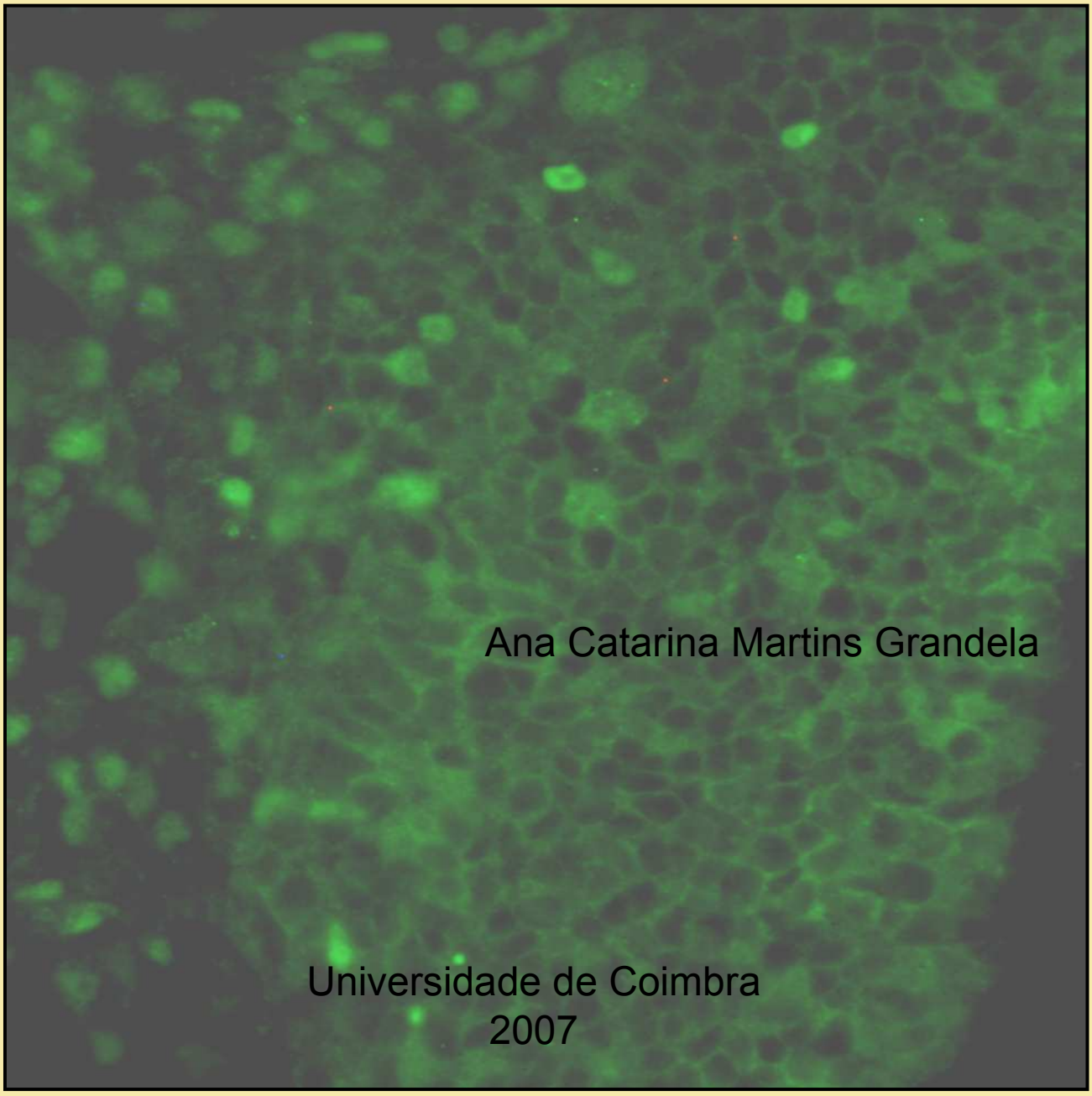
Regulation of **A**poptosis and **D**ifferentiation by **p53** in **H**uman **E**mbryonic **S**tem **C**ells



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Universidade de Coimbra
2007

REGULAÇÃO DA APOPTOSE E DIFERENCIAÇÃO PELO P53 EM CÉLULAS ESTAMINAIS EMBRIONÁRIAS HUMANAS

REGULATION OF APOPTOSIS AND DIFFERENTIATION BY P53 IN HUMAN EMBRYONIC STEM CELLS

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Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para a prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Molecular

Dissertation presented to Faculty of Science and Technology of the University of Coimbra in partial fulfillment of the requirements for a Doctoral degree in Molecular Biology

Universidade de Coimbra
2007

Este trabalho foi realizado no Monash Institute of Medical Research da Monash University, Australia, sob a orientação do Professor Associado Martin Pera e Professor Ernst Wolvetang e co-orientação do Professor Associado João Ramalho de Sousa Santos (Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Portugal). A sua realização foi suportada pela bolsa SFRH/BD/1189/2003 da Fundação para a Ciência e a Tecnologia, Portugal.

