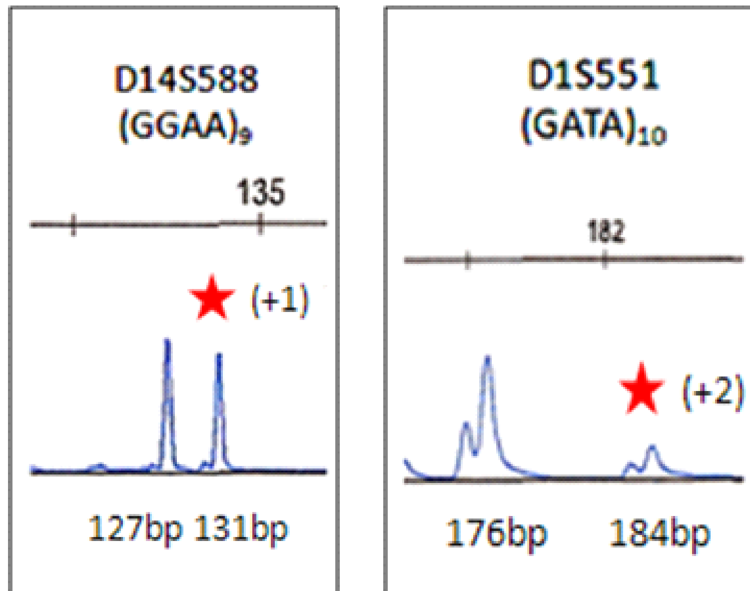

Figure 4.1 Continued**.**

Notes: Normal and mutated alleles are identified according to repeat motif shifts. Tetranucleotide markers (D14S588 and D1S551) show the corresponding normal allele, as well as the mutated allele that was shifted greater than 1 or less than 2 repeat motifs. Mutated alleles are indicated with a red star and the number of repeat motif shifts is in parentheses. (-) indicated a loss of repeat units, while (+) indicated a gain of repeat units. Normal alleles are shown next to their mutated alleles. Each set of peaks is identified by the marker name and repeat motif (top row). Shown below each peak is the size of each allele in base pair (bp). Markers were labeled with 6-FAM (blue) fluorescent dyes.

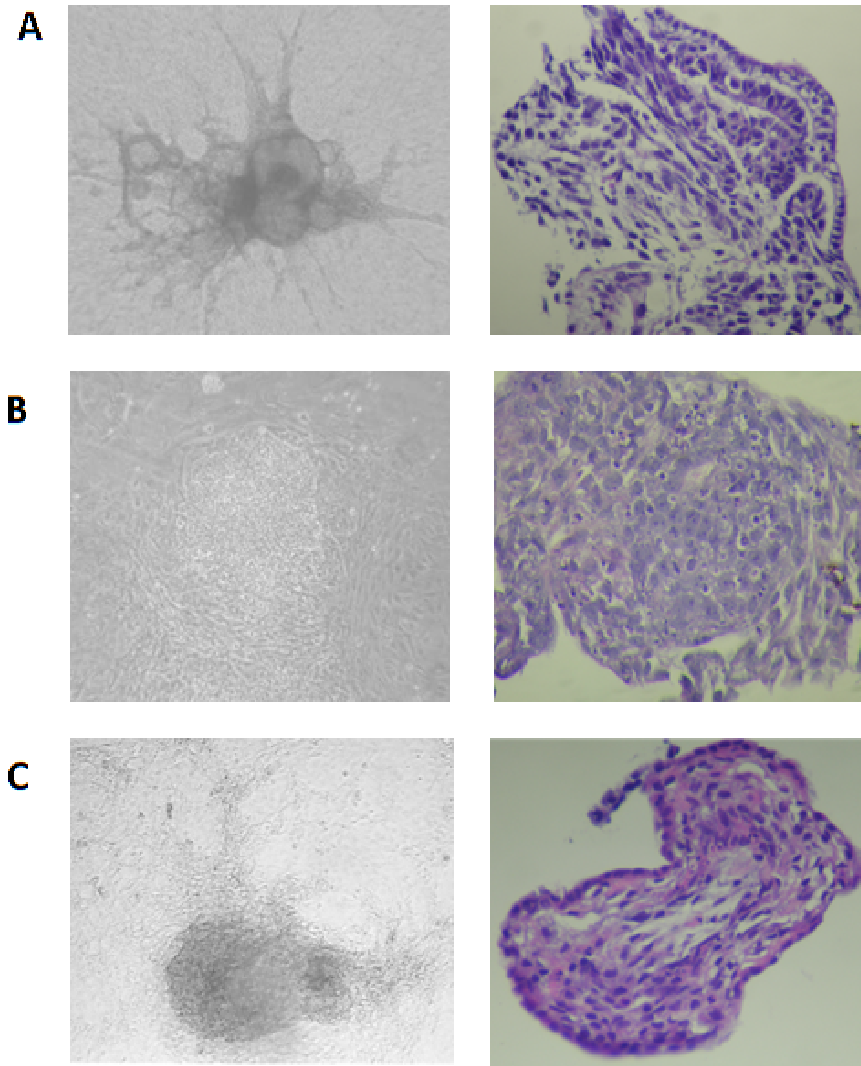

Figure 4.2 Histological characterizations of embryoid bodies from ES cells

Notes: A, B, and C are from EBs at 14 days after *in vitro* differentiation showing typical morphology and histology characteristics of differentiated tissues from the three germinal layers. (A) Neural epithelium characteristics in an ectoderm layer. (B) Mesenchymal characteristics in a mesoderm layer. (C) Pseudostratified columnar epithelium characteristics in an endoderm layer. Phase contrast images (left panels) are shown with 10X magnification. Haematoxylin and Eosin stained images (right panels) are shown at 60X magnification.

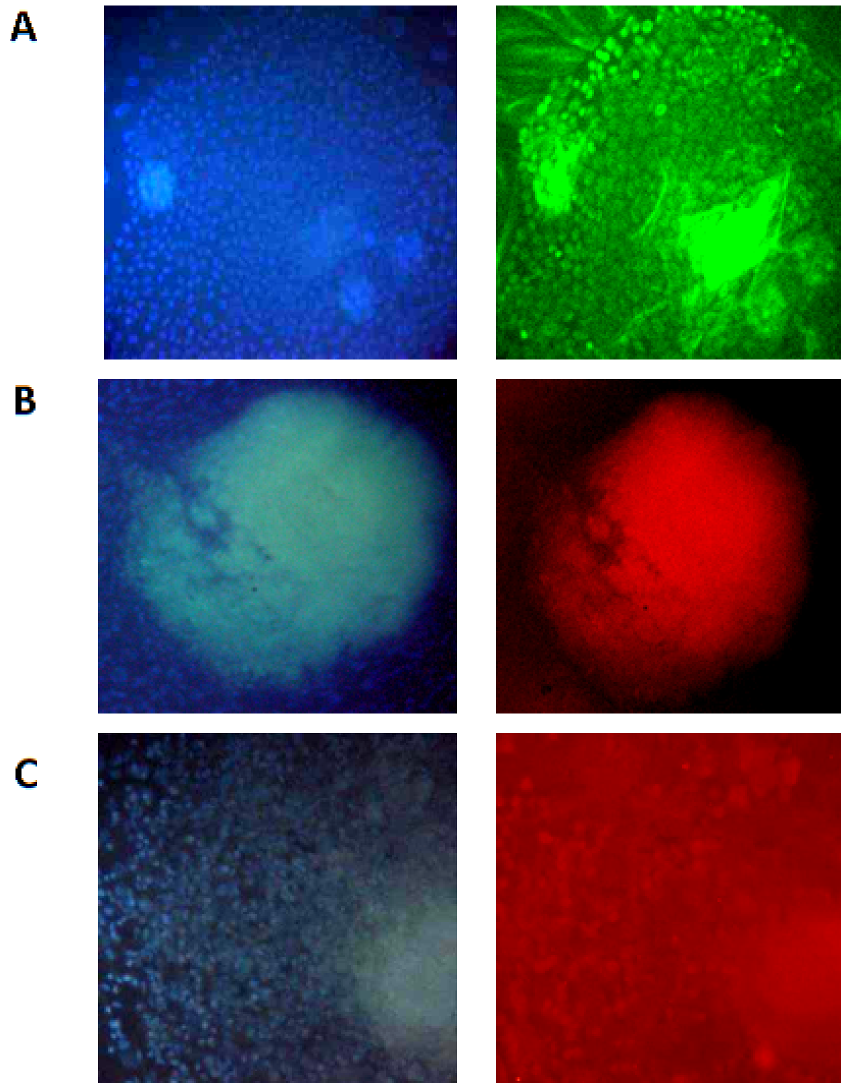

Figure 4.3 Immunofluorescent characterization of embryoid bodies differentiated from ES cells

Notes: A, B, and C are from EBs at 14 days after *in vitro* differentiation. (A) EB showing βIII-tubulin positive expression (green) which is characteristic of neuroectoderm differentiation. (B) EB showing myosin positive expression (red) which is characteristic of cardiac muscle (mesoderm differentiation). (C) EB showing GATA positive expression (orange) which is characteristic of endoderm differentiation. Nuclei were visualized with DAPI stain (blue) (left panels). Fluorescence images are show in magnification of 60X.

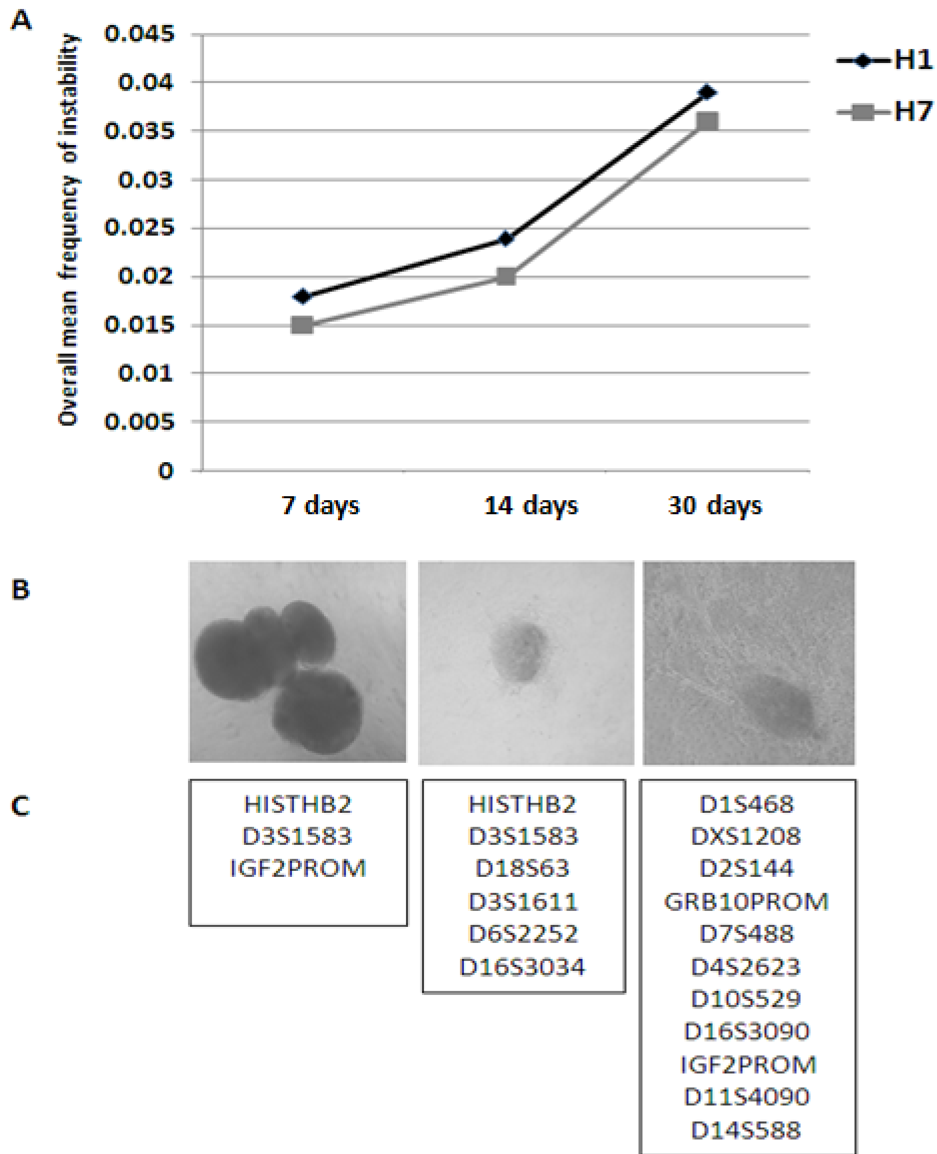

Figure 4.4 Mean mutation frequencies of unstable markers across EB samples at three points of time

Notes: (A) EBs from H1 and H7 ES cell lines showed statistically significant differences of frequencies of unstable markers at 7 days after *in vitro* differentiation when compared to frequencies of unstable markers at 30 days after *in vitro* differentiation ($p<0.05$). Values represent the overall mean frequency of unstable markers over three points of time. (B) Phase contrast image of EBs in suspension at 7, 14, and 30 days after *in vitro* differentiation (magnification of 10X) (C) List of unstable markers at 7, 14, and 30 days after *in vitro* differentiation.

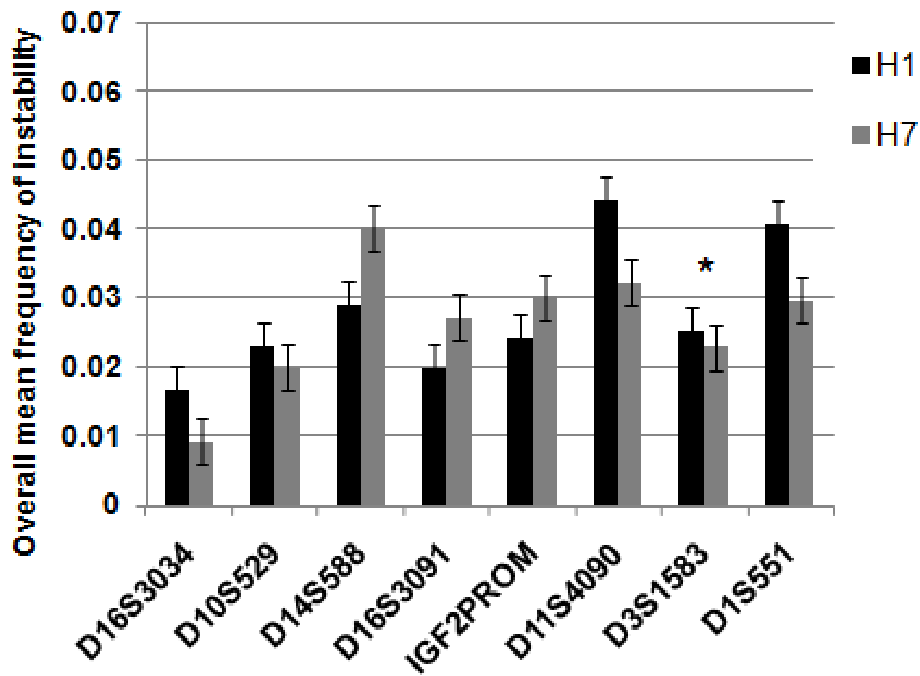

Figure 4.5 Unstable markers in embryoid bodies differentiated from H1 and H7 ES cell lines

Notes: Differences in overall mean mutation frequencies were observed between EBs from H1 and H7 ES cell lines. Values represent the overall mean mutation frequency of EB sample replicates (n=144) per marker that was calculated with SP-PCR software (MD Anderson Cancer Houston, TX). D16S3091 and IGF2-PROM markers showing a highly statistically significance differences ($p<0.001$). D16S3034, D10S529, D14S588, D11S4090, and D1S551 markers show high statistically significant differences ($p\leq0.05$). The D3S1583 marker did not show any significance differences (*).

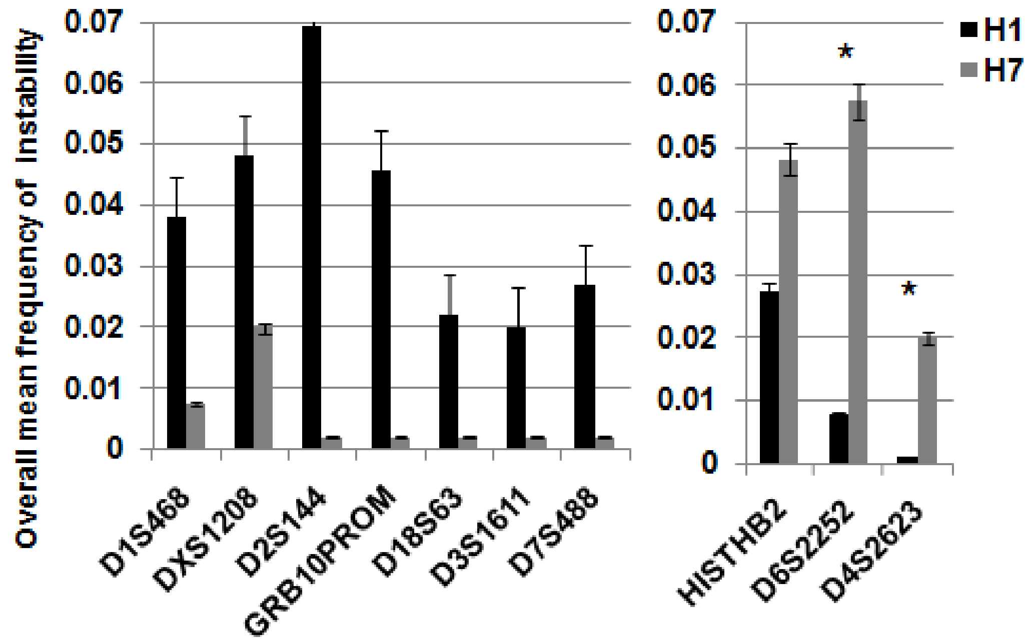

Figure 4.6 Unstable markers of embryoid bodies differentiated from H1 and H7 ES cell lines

Notes: Statistically significant differences in overall mean mutation frequencies were observed between EBs from H1 and H7 ES cell lines. (A) Overall mean values for mutation frequencies of unstable markers observed in EBs from the H1 ES cell line. (B) Overall mean values for mutation frequencies of unstable markers observed in EBs from the H7 ES cell line. Values represent the overall mean mutation frequency of EB sample replicates (n=144) per marker that was calculated with SP-PCR software (MD Anderson Cancer Houston, TX). Markers show statistically significant differences $p \leq 0.05$ except for D6S2252 and D4S2623 that show marginally significant differences $^{*}p \leq 0.10$.

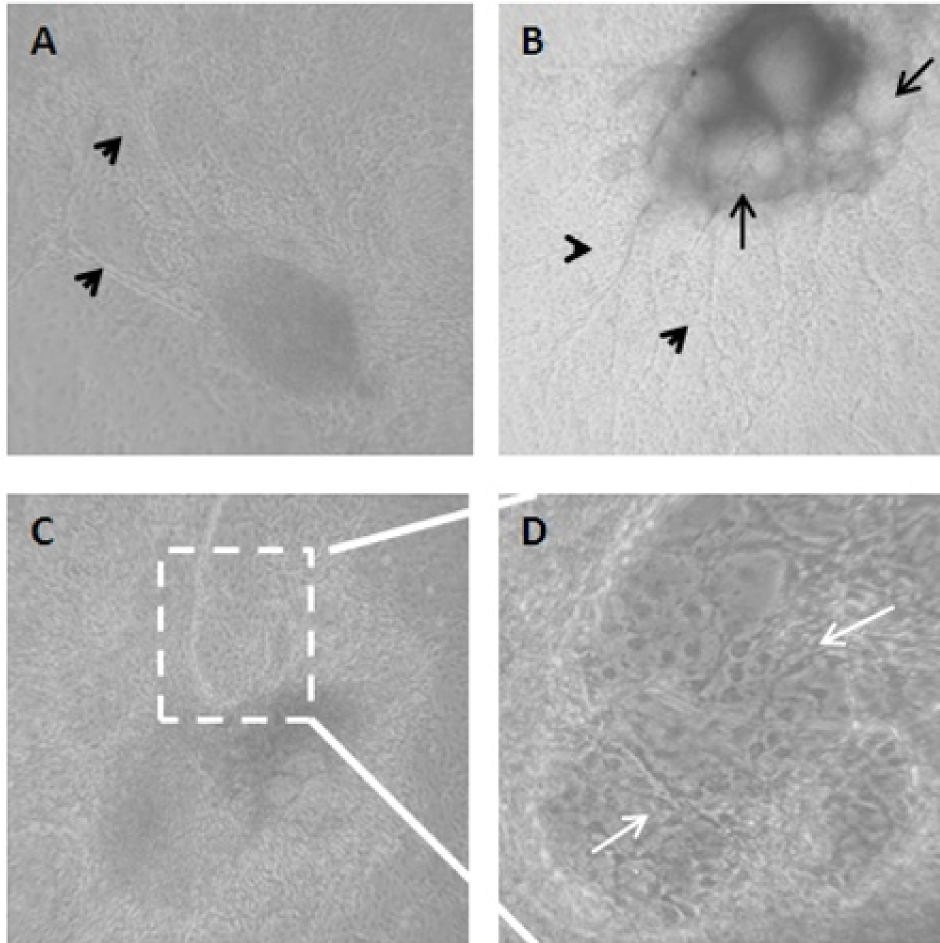

Figure 4.7 Embryoid bodies from the H1 ES cell line differentiated into early neuroectodermal tissue after 30 days

Notes: (A-B) Neural progenitor spheres with extensive cell growth around the clusters and neurite grew radially from the middle EB sphere (black arrow heads). (B) Neural rosettes are observed inside the floating spheres (black arrows). (C) Neural rosette with high confluence of early progenitors that appear after 3 weeks of *in vitro* differentiation from the H1 ES cell line. (D) Boxed region from C panel, shown in 60X magnification and displays neuronal generation in the outgrowth area. Cells generated are in migration status (white arrows). Phase contrast images (A-C) are at a magnification of 10X.

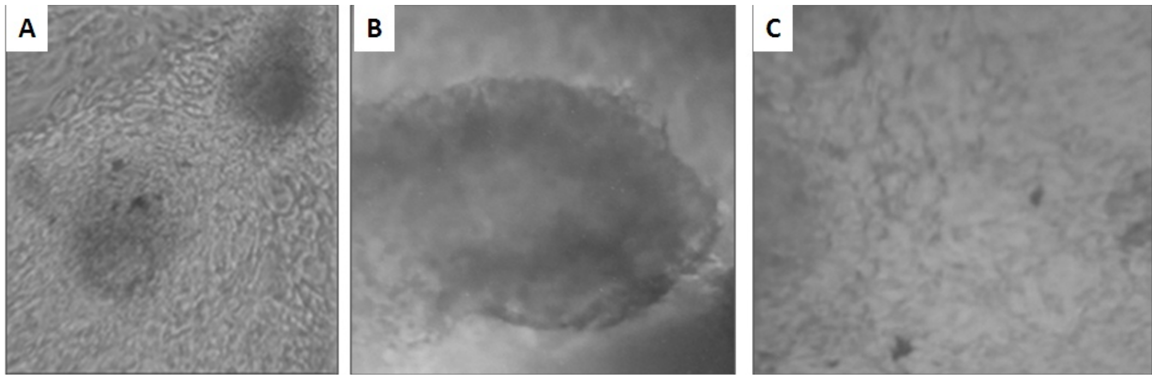

Figure 4.8 Embryoid bodies from the H7 ES cell line differentiated into cardiac muscle tissue (early mesoderm) 14 days after in *vitro* differentiation induction

Notes: (A) EBs differentiated into mesoderm, showing characteristic morphology of a confluent cardiac lineage. (B-C) EBs differentiated into functional mesoderm, showing contractile cardiac muscle. Contraction rate frequency increased over time during *in vitro* differentiation. (B) Initially, the rate was 50 contractions per minute (cpm) at 14 days of *in vitro* differentiation induction (see supplemental file Video005). (C) Contractions increased to 70 cpm after 30 days of *in vitro* differentiation induction (see supplemental file Video007). Phase contrast images are at a magnification of 10X.

Table 4.1 List of single tandem repeat markers analyzed in samples of embryoid bodies from H1 and H7 ES cell lines

Pluripotency	Differentiation			Imprinting	
OCT 4	D16S3034	D6S1698	D16S3091	D7S488	DNMT3
D1S1656	D12S1719	D10S1653	D1S468	D6S1001	GRB10PROM
D1S551	D4S2623	D11S909	TNFa3	HISTH4A	D20S821
D12S1682	D2S134	D5S2021	D15S983	HISTHB2	IGF2R
D1S2630	D11S1331	D18S63	DXS1208	D10S529	DIRAS3PROM
D6S2384	D4S1625	D4S1542	D5S426	D22S447	PEG10PROM
D6S416	D1S430	DXS981	D3S1541	D8S11268	SNURF10PROM
D2S2327	D2S290	D14S588	G60405	D22S941	IGF2PROM
NANOG	D3S1583	D3S2459	D3S1611	D7S638	IGF
D9S1840	DXS458	D17S2180	D11S2179	D6S2252	
	D21S1909	EGFR		D2S144	

Notes: Ten markers were related to pluripotency genes, 33 were related to differentiation genes, and 20 were related to imprinting genes.

Table 4.2 Mean mutation frequencies of eight unstable markers in embryoid bodies differentiated from H1 and H7 ES cell lines

ES cells	Day	D16S3034			D10S529			D14S588			D16S3090			IGF2-PROM			D11S4090			D3S1583			D1S551		
		n	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*
H1	7	44	2	0.0166	39	2	0.0245	46	0	0.0000	41	0	0.0000	51	0	0.0000	50	0	0.0000	38	1	0.0132	49	0	0.0000
	14	45	0	0.0000	43	4	0.0360	69	0	0.0000	47	1	0.0121	63	1	0.0151	56	0	0.0000	32	2	0.0369	52	0	0.0000
	30	59	0	0.0000	45	1	0.0080	50	2	0.0278	73	3	0.0276	47	3	0.0330	37	1	0.0408	34	0	0.0000	31	1	0.0408
Total		148	2	0.0166	127	7	0.0228	165	2	0.0278	161	4	0.0199	161	4	0.0241	133	1	0.0408	104	3	0.0251	122	1	0.0408
H7	7	42	0	0.0000	40	1	0.0131	43	0	0.0000	72	0	0.0000	49	0	0.0000	62	0	0.0000	35	2	0.0312	61	0	0.0000
	14	43	1	0.0092	40	1	0.0116	42	0	0.0000	63	0	0.0000	52	0	0.0000	64	0	0.0000	37	1	0.0141	73	0	0.0000
	30	34	0	0.0000	37	3	0.0349	40	1	0.0390	83	3	0.0818	75	3	0.0300	50	1	0.0322	47	0	0.0000	65	2	0.0297
Total		119	1	0.0092	117	5	0.0199	125	1	0.0390	218	3	0.0272	176	3	0.0300	176	1	0.0322	119	3	0.0226	199	2	0.0297
p-value				0.0580			0.0308			0.0410			<0.001			<0.001			0.0500			*NS			0.0100

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated by SP-PCR software with SP-PCR software (MD Anderson Cancer Houston, TX). *NS Indicates no statistical significance.

Table 4.3 Mean mutation frequencies of seven unstable markers that displayed statistically significant differences in EBs from the H1 ES cell line compared to EBs from the H7 ES cell line

ES cells	Day	D1S468			DXS1208			D2S144			GRB10-PROM			D18S63			D3S1611			D7S488		
		n	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*
H1	7	37	0	0.0000	20	0	0.0000	41	0	0.0000	40	3	0.0347	17	0	0.0000	30	0	0.0000	59	1	0.0109
	14	47	0	0.0000	42	0	0.0000	46	0	0.0000	48	0	0.0000	47	4	0.0219	46	3	0.0199	46	0	0.0000
	30	35	3	0.0381	25	2	0.0480	30	2	0.0694	34	3	0.0549	17	0	0.0000	34	0	0.0000	70	2	0.0160
Total		119	3	0.0381	87	2	0.0480	117	2	0.0694	122	6	0.0448	81	4	0.0219	110	3	0.0199	175	3	0.0269
H7	7	68	0	0.0000	35	0	0.0000	36	0	0.0000	42	0	0.0000	70	0	0.0000	42	0	0.0000	80	0	0.0000
	14	73	0	0.0000	41	0	0.0000	27	0	0.0000	34	0	0.0000	37	0	0.0000	42	0	0.0000	78	0	0.0000
	30	84	1	0.0075	46	1	0.0198	46	0	0.0000	64	0	0.0000	19	0	0.0000	33	0	0.0000	39	0	0.0000
Total		225	1	0.0075	122	1	0.0198	109	0	0.0000	140	0	0.0000	126	0	0.0000	117	0	0.0000	197	0	0.0000
p-value				<0.0010			0.0500			0.0081			0.0015			0.0261			0.0324			0.0419

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated with SP-PCR software (MD Anderson Cancer Houston, TX).

Table 4.4 Mean mutation frequencies of three unstable markers that displayed statistically significant differences in EBs from the H7 ES cell line compared to with EBs from H1 ES cell line

ES cells	Day	HISTHB2			D6S2252			D4S2623		
		n	*m*	*f*	*n*	*m*	*f*	*n*	*m*	*f*
H1	7	42	1	0.0100	32	0	0.0000	51	0	0.0000
	14	43	5	0.0446	69	1	0.0078	63	0	0.0000
	30	28	0	0.0000	38	0	0.0000	25	0	0.0000
Total		113	6	0.0273	139	1	0.0078	139	0	0.0000
H7	7	44	5	0.0408	74	0	0.0000	40	0	0.0000
	14	40	5	0.0557	42	0	0.0000	31	0	0.0000
	30	44	0	0.0000	42	2	0.0575	36	1	0.0204
Total		128	10	0.0483	158	2	0.0575	107	1	0.0204
p-value				**0.0118**			*0.1*			*0.069*

Notes: Number of normal alleles (*n*), number of mutated alleles (*m*), and mean value of mutation frequency (*f*) calculated with SP-PCR software (MD Anderson Cancer Houston, TX). p-value ≤ 0.05 in **bold.** p-value ≤ 0.10 are in *italic.*

Table 4.5 List of genes associated with unstable markers

STR	Gene	Gene Name	Gene Function	References
D16S3034	CHD9	Chromodomain helicase DNA binding protein 9	Embryo development	Shur I, 2006
D10S529	H2AFY2	H2A histone family, member Y2	Nucleosome assembly	Matthew J, 2010 Marcus B, 2009
D14S588	PTGDR	Prostaglandin D2 receptor (DP)	Signal transduction regulator	Boie Y, 1995
D16S3091	CDH13	Cadherin 13, H-cadherin (heart)	Growth factor activity	Li L, 2010
IGF2PRO	IGF2	Insulin-like growth factor 2 (somatomedin A)	Growth factor activity	Tabano S, 2010 Demars J, 2010
D11S4090	NCAM	Neural cell adhesion molecule 1	Signal transduction regulator	Kleene R., 2010
D3S1583	RARB	Retinoic acid receptor, bet	Embryo development	Elizalde C, 2011 Sheng N, 2010
D1S551	RGS4	Regulator of G-protein signaling 4	Signal transduction regulator	Charlesworth P, 2006 Ebert PJ, 2006
D1S468	TP73	Tumor protein p73	Transcription factor	Berna S, 2010 Kim KP, 2007

Notes: Summary of gene characteristics located in close proximity to unstable markers involved in embryonic development. These eighteen unstable markers were identified in EBs differentiated from H1 and H7 ES cell lines

Table 4.5 continued

STR	Gene	Gene Name	Gene Function	References
DXS1208	HSPB1	Heat shock termic protein	Transcription factor	Asangi RK, 2010 Schwarz L, 2010
D2S144	DNMT3	DNA (cytosine-5-)- Methyltransferase 3 alpha	DNA methylation	Taiping C, 2002 Bethany L, 2010
GRB10PRO	GRB10	Growth factor receptor- bound protein 10	Growth factor activity	Norio T, 2007 Monk D, 2009
D18S63	TGIF1	TGFB-induced factor homeobox 1	Growth factor activity	Hamid R, 2009 Pazmany T, 2006
D3S1611	MLH1	MutL homolog 1, colon cancer	DNA repair	Rodriguez Jimenez FJ., 2008
D7S488	HDA 9	Similar to histone deacetylase 9 isoform 3	Nucleosome assembly	Karamboulas C, 2006
HISTHB2	HISTHB2	H2A histone family, member Y2	Nucleosome assembly	Lee MG, 2007
D6S2252	HIST1 H2AH	Histone cluster 1, H2ah	Nucleosome assembly	Hengbin W, 2004 Zhang R, 2005
D4S2623	EGF	Endoderm growth factor	Embryo development	Chu J, 2010

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CHAPTER V

UNSTABLE REPETITIVE SEQUENCES LOCATED NEAR GENE PROMOTER COULD BE A SIGNAL FOR REGULATION OF GENE EXPRESSION DURING TUMORIGENESIS

5.1 Abstract

Single tandem repeats are sequences of DNA that have been implicated in the deregulation of gene expression in human conditions such as fragile X syndrome, neurodegenerative diseases, and tumorigenesis. Understanding the origin of repetitive sequence instability and functions on the genome allow us to describe early steps of genomic instability signals in cell differentiation and tumor transformation mechanisms. Here we show how instability in repetitive sequences located distal or proximal distances to particular genes could be a signal for deregulation of gene expression after DNA damage accumulation in ovarian cancer cells and normal ovarian cells. Significant instability was shown in five single tandem repeat markers (BAT26, BAT60, D7S3046, DXS9902, and DXS6801) nine days post-exposure to high concentrations of H_2O_2. Genes located near these unstable repetitive markers were identified. Our results from gene expression analyze reported significant up-regulation of five genes (MSH2, CHMP4C, STAG3L4, AUTS2, and PMS2L4), and significant down-regulation of four genes (EPCAM, ASB9, FIGF, and PCDH11X) in ovarian cancer cells in comparison to normal ovarian cells after DNA damage. These observations are consistent with our hypothesis that genomic instability in repetitive regions of the genome is a signal for

differential gene expression that leads to tumor transformation and maintains cell survival after accumulation of DNA damage. Genetic modification patterns in specific target genes involved in tumor cell transformation are useful tools for testing tumor progression and improving cancer therapy sensitivity.

5.2 Introduction

Developmental genes are usually deregulated during neoplastic transformation, leading to cellular responses such as proliferation, differentiation, migration, invasion, and angiogenesis, ensuring a perfect environment for tumor transformation (Gupta *et al.* 2005; Ince *et al.* 2007). Aggressiveness and invasiveness are fundamental characteristics of ovarian tumor progression. Several authors suggest that the ability to metastasize rapidly to different organs is due to developmental signals because tissues of germinal origin are deregulated or reactivated during tumor transformation (Gupta *et al.* 2005; Karakosta *et al.* 2005; Proia *et al.* 2011). Primordial germinal cells migrate into the genital ridge, and their differentiation into the female gonad is the result of coordinated molecular signals early in embryo development. Similarities have also been found in the neoplastic phenotype on ovarian tumorigenesis.

Cell differentiation and tumor transformation share several molecular signaling pathways, including gene expression and epigenetic modifications (Karakosta *et al.* 2005; Proia *et al.* 2011). Tumor cells show losses in genome integrity due to the accumulation of DNA damage (Fearon and Vogelstein 1990). Instability in single tandem repeats originate frame shift mutations in coding and non-coding regions in the DNA, inducing failures in cellular regulatory pathways such as cell cycle control, apoptosis, and DNA repair that are responsible for avoiding cell transformation (Imai *et al.* 2008). We

hypothesize that unstable repetitive regions located in the 5'untranslated region of a gene could be the earliest molecular signal for transcription deregulation that allows tumorigenesis. Instability and gene expression profile determination could be detectors of initiation, progression, and prognostics for ovarian tumorigenesis.

The aim of the present study was to characterize single tandem repeat signaling, in relation to nearby genes that induce gene expression deregulation after DNA damage accumulation in both cancer and normal ovarian cells. Detection of five unstable repetitive sequences in the genome allows for the identification of sixteen neighboring possible target genes involved in ovarian tumorigenesis. Gene expression status of these target genes was determined by real time PCR in cancer and normal ovarian cells 9 days post-exposure to H_2O_2 (30µM). Gene expression deregulation was observed in genes involved in cell stress responses, such as DNA repair, cell growth, and tumor progression. Our results support our hypothesis that instability in repetitive regions could be a signal of gene expression modifications that lead to tumor transformation and progression after DNA damage accumulation.

Additionally, determination of genomic instability and gene expression interaction aid in our understanding of the earliest steps in tumorigenesis that lead to the impaired gene functions involved during cell transformation. Our analysis revealed five novel candidate genes that showed gene expression deregulation after DNA damage in ovarian cancer cells in comparison to ovarian normal cells. Identification of novel and potential target genes provide a systematic validation of biomarkers for the characterization of ovarian carcinoma. New therapeutic approaches are needed for ovarian cancer treatments and understanding the mechanisms of initiation and progression could help develop and validate new treatments in favor of the patients.

5.3 Materials and Methods

5.3.1 Cell culture

Human cancer cell line (SKOV) was purchased from American Type Culture Collection, ATCC (Rockville, MD) and human ovarian cell line established from ovarian tissue removed from a normal woman. This tissue was donated for this research by informed consent (IRB approval number 11-088). Cancer and normal ovarian cells were cultured routinely in T-25 culture flasks (Falcon, Becton Dickinson, Labware, NJ) in Dulbecco's modified eagle medium (DMEM) high glucose (Invitrogen, Carlsbad, CA), supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 30mg/ml of L-glutamine (Invitrogen, Carlsbad, CA), and 1% of antibiotic/antimycotic (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified atmosphere at $37^{\circ}C$ in 5% CO_2. Medium was changed every 72 hours.

5.3.2 *In vitro* exposure to hydrogen peroxide

When cancer and normal ovarian cells reached 80% confluency, they were trypsinized and treated with 0 or 30μM concentration of H_2O_2 (Fisher Scientific, Houston, TX) in 1X PBS (Invitrogen, Carlsbad, CA) for 1 hour in a humidified atmosphere at $37^{\circ}C$ in 5% CO_2. Cells were then centrifuged at 1000 x g for 5 minutes. The supernatant was removed and cells were washed twice with fresh 1X PBS. Untreated and treated cells were then plated in triplicate at a density of 1×10^5 cells/ml and cultured in a humidified atmosphere at $37^{\circ}C$ in 5% CO_2. Medium was changed every 72 hours until 9 days post-exposure. Concentrations of H_2O_2 and determination of time post-exposure was reported from previous experiments in our lab that showed increased

mutation frequencies in specific microsatellites under these conditions (Moreno-Ortiz 2011).

5.3.3 RNA isolation

At 9 days post-exposure, cancer and normal ovarian cells were trypsinized. Cells were then centrifuged at 1000 x g for 5 minutes, and after PBS washing, total RNA was extracted from cell suspensions using RNeasy plus mini kit (Qiagen, Ontario, Canada) following the manufacturer's protocol. cDNA was synthesized by using QuantiTect reverse transcription kit (Qiagen, Ontario, Canada) according to the manufacturer's recommendation. Briefly, 1μg of total RNA was treated with DNA wipeout buffer for 2 minutes at 42°C, Quantiscript reverse transcriptase master mix was added to the RNA sample and incubation was carried out at 42°C for 30 minutes, followed by incubation at 95°C for 3 minutes to inactivate the reaction. cDNA samples were stored at -20°C until real time PCR was performed.

5.3.4 Real time PCR

Real time PCR was performed to assess transcripts of 16 genes that are in, or near, five unstable single tandem repeat markers that previously showed high mutation frequencies in these ovarian cell lines (Moreno-Ortiz 2011). The validated primer sets, QuantiTect primer assays, were used for the genes EPCAM, MSH2, ASB9, ASB11, FIGF, PIR, E2F5, CA2, CHMP4, FABP4, PAG1, STAG3L4, PMS2L4, AUTS2, PCDH1X, and NAP1L3 (Qiagen, Ontario, Canada) (Table 5.1). All primers were designed to span exon - intron boundaries to avoid non-coding genomic DNA amplification (Qiagen, Ontario, Canada). Expression changes of genes were evaluated in relative expression with respect to the gene β-Actin (ACTB). RNA amplification was

performed with 2µl of cDNA samples and detected by QuantiFast SYBR Green PCR kit (Qiagen, Ontario, Canada). Primer concentration used was 1µM. Real time PCR was performed in an ABI Prism 7000 sequencer detector (Applied Biosystemss, Foster City, CA) using the following protocol: 1 cycle of 95°C for 5 minutes for initial activation step, and 40 cycles of two step cycling 95°C for 10 seconds for denaturation and 60°C for 30 seconds for combined annealing/extension steps. A melting curve was performed starting at 55°C with a 0.5°C increased over 10 seconds in 80 cycles. Negative controls (no cDNA) were included to check for contamination and positive controls of the ACTB housekeeping gene were included as an amplification control. Reactions were replicated three times for each sample.

5.3.5 Real time gene expression statistical analysis

Real time PCR data was calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$ method) between target genes and the internal control gene per sample (Schmittgen and Livak 2008). The data is reported as a gene expression fold change due to H_2O_2 exposure on ovarian cancer and ovarian normal cell lines. Differences in gene fold change values after treatment were calculated and analyzed with a two tailed *t*-test using a statistical package SAS/win 9.2 (SAS Institute, Cary, NC). Gene expression changes were considered statistically significant when the p value was ≤0.05 and were considered marginally significant if the p value was ≤0.10.

5.4 Results

5.4.1 Chromosome location of unstable markers and neighboring genes

Five single tandem repeat markers that showed high frequencies of instability were previously selected in our lab (Moreno-Ortiz 2011). We determined the genomic

distance in mega bases (Mb) of the neighboring genes located upstream or downstream of each unstable marker. We identified sixteen genes associated with these particular unstable markers (Table 5.1). Marker BAT26 has two genes upstream from it (EPCAM and MSH2) (Figure 5.1), marker BAT60 has three genes upstream from it (CHMP4C, FABP4, and PAG1) in addition to two genes downstream from it (E2F5 and CA2) (Figure 5.2). Marker DXS9902 has four genes upstream from it (ASB9, ASB11, FIGF, and PIR) (Figure 5.3), Marker D7S3046 has two genes upstream from it (STAG3L4 and PMS2L4) in addition to one gene located downstream from it (AUTS2) (Figure 5.4). Marker DXS6801 has two genes located downstream from it (PCDH11X and NAP1L3) (Figure 5.5).

5.4.2 Instability is a signal of gene expression changes in ovarian cells

Determination of genomic instability in single tandem repeats is a normal molecular pathway studied widely during tumorigenesis (Boland *et al.* 1998; Berg *et al.* 2000). We were interested in determining whether instability found in single tandem repeats located near specific genes could be the signal of gene expression changes after DNA damage in ovarian cancer cells. To identify novel genes that contribute to the malignant progression of ovarian carcinoma, normal and cancerous ovarian cells were treated with a high concentration of H_2O_2 (30µM) as a source of DNA damage. Instability was found in five single tandem repeat markers (BAT26, BAT60, D7S3046, DXS9902, and DXS6801) after 9 days post-exposure. This approach revealed sixteen distinct genes that were near the unstable markers found previously in our lab. We evaluated by real time PCR two DNA repair genes (MSH2 and PMS2L4), four genes involved in cell growth pathways (FIGF, EPCAM, CHMP4C, and PCDH11X), two genes

associated with tumorigenesis and metastasis (ASB9 and STAG3L4), and one candidate gene for autism (AUTS2). A total of nine out of sixteen genes showed differential expression (EPCAM, MSH2, CHMP4C, ASB9, FIGF, STAG3L4, AUTS2, PMS2L4, and PCDH11X) (Table 5.2) in ovarian cancer cells in comparison with normal ovarian cells in response to DNA damage accumulation. These results showed a differential expression on more than 1.5 fold higher expression changes in tumor ovarian cells relative to the normal ovarian cells. Non-significant differences in gene expression were shown in seven genes associated with unstable repetitive sequences after H_2O_2 exposure (E2F5, CA2, FABP4, PAG1, ASB11, PIR, and NAP1L3). Our results suggest that instability in markers located in close proximity to target genes could be an early signal for differential expression during ovarian cancer evolution.

5.4.3 DNA repair genes deregulate as a cause of instability of the upstream single tandem repeats

Microsatellite instability has been associated with colon cancers resulting from deficiencies in DNA repair mechanisms, specifically in mismatch repair (MMR) proteins (Thibodeau *et al.* 1993; Boland *et al.* 1998). In ovarian cancer, MMR deficiencies are involved in tumor initiation (Begum *et al.* 2008; Yoon *et al.* 2008). We asked if instability of repetitive sequences located upstream from the DNA repair genes could be deregulated in normal ovarian and cancerous ovarian cells after H_2O_2 exposure. Consequently, we detected instability in two markers (BAT26 and D7S3046) located upstream from MSH2 and PMS2L4, which are DNA repair genes responsible for MMR mechanisms after DNA damage. Significant gene expression changes were found in these genes after 9 days post-exposure (Table 5.2). The MSH2 gene showed increased expression in cancerous ovarian cells compared to normal ovarian cells ($p= 0.059$)

(Figure 5.6). Additionally, the PMS2L4 gene showed increased expression in cancerous cells compared to normal cells after H_2O_2 exposure (p=0.003) (Figure 5.6). Significant differential gene expression was found in two MMR proteins and could be a mechanism for ovarian tumorigenesis.

5.4.4 Genes involved in cell growth signals are expressed differentially in ovarian cancer cells

Cell survival, invasion, angiogenesis, and migration are characteristics of tumorigenesis. We asked if instability in the markers DXS9902, BAT26, BAT60, and DXS6801, which are located in the neighboring regions of cell growth regulator genes, induced gene expression changes that are involved during tumor initiation and progression. We found differential expression in four genes involved in cell growth pathways (FIGF, EPCAM, and PCDH11X, and CHMP4C). FIGF, EPCAM, and PCDH11X were down-regulated in both cancerous and normal ovarian cells after exposure (Table 5.2). Up-regulation was observed in the CHMP4C gene due to H_2O_2 exposure in ovarian cancer cells compared to normal ovarian cells (p= 0.023) (Figure 5.7). Cell growth mechanisms become imbalanced by genomic instability and gene expression changes resulting for accumulation of DNA damage in cancerous and normal ovarian cells.

5.4.5 Instability is present in markers linked to deregulated genes involved in intracellular signals during tumorigenesis

Aggression and invasion are main characteristics of carcinomas with poor prognosis. Deregulation of particular genes lead to ovarian cancer evolution. Cellular homeostasis is crucial in resistance to tumor treatment. Our study was designed to determine if instability in markers DXS9902 and D7S3046, located near the ASB9 and

STAG3L4 genes, is a signal for differential gene expression after H_2O_2 exposure of cancerous and normal ovarian cells. We found that tumor and normal ovarian cells displaying instability in single tandem repeats located upstream of ASB9 gene, also displayed significant down-regulation of this gene after DNA damage (p=0.013) (Figure 5.8). In contrast, the STAG3L4 gene that is located downstream of the repetitive region showed significant up-regulation in cancerous ovarian cells compared with normal ovarian cells after 9 days post H_2O_2 exposure (p=0.003) (Figure 5.8). Taken together, these results indicate that gene expression changes are present after DNA damage and could signal of cell survival.

5.4.6 AUTS2 gene is deregulated during ovarian cancer

The repetitive marker D7S3046 was unstable in ovarian cancer cells and is located upstream of the autism susceptibility candidate 2 (AUTS2) gene. This gene has been studied in patients with bipolar schizoaffective disorder, autism, and attention-deficit/hyperactivity disorder (ADHD) (Sultana *et al.* 2002; Hamshere *et al.* 2009). No report has linked this gene to cell transformation or tumorigenesis. The AUTS2 gene showed significantly increased up-regulation in ovarian cancer cells after 9 days post H_2O_2 exposure (p=0.09) (Figure 5.9). We speculate that this gene could be involved during early neural development but can be deregulated and targeted for involvement during cell differentiation and tumor transformation (Sultana *et al.* 2002; Gratacòs *et al.* 2009).

5.5 Discussion

Ovarian carcinomas start on the external epithelial layer, and then cortical inclusions move it to the internal epithelium through the formation of cysts. Ovarian cells

gradually acquire a series of genomic abnormalities leading to invasive tumors. Carcinomas result from disruption of the gene complex responsible for cellular DNA damage response. These and other stress response genes play a role in different cellular functions including development, differentiation, and tumor transformation. The origin and pathogenesis of ovarian tumorigenesis is poorly understood.

Unraveling the complex molecular regulatory systems should allow better understanding of the signals that could trigger ovarian carcinogenesis. Analysis of single tandem repeat instability combined with real time PCR is an approach to identify novel genes involved in ovarian tumor initiation and progression. Our study showed that instability in single tandem repeat markers located near particular genes could be the signal of gene expression changes observed in cancerous and normal ovarian cells after DNA damage. We identified nine novel target genes involved in stress responses during ovary tumor formation (ASB9, PMS2L4, MSH2, AUTS2, STAG3L4, EPCAM, FIGF, PCDH11X, and CHMP4C). Genes identified play roles in cellular processes including cell survival, DNA repair, and growth signals. Normally, DNA sequences have non-coding regions called heterochromatin that are responsible for chromosomal integrity. These specific regions are targets for genomic instability after DNA damage accumulation in the cells. All genes identified in our study have an unstable repetitive sequence upstream of the gene start site, which may be the mechanism that triggers differential expression by regulatory elements involved in transcriptional deregulation during tumorigenesis (Panne *et al.* 2007; Kuwabara *et al.* 2009; Montoya-Durango *et al.* 2009).

We hypothesized that genomic instability could be the signal that regulates gene expression in specific genes responsible for cell cycle, differentiation, and cell growth

pathways during tumor transformation. Our results demonstrated that instability in both proximal and distal repetitive sequences upstream or downstream of specific genes could be the signal for deregulation of gene expression. Several studies report the presence of regulatory elements localized in neighboring genes as enhancer or repressor sequences responsible for transcription modulation (Panne *et al.* 2007). Regulatory elements are located upstream or downstream of transcription start sites. Some authors report that they are within a 5 kb distance, while others report distances up to 1 Mb (Lettice *et al.* 2003; Kleinjan *et al.* 2006; Visel *et al.* 2009). We suggest that instability in single tandem repeats could be a regulatory element signal important in cell differentiation and tumor transformation. Misbalance of gene expression is a signal for ovarian tumor invasion, metastasis, and resistance of cancer cells *in vivo*. For example, our data indicates that marker DXS9902, a proximal unstable repeat marker located 0.03Mb from the promoter region of ASB9 gene, induces ASB9 differential gene expression after DNA damage in ovarian cancer cells. The Ankyrin repeat and SOCS box-containing 9 (ASB9) gene is an E3 ubiquitin ligase that mediates the degradation of proteins. This gene is a prognostic indicator in patients with colorectal cancer. Low expression of ASB9 is associated with increased invasiveness and poor prognostics (Tokuoka *et al.* 2010). In addition, ASB9 is responsible for regulation of proliferation and differentiation when it interacts with the creatine kinase system, which negatively regulates cell growth (Kwon *et al.* 2010). Specifically, in ovarian cancer, the Ankyrin gene has been directly associated with aggressiveness of the tumor and poor prognostics (Scurr *et al.* 2008). Down-regulation of the ankyrin gene is a strategy to improve the treatment outcome by the induction of chemotherapy sensitivity in patients with ovarian cancer (Scurr *et al.* 2008).

DNA damage triggers different cellular pathways, including DNA repair, that involve large numbers of genes. MMR deficiency was identified initially as a potential cancer initiating pathway in colon cancer but nowadays is also linked to several other cancers, including ovarian and endometrial cancer (Thibodeau *et al.* 1993; Boland *et al.* 1998; Yoon *et al.* 2008). The MMR complex is formed by MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2 proteins. MMR proteins are intimately involved in maintaining genomic integrity by repairing nucleotides losses or gains in single tandem repeat motifs across the genome after DNA damage. DNA repair mechanisms are differentially regulated in ovarian cancer cells by silencing MLH1 through hypermethylation (Swisher *et al.* 2009). Differential repair responses in the gene expression of MMR proteins such as MSH2 and PMS2 were observed in ovarian cancer cells. We found changes of expression in these two important MMR genes in our cancer cells after H_2O_2 exposure compared to normal cells. Normal ovarian cells showed no expression of MSH2 and PMS2L4, suggesting that normal cells may be defective in the ability to repair the sequence of unstable DNA sequences after H_2O_2 exposure. The MutS homolog 2, colon cancer, nonpolyposis type 1(MSH2) gene has been widely linked to tumorigenesis. When MMR genes are deregulated, DNA damage accumulation occurs in the cells, contributing to tumor initiation and progression (Boland *et al.* 1998). In addition to MSH2, we found that changes in differential expression in the postmeiotic segregation increased 2 pseudogene 4 (PMS2L4) gene that is involved in DNA repair mechanisms. Mutations in the PMS2 genes are characteristic of tumorigenesis. Germline mutations are associated with lymphomas and neuroectodermal tumors in children (Hendriks *et al.* 2006). Our results indicate that instability in repetitive regions near DNA repair genes are responsible for gene expression deregulation after DNA damage accumulation.

The STAG3L4 and CHMP4C genes show differential expression changes in genes involved in cell growth, as well as intracellular signals that regulate cellular processes. The Stromal antigen 3-like 4 (STAG3L4) gene is a meiosis specific cohesion that stabilizes sister chromatid cohesion protein (Prieto *et al.* 2004). Allele specific imbalances of the STAG3 gene in primary epithelial ovarian tumors by single nucleotide polymorphism (SNP) have been reported (Notaridou *et al.* 2011). In lymphomas, this gene is inactivated after irradiation exposure (Kalejs *et al.* 2006). Chromosomal instability in colon and testicular cancer has been linked to STAG3 gene mutations (Skotheim *et al.* 2005; Barber *et al.* 2008). In our study, this gene was up-regulated in ovarian cancer cells after DNA damage due to H_2O_2 exposure compared to with normal ovarian cells that did not show expression of this gene. The chromatin modifying protein 4C (CHMP4C) gene is involved in endosomal degradation of receptors. For example, it is responsible for degradation of the epidermal growth factor (EGF) receptor (Bowers *et al.* 2006). This gene is possibly involved in stress responses through interaction with the p53 protein that prevents accumulation of DNA damage by regulating cell growth. The CHMP4C transcript is regulated by the p53 protein, enhancing exosome production that induces a quick degradation of epidermal growth factor receptors from the plasma membrane (Katoh *et al.* 2004; Yu *et al.* 2009). Our report is the first documentation of CHMP4C gene up-regulation during ovarian cancer development as a co-modulator of the p53 tumor suppressor pathway.

In conclusion, the mechanisms underlying gene expression deregulation of ASB9, PMS2L4, MSH2, STAG3L4, and CHMP4C in ovarian tumors still needs to be elucidated. Our observation of differential expression may indicate epigenetic modification play a role in gene silencing or in activation by methylation and histone

changes during DNA damage response signals during ovarian tumorigenesis. We used a functional approach to study whether genomic instability, is an early disruptor of gene expression, leads to cellular differentiation and the development of ovarian carcinomas. This approach allows the identification of novel gene candidates, useful for diagnostics and prognostics of ovarian cancer. Our results add to the elucidation of functional genetic events that may induce ovarian carcinoma progression and offers potential biomarkers for cancer testing.

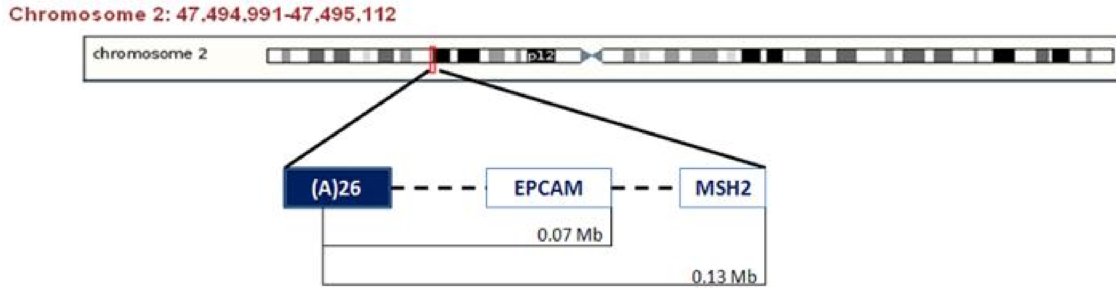

Figure 5.1 BAT 26 single tandem repeat marker

Notes: (A) Marker location on chromosome 2 (2p22-p21) (open red box). (B) Repeat motif of marker BAT 26 $(A)_{26}$ (filled blue box) and two genes located downstream (open blue boxes). The name of the genes and the associated distance in mega bases (Mb) from the marker is shown below each gene.
(http://www.ensembl.org/Homo_sapiens/Location/View?r=2%3A47494991-47495112)

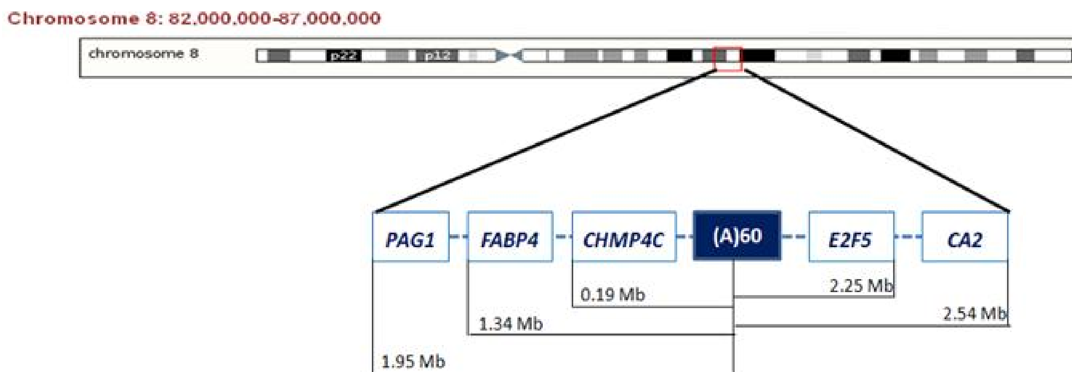

Figure 5.2 BAT 60 single tandem repeat marker

Notes: (A) Marker location on chromosome 8 (8q21) (open red box). (B) Repeat motif of marker BAT 60 $(A)_{60}$ (filled blue box), three genes located upstream (open blue boxes on the left), and two genes located downstream (open blue boxes on the right). The name of the genes and the associated distance in mega bases (Mb) from the marker is shown below each gene.
(http://www.ensembl.org/Homo_sapiens/Location/View?r=8%3A83732830-83733122)

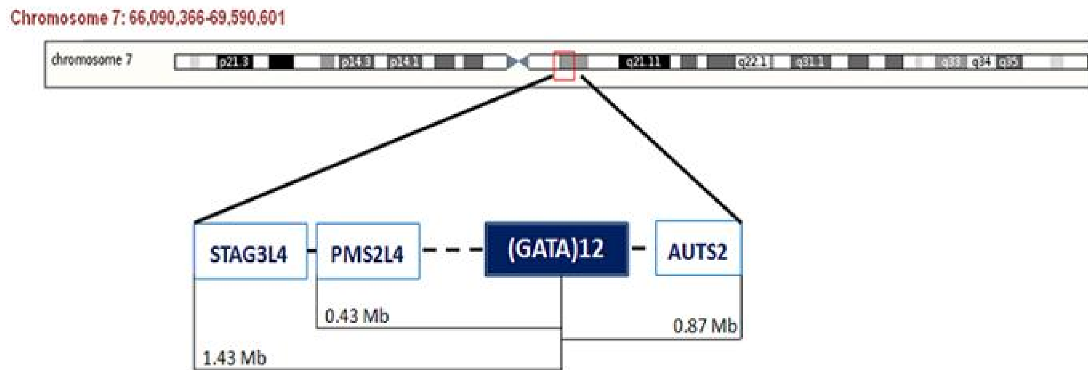

Figure 5.3 D7S3046 single tandem repeat marker

Notes: (A) Marker location on chromosome 7 (7q21.1) (open red box). (B) Repeat motif of marker D7S3046 $(GATA)_{12}$ (filled blue box), two genes located upstream (open blue boxes on the left), and one gene located downstream (open blue box on the right). The name of the genes and the associated distance in mega bases (Mb) from the marker is shown below each gene. (http://www.ensembl.org/Homo_sapiens/Location/View?db=core&r=7%3A66551985-69552321)

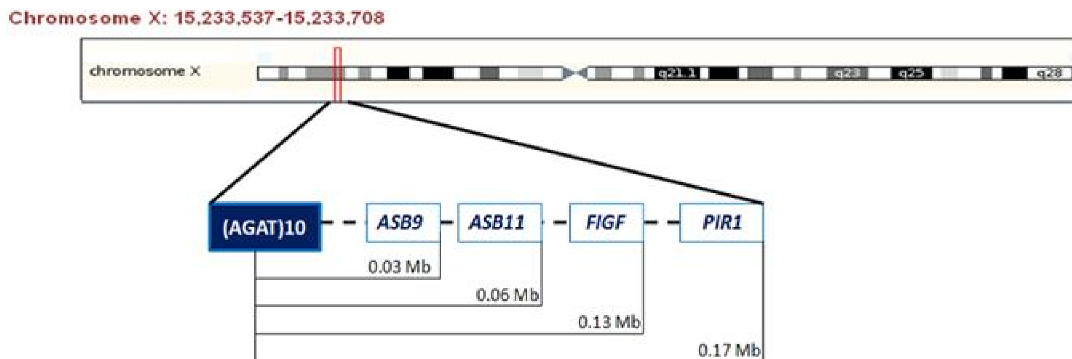

Figure 5.4 DXS9902 single tandem repeat marker

Note: (A) Marker location on chromosome X (Xp22.31) (open red box). (B) Repeat motif of marker DXS9902 $(AGAT)_{10}$ (filled blue box) and four genes located downstream (open blue boxes). The name of the genes and the associated distance in mega bases (Mb) from the marker is shown below each gene. (http://www.ensembl.org/Homo_sapiens/Location/View?r=X%3A15233537-15233708)

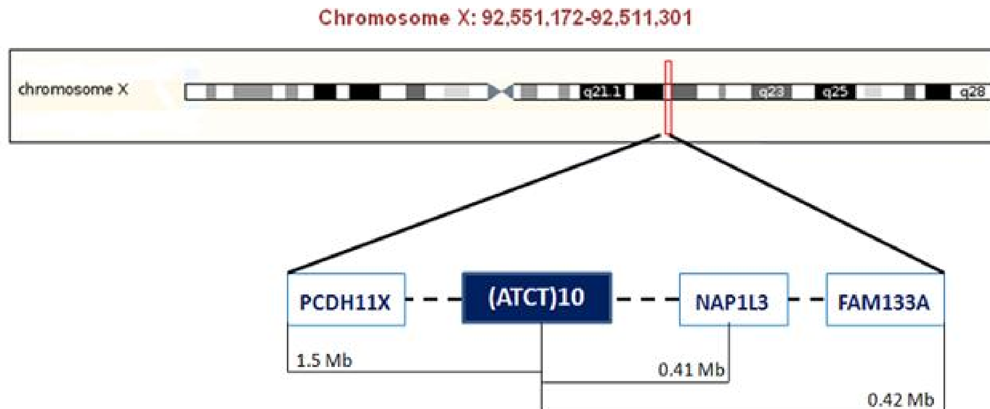

Figure 5.5 DXS6801 single tandem repeat marker

Note: (A) Marker location on chromosome X (Xq21.32) (open red box). (B) Repeat motif of marker DXS6801 $(ATCT)_{10}$ (filled blue box), one gene located upstream (open blue box on the left), and two genes located downstream (open blue boxes on the right). The name of the genes and the associated distance in mega bases (Mb) from the marker is shown below each gene.
(http://www.ensembl.org/Homo_sapiens/Location/View?db=core&r=X%3A91511301-93551172)

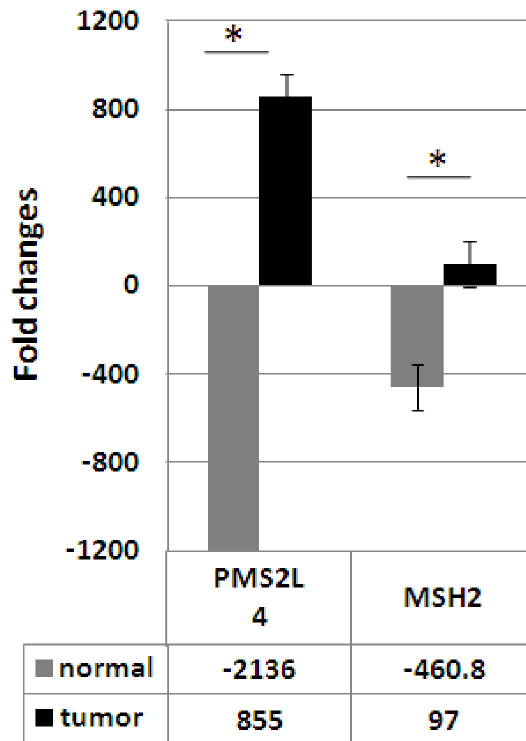

Figure 5.6 Changes in expression of DNA repair genes

Note: Real time PCR for PMS2L4 and MSH2 genes. Nine days after H_2O_2 exposure, total RNA was extracted from normal and cancerous cells, reverse transcribed, and amplified with specific primers per gene. Quantitative data were normalized to the level of the housekeeping gene ACTB. Error bars show SD, (n=3) *$p<0.05$. The table below the graph shows the fold change values of gene expression per sample and gene. Fold change was calculated using the comparative C_T method.

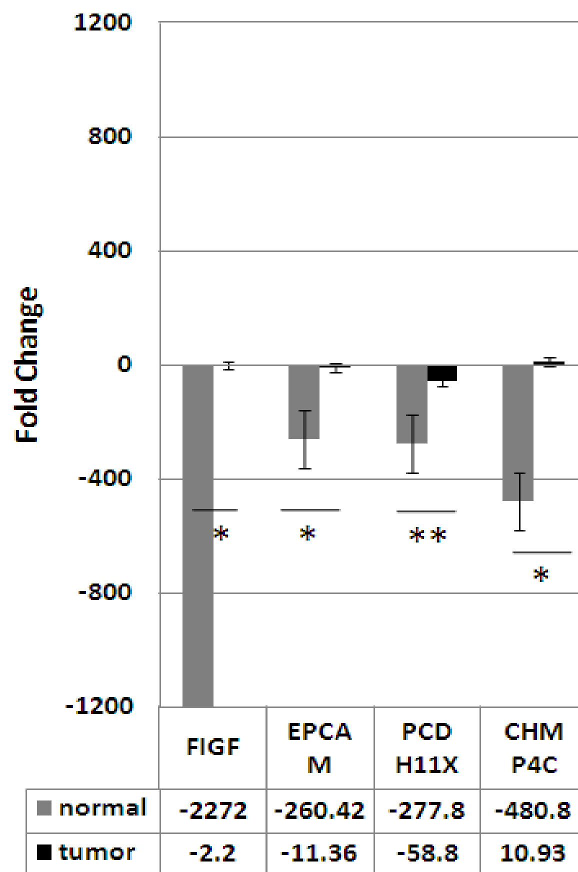

Figure 5.7 Changes in expression of genes responsible for cell growth.

Note: Real time PCR for FIFG, EPCAM, PCDH11X, and CHMP4C genes. Nine days after H_2O_2 exposure, total RNA was extracted from normal and cancerous cells, reverse transcribed, and amplified with specific primers per gene. Quantitative data were normalized to the level of the housekeeping gene ACTB. Error bars show SD, (n=3) *p<0.05 **p<0.10. The table below the graph shows the fold change values of gene expression per sample and gene. Fold change was calculated using the comparative C_T method.

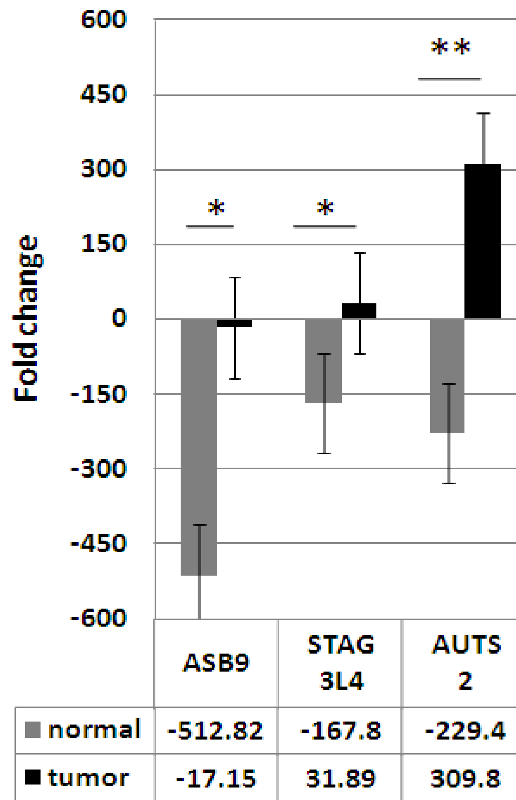

Figure 5.8 Changes in expression of genes involved in tumorigenesis and one gene candidate for susceptibility of autism

Notes: Real time PCR for ASB9, ATAG3L4, and AUTS2 genes. Nine days after H_2O_2 exposure, total RNA was extracted from normal and cancerous cells, reverse transcribed, and amplified with specific primers per gene. Quantitative data were normalized to the level of the housekeeping gene ACTB. Error bars show SD, (n=3) *p<0.05 **p<0.10. The table below the graph shows the fold change values of gene expression per sample and gene. Fold change was calculated using the comparative C_T method.

Table 5.1 List of genes evaluated by real time PCR

Gene Symbol	Gene Name	Gene ID	Function	size (bp)
ASB9	Ankyrin repeat and SCOS box- containing 9	140462	Protein degradation	177
PMS2L4	Postmeiotic segregation increased 2 like 4 pseudogene	5382	DNA repair	138
MSH2	mutS homolog 2, colon cancer,non polyposis.	4438	DNA repair	80
AUTS2	Autism susceptibility candidate 2	26053	neural cell differentiation	83
STAG3L4	Stromal antigen 3 like 4	64940	Sister chromatides cohesion	109
EPCAM	Epithelial cell adhesion molecule	4072	Cell adhesion protein	100
FIGF	c-fos induced growth factor	2277	Growth factor	138
PCDH11X	Protocadherin 11 X-linked	27328	Cell-cell recognition	146
CHMP4C	Chromatin modifying protein 4C	92421	Endosomal degradation	81
ACTB	Actin beta	60	Housekeeping gene	104

Notes: Sequences of the primers are available in https://www.qiagen.com/geneglobe/qtprimerview.aspx?

Table 5.2 Changes in gene expression for normal and cancerous ovarian cells

Gene symbol	Fold Change		*p-value*
	Normal	Tumor	
ASB9	-413	-17	**0.013**
PMS2L4	-1037	855	**0.003**
MSH2	-481	97	**0.059**
AUTS2	-229	310	*0.09*
STAG3L4	-168	32	**0.003**
EPCAM	-250	-11.4	**0.001**
FIGF	-533	-2.2	**0.03**
PCDH11X	-278	-59	*0.074*
CHMP4C	-481	21	**0.023**

Notes: Fold changes were calculated using the comparative C_T method. P-values ≤ 0.05 **(bold)** $p<0.10$ *(italic)*

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APPENDIX A

TANDEM REPEAT MOTIF LOCATED IN PROXIMAL PROMOTER REGIONS OF PLURIPOTENCY, SELF-RENEWAL, AND DIFFERENTIATION GENES

	Gene Symbol	Repeat Motif
1	AATF	(CA)18
2	ACTL6A	(CA)17
3	ACTN4	(GATA)15
4	ADCY2	(CA)16
5	ADCY2	(CA)15 (TA)7
6	ADNP2	(CA)18
7	AFMA044XE1	(CA)21
8	AGPAT2	(CA)12
9	AGPAT2	(CA)17
10	AKAP12	(CTAT)11
11	AMPA3	(CA)17
12	ANKHD1	(CA)15
13	ANKS1B	(CA)14
14	ANKS1B	(GATA)13
15	AP1M2	(GAAA)17
16	ARID48	(CA)16
17	ATF3	(GATA)8
18	ATG10	(GATA)13
19	ATP1A3	(GATA)12
20	ATP9A	(CA)19
21	ATR	(CA)22
22	ATR	(CA)19
23	ATXN1	(CA)19
24	B3GALT1	(CA)18
25	B4GALT1	(CA)18
26	B4GALT1	(CA)12
27	BANK1	(CA)21
28	BCAT1	(CA)22
29	BCOR	(A)21 (TA)14
30	BCS1L	(GATA)13
31	BCS1L	(CTAT)12
32	BMP2	(CA)17
33	BMP2K	(CA)23
34	BMP2K	(CA)19
35	BRCA1	(CA)21
36	BRIP1	(CA)13
37	BTRC	(CA)23 (TA)8
38	BUB1	(CA)18
39	BXDC1	(CA)17
40	C11orf75	(GAT)12
41	C16orf53	(GT)7
42	C20orf30	(CA)20
43	C6orf165	(TTA)6
44	C3orf26	(TAA)15
45	CA4	(CA)19
46	CACNA1A	(GT)15
47	CACNA2D2	(CA)30
48	CADPS	(CA)27
49	CADPS2	(CA)20
50	CAMK1D	(TATC)11
51	CAPN1	(CA)18
52	CCDC22	(CA)15
53	CDC2L6	(CA)21
54	Cdc42bpa	(CA)18
55	CDKN2A	(CA)18 (GA)11
56	CEBPE	(CA)20
57	CFDP1	(A)10
58	CHD9	(CA)11
59	CHN2	(CA)13
60	CHST11	(CATC)11
61	CIT	(CTAT)13
62	CIT	(CA)20
63	CLN3	(GT)18
64	CMM4	(CA)21
65	CNBP	(GAAA)16
66	CNOT6	(CA)18
67	COBL	(CA)16
68	CPNE4	(GAAA)16
69	CSRP2	(CA)24
70	CSRP2BP	(TAA)7

	Gene Symbol	Repeat Motif
71	CTDP1	(CA)18
72	CTNNA1	(CA)18
73	CUL4B	(CA)17
74	CYFIP1	(CA)27
75	DAP3	(CTAT)10
76	DAP3	(GATA)11
77	DCN	(CTAT)9
78	DDX50	(CA)16
79	DHRS3	(CA)14 (CT)11
80	DIAPH2	(CA)16
81	DIAPH3	(CA)17
82	DICER1	(CA)19
83	DLG3	(CA)14
84	E2F6	(CCTT)9
85	EGF	(GATA)8
86	EIF2C2	(CA)16
87	EN2	(GATA)13
88	EZH2	(CA)22
89	FBXL7	(CA)20
90	FBXW11	(CA)21
91	FGD1	(CA)16
92	FGF12	(TAA)10
93	FGFR2	(CA)22
94	FHIT	(CA)18
95	FILIP1L	(TAA)15
96	FOXN3	(CA)16 (GA)9
97	FOXP1	(CA)20
98	FUBP3	(CA)21
99	FXYD2	(CTAT)12
100	GABRR3	(GAT)15
101	GALK2	(CA)16
102	GARS	(GGAA)11 (GGCA)6
103	GPM6B	(CA)20
104	GPR98	(CA)17

	Gene Symbol	Repeat Motif
106	GRAMD1A	(CA)17
107	GRIA3	(CA)18
108	GRID2	(GATA)7
109	GRID2	(CATT)6
110	GRIK2	(TAA)14
111	GRM5	(TCTA)12
112	HAS2	(GAT)9
113	HLA-C	(GATA)10
114	HOXB3	(CA)20
115	HRASLS3	(CA)18
116	HS6ST3	(CA)19
117	ICMT	(CA)20
118	ICMT	(CA)19
119	IGFBP2	(CA)17
120	IL12B	(TAA)14
121	IL1RAPL1	(GATA)10
122	IRS1	(CA)20
123	ITMB2B	(CA)11
124	JARID2	(CA)14
125	JARID2	(CA)27
126	JARID2	(TG)20
127	kazrin	(CA)20
128	KCNK5	(CA)17
129	KCNN2	(CA)19
130	KCNQ5	(CTAT)10
131	KIT	(GATA)10
132	LAMA4	(CA)18
133	LAMB1	(CA)19
134	LECT1	(CA)15 (GT)15
135	LHPP	(CAA)11
136	LIN52	(CA)18
137	Lipa	(CA)14 (CT)12
138	LITAF	(GATA)10
139	LITAF	(CA)20

	Gene Symbol	Repeat Motif
141	LOC344967	(CATC)11
142	LOXL1	(GATA)10
143	LPHN2	(CA)11
144	LRRC40	(CA)12
145	LY9	(CA)13
146	MAFD1	(CA)17
147	MAP1B	(GATA)11
148	MAP2K1	(TAAA)11
149	MP3K12	(CA)48
150	MCC	(CA)15
151	MCC	(CA)22
152	MEIS1	(CA)12
153	MGAT4C	(CA)18
154	MLLT10	(CA)19
155	MMAB	(CA)21
156	MMVP2	(CA)20
157	MNAT1	(TTA)11
158	MOPCB2	(CA)24
159	MTHFD1L	(CA)18
160	MTHFD2	(CA)18
161	MTSS1	(CA)14
162	MUTED	(CT)9 (CA)20
163	MYH9	(CTAT)14
164	MYO3A	(CA)14
165	NAP1L1	(CA)21
166	NCAM1	(CA)23
167	NCOR1	(A)14 (CA)13
168	NCOR2	(CA)22
169	NECAP1	(CTAT)11
170	NIDDM3	(CA)18
171	NKTR	(CA)17
172	NLGN4X	(CA)25
173	NOD2	(CA)19
174	NOL4	(GATA)10

	Gene Symbol	Repeat Motif
211	POLQ	(CA)16
212	POLR2H	(CA)16
213	POU2F1	(CA)15
214	POU4F1	(CA)17
215	POU6F2	(CA)17
216	PPFIA2	(CA)11
217	PPP2R5C	(CA)13
218	PQBP1	(CA)19
219	PRKCG	(GT)23
220	PRKDC	(CA)16 (CT)15
221	PRMT3	(CA)18
222	PROM1	(CA)13
223	PROM1	(CA)22
224	PRPF31	(CA)15
225	PSMC2	(CA)16
226	PSMD8	(CA)12 (CT)8
227	PTER	(GATA)10
228	PTGDR	(CTA)11
229	RNMT	(CA)17
230	ROR1	(CA)18
231	SBF2	(CA)23
232	SBF2	(CT)9 (CA)17
233	SEMA3A	(GATA)13
234	SEPT	(CA)18
235	SETBP1	(TTA)13
236	SFRS11	(CA)13
237	SFXN1	(CA)25
238	SHANK2	(GAAT)9
239	SHANK2	(CA)18
240	SHANK2	(TC)12 (CA)14
241	SLC44A1	(CA)18
242	SLC8A3	(CTAT)12
243	SLCO3A1	(TAA)11
244	SMAD4	(CA)16

	Gene Symbol	Repeat Motif
246	SMOC1	(TAA)9
247	SNRPG	(CA)18
248	SORBS1	(CA)14
249	SOS1 -D3S1348	(TTA)12
250	SOS1 -D2S2186	(CA)18 (GT)8
251	SOS1 -D2S1356	(TAA)11
252	SOS1 -D2S441	(CTAT)12
253	SOS1 -D2S1346	(TAA)12
254	SOX6	(GGAT)10
255	SSPO	(CA)17
256	ST5	(CA)16
257	RAI14	(CA)22
258	RALA	(CA)12
259	RARB	(CA)12
260	RASGRF2	(TTA)12
261	RB1	(CA)32
262	RBM9	(CA)18
263	RBM9	(CA)28
264	RBM9	(CA)19 (CTA)6
265	RGS4	(TAA)12
266	RGS4	(GATA)16
267	RGS4	(GATA)10
268	RNF182	(TTA)13
269	RNF24	(CA)24
270	TBX21	(CA14)
271	TBX5	(CA)20
272	TCF12	(TAA)9
273	TCF4	(GT)16
274	TCF7L1	(CA)17
275	TEP1	(CA)24
276	TFEB-BP4	(GGAT)8
277	TGFBR3	(TAA)10
278	THAP8	(CA)16

	Gene Symbol	Repeat Motif
280	TIAM1	(CA)12
281	TLK1	(CA)14
282	TLK2	(CA)20
283	TMEM67	(GATA)9
284	TNPO1	(CA)18
285	TP63	(GATA)9
286	TPD52	(CA)22
287	TPTE	(TAA)14
288	TRA2	(CTAT)17
289	TRAP1	(GATA)7
290	TRIT1	(CTAT)7
291	TRPC6	(GATA)11
292	TRRAP	(CA)18
293	TTC28	(CTAT)14
294	TTK	(TAA)11
295	TXNDC5	(CA)22
296	UBAP1	(CA)18
297	UCK2	(TTA)8
298	UGP2	(CA)22
299	UNC5D	(CA)14
300	USP34	(CA)18
301	VAPB	(CA)25
302	WARS52	(GATA)9
303	XRCC1	(CA)12
304	XRCC5	(CA)14
305	XRN2	(CA)18
306	YAP1	(CA)14
307	YAP1	(CA)12
308	ZCCHC7	(CA)18
309	ZNF423	(CA)18
310	ZNF432	(TA)27
311	ZNF473	(CA)9
312	ZNF657	(CA)22

APPENDIX B

H1 (WA01), H7 (WA07) HUMAN EMBRYONIC STEM CELL LINES AND MEF CF-1 MOUSE FIBROBLAST CELL LINE INFORMATION AND CERTIFICATES OF ANALYSIS

Certificate of Analysis

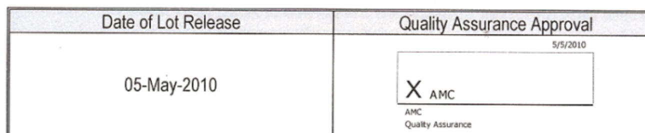
Product Description	WA01 Distribution Lot		
Cell Line Provider	WiCell		
Parent Material	WA01-MCB-04[1]		
Lot Number	WA01-DL-09		
Date Vialed	17-September-2009		
Passage Number	P27		
Culture Platform	Feeder dependent - MEFs Feeder Independent:		
	Media: hES Medium	Matrix: MEFs	Passaging Reagent: Collagenase

The following testing specifications have been met for the specified product lot:

Test Description	Test Provider	Test Method	Test Specification	Result
Post-Thaw Viable Cell Recovery	WiCell	SOP-CH-305	≥ 15 Undifferentiated Colonies, ≤ 30% Differentiation	Pass
Identity by STR	UW Molecular Diagnostics Laboratory	PowerPlex 1.2 System by Promega	Positive identity	Pass
Sterility - Direct transfer method	Apptec	30744	No contamination detected	Pass
Mycoplasma	Bionique	M250	No contamination detected	Pass
Karyotype by G-banding	WiCell	SOP-CH-003	Normal karyotype	Pass
Flow Cytometry for ESC Marker Expression	UW Flow Cytometry Laboratory	SOP-CH-101 SOP-CH-102 SOP-CH-103 SOP-CH-105	Report - no specification	See report

Appropriate biosafety precautions should be followed when working with these cells. The end user is responsible for ensuring that the cells are handled and stored in an appropriate manner. WiCell is not responsible for damages or injuries that may result from the use of these cells.

Please contact technical service via the website to request test methods and other assistance with your cells. The knowledgeable technical support staff can assist with cell culture concerns, training, and any other customer service concerns.



Date of Lot Release	Quality Assurance Approval
05-May-2010	5/5/2010 X AMC AMC Quality Assurance

~~P.O. Box 7366 • Madison~~, WI 53707-7365 • Phone: 888.204.1782 • Fax: 608.441-8011
www.wicell.org

[1] WA01-MCB-04 was tested as a DDL, not a MCB.

Fast Track Distribution Lot

Certificate of Analysis

Product Description (Cell Line)	WA07 Fast Track Distribution Lot
Cell Line Provider	WiCell Research Institute (Madison, WI, USA)
Lot Number	WA07-FTDL-01
Date Vialed	20-February-2009
Passage Number	P26

The following testing specifications have been met for the specified product lot:

Test Description	Test Method	Test Specification	Result
Post-Thaw Viable Cell Recovery	SOP-CH-305C	Viable cells recovered	Pass
Sterility	Apptec Protocol 30744 Rev. 1	Negative	Pass
Identity by STR	SOP-SS-006A	Positive identity	Pass*
Mycoplasma	Bionique Method M250	No contamination detected	Pass
Karyotype by G-banding	SOP-CH-003B	Normal karyotype	Pass

Electronic versions of this certificate of analysis (CoA) complete with electronic copies of individual reports, results, and procedures are available on our website, www.nationalstemcellbank.org. There are also archived CoAs for past cell lots.

Cells distributed by the National Stem Cell Bank are intended for research purposes only and are not intended for use in humans. These cells have undergone testing and are not known to harbor pathogens. However, appropriate biosafety precautions should be followed when working with these cells. The end user is responsible for ensuring that the cells are handled and stored in an appropriate manner. The NSCB is not responsible for damages or injuries that may result from the use of these cells.

Please visit the technical service portion of the website for assistance with your human ES Cells. The knowledgeable technical support staff can assist with embryonic stem cell culture concerns, training, and any other customer service concerns you may encounter.

Date of Lot Release: 07OCT09

WiCell Quality Assurance: [signature] Date: 07OCT09

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ATCC™

Product Information Sheet for SCRC-1040

Lot number: 58551798

ATCC Catalog No.: SCRC-1040
Lot No.: 58551798
Designation: MEF (CF-1)
Description: Mouse fibroblast
Total Cells/mL: 4.5 x 10(6)
Expected Viability: 94.9% to 96.9%
Ampule Passage No.: 3
Population Doubling (PDL): N/A
Dilute Ampule Content: Seed at 8.0 x 10(3) viable cells/cm(2)
Volume/Ampule: 1 mL
Date Frozen: 06/18/2009

A T-25 set up at a seeding density of 8.0 x 10(3) viable cells/cm(2), using culture medium as described in the product information sheet, reaches approximately 60% to 70% confluence in 2 days.

Copyright ATCC, 1998; ATCC is a registered trademark of the American Type Culture Collection 9-98

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110-2209

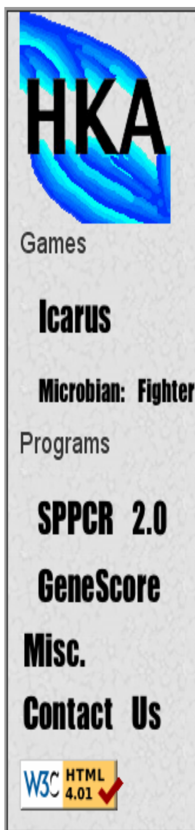
Ordering info: 800 638-6597 (USA and Canada)
703 365-2700 (elsewere) Fax: 7033652750
email: sales@atcc.org web site: www.atcc.org

APPENDIX C

SP-PCR SOFWARE VERSION 2.0 FROM M.D. ANDERSON CENTER

INTRUCTIONS AND MUTATION FREQUENCIES REPORTS

(http://www.hkasoftware.com/index.php?object=SPPCR)



SPPCR 2.0

SPPCR 2.0 is a port of the Fortran program written by Barry Brown designed to calculate theoretical g.e., Mutant Frequency, and Significance of Two Numbers.

Mac

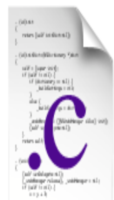
Download: sppcr.zip
To Run: Double click the program
To Install:

1. Open Terminal
2. type *cd the_directory_you_downloaded_sppcr_to*
3. type *sudo cp sppcr /usr/bin/*
4. enter your admin password

Windows

Download: sppcr.zip
To Run: Double click the program
To Install: Drag it to your system folder
Troubleshooting: If it claims there is a missing DLL, try installing the Microsoft Visual C++ 2010 Redistributable Package (x86)

Documentation HTML PDF RTF



Mac Source Code: SPPCR 2.0.zip
Windows Source Code: sppcr-win32.zip

All concerns/bugs/feature request should be addressed to Brian Ramagli

---

Documentation at http://www.hkasoftware.com/index.php?object=SPPCR, is a continuation.

SPPCR 2.0

The Final Frontier

Table of content

1) Intro

2) Synopsis

3) Input format and meanings

3a) Assumption about input

4) Output format and meanings

5) Known problems

6) Frequently Asked Questions

1) Intro

In the course of every program's life, it must be ported. To a new and better language, operating system, or platform. SPPCR 2.0 is a complete port, with bug fixes throughout, of Barry W. Brown's SPPCR 1.0.

2) Synopsis

The general use for this program is to calculate what we presume the actual ge, the mutation frequency, and the significance of a given data set, or pair of data set.

3) Input format and meanings

When using this program as a tool from Excel/Filemaker or other programs that export via applescript or what-have-you, you need to give it an initial argument of 4.The initial argument allows the program to be run several different ways, and allows me to give several types of specific, need-based output.

current initial arguments are:

0 - do nothing

2 - run hardcoded test data to test the program and make sure it is running

3 - interactively input data by hand responding to command prompts.

example:

```
Enter the number of runs (number of dna amounts):2
Enter the number of alleles seen:5
Enter the sizes of the 5 alleles:140 142 144 146 148
Enter the size of the 2 progenitor alleles. If the subject
is homozygous, enter the size of the 1 progenitor twice.144
144
For each run, enter the expected genomes.
Expected ge for run 1:.8
Expected ge for run 2:.4

For each run, enter the number of replicates.
```

```
Replicates for run 1:
```

and so on. Terminology that the program used is all explained at the end of this section, as well as above the prompts.

4 - read in data in the following format:

Num runs

Num alleles

allele sizes (there needs to numallele of them)

progenitor allele's sizes, there needs to be 2 of them, if homozygous, repeat it twice

then, for each run/row

a) observed/expected ge

b) number of replicates

c) number of alleles saw at each allele size

example:

```
4 2 19 144 146  148  150  152  154  156  158  160  162  164
166  168  170  172  174  176  178  180
154  156
0.81 96 0 0 0 1 1 52 53 0 0 0 0 0 0 0 0 0 0 0 0
0.58 32 0 0 0 1 2 15 11 0 0 0 0 0 0 0 0 0 0 0 0
```

Now lets look at this in details. 4 means we are using this format of input/output. The 2 means there are 2 different runs being looked at. The next number states that there at 19 possible allele sizes that have observed alleles in them. The next 19 numbers are of course the allele sizes. 154 and 156 are the 2 progenitors in this case.
After that, we have our first run, which has an expected ge of .81, 96 replicates, and the next 19 numbers are the observed alleles. Our second run has an expected ge of .58 and 32 replicates, and the next 19 numbers are of course the observed allele.

5 - This is the quiet version of option 6. It is used to calculate significance between 2

frequencies. The format to pipe your data in is:

Frequency 1

Frequency 2

Standard Error 1

Standard Error 2

6 - Like option 5, this will calculate the significance, but it is intended for interaction between the user and it. Just follow the prompt.

```
Enter the first mutant frequency: .046
Enter the second mutant frequency: .047
Enter the first standard error: .003
Enter the second standard error: .0042
```

7 - exactly like option 4, but has a verbose output

terminology:

run: A PCR experiment at one sample

allele size: PCR fragment size

replicates: number of wells examined

expected ge: what you think you put into the reaction

progenitors: parental alleles

3a) Assumptions about input

a) The first input to the program must be a single character, preferably of the numerical type from 0-6

b) At least one progenitor has been seen.

4) Output format and meanings

The computations are made for the whole. Meaning that if you do 4 runs, the ge and frequencies are calculated as if all the runs were one giant single run.

for mode:

2 - The output to the hardcoded data should be just a standard listing. It changes from

build to build so that the developer may fine tune aspects and perhaps even discover bugs. It is not intended for the consumer's use.

```
3-d0 = 0.7106
```

The d0 is an antiquated statistical output used for legacy reason (hangover from sppcr 1.0 and previous incantations). In sppcr 2.0, the ge is already calculated for you.

```
95% CI (0.6417,0.7961)
```

This the the 95% Confidence Interval for the d0.

The 1/d0 and the confidence interval for that are exactly what they sound like.

```
estimated ge for run 0 = 1.0555
```

This is the statistically calculated estimate of what the ge is.

```
Mutant Frequency
estimate: 0.016160
bootsrap SE: 0.004675
```

This is gives the mutant frequency, and the resampled bootstrap error (to be used to determine the significance between 2 mutant frequencies)

4 - Since this is used strictly for connecting with outside programs via piping, this only outputs <# of runs> G.E.s, followed by the mutant frequency, followed by the standard error.

5 - Returns 1 number, that being your significance.

6 - The Z value is the statistical Z value used. If you wish to use a standard lookup table to confirm yourself, you can. If you are a normal person, and expect this program to do everything for you, it does. The calculated significance is provided on the next line.

```
Z = -0.193746
significance = 4.231874E-01
```

5) Known Problems

• Does not give proper results in the event of double progenitor loss. Single progenitor loss appears to have correct results, but it has not been thoroughly tested.

6) Frequently Asked Questions

Q) I have inputted several runs, each with the same expected ge, but different overall traits. Why do I get the same ge for all my runs?

A) The program calculates all "runs" as a single experiment. What you are seeing is the ge for all the runs together. If you wish to obtain a better estimate of the ge, do each rune individual.