This work was performed at the Monash Institute of Medical Research, Monash University, Australia, under the supervision of Associate Professor Martin Pera and Professor Ernst Wolvetang and co-supervision of Associate Professor João Ramalho de Sousa Santos (Department of Zoology, Faculty of Science and Technology of the University of Coimbra). Its execution was supported by grant SFRH/BD/1189/2003 from Fundação para a Ciência e a Tecnologia, Portugal.

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Financiamento no âmbito do III Quadro Comunitário de Apoio, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCES

ACKNOWLEDGMENTS

I would like thank:

- The Fundação para a Ciência e a Tecnologia (FCT) for financial support (scholarship and bench fees) crucial to develop this work;
- The Australian Stem Cell Centre (ASCC) for financial and material support;
- The BEB PhD Programme coordinators for the opportunity of doing this PhD;
- The BEB PhD Programme students that with motivation, intelligence and friendship provided me the best environment to learn;
- Linh Nguyen, Karen Koh, Irene Tellis, Tracey Lomas, Lisa Kass, Sheetal Saini and specially Pegah Jamshidi for expert technical assistance in hESC cell lines culture and be always there when I forgot to order cells or whenever I needed help;
- Andrew Fryga and Darren Ellemor (ASCC) for help and discussion on flow cytometry;
- Sean Grimmond and Gabriel Kolle for their transcriptome and bioinformatic analysis;
- Richard Davis for his help, patience and love;
- Students and Post-docs of Pera's lab for their help and friendship;
- Professor Andy Laslett for his help which will be paid for the rest of my life with warm and fresh Portuguese custard tarts;
- Nick Hannan and Tom Chung for stimulating scientific meetings and their precious help, especially in the end of PhD;
- Professor Stephen Fox for help in teratomas examinations;
- Professor Joseph Sambrook for critical reading of the manuscript's preparation;
- Professor João Ramalho-Santos for facilitating this research and being always there;
- Professor Martin Pera for giving me the opportunity to enter in human embryonic stem cell world and being a mentor;
- Professor Ernst Wolvetang (my Boss) for constant motivation, putting up with my dramas and let me do pretty much everything I wanted to do. After all, "The PhD is the only time we are free"…
- Aos meus pais por todas as razões óbvias, mas principalmente por aceitarem as minhas longas ausências e de me terem "dado a cana"… (To My parents for all the obvious reasons, but especially for being able to accept my long absence).

TABLE OF CONTENTS

ABBREVIATIONS

Ac- Activin
Apaf-1-Apoptotic Protease Activating Factor-1
APS- Ammonium persulfate
BA- Bongkrekic acid
BH- Bcl2
BMP- Bone morphogenetic proteins
BSA- Bovine serum albumin
BrdU- Bromodeoxyuridine
CARD- Caspase activation and recruitment domain
cDNA- Complementary DNA
CM- Conditioned medium
CGH- Comparative genomic hybridization
CCCP- Carbonyl cyanide 3-chlorophenylhydrazone
cPFT- Cyclic pifithrin-α
CHX- Cycloheximide
Cyclosp- Cyclosporin
DAPI- 4',6-Diamidino-2-Phenylindole
ddH_2O- Double distilled water
DED- Death effector domain
DISC- Death-inducing signal complex
DMEM- Dulbeco's modified Eagle's medium
DNA- Deoxyribonucleic acid
DMSO- Dimethylsulfoxide
DRAM- Damage-regulated autophagy modulator
dsRNA- Double stranded RNA
EB- embryoid-bodies
EHS- Engelbreth-Holm-Swarm
ERK- Extracellular signal-regulated kinases
ESC- Embryonic stem cells
Et- Etoposide
EtOH- Ethanol
FACS- Fluorescence-activated cell sorting
FADD- Fas-associated death domain protein
FasL- Fas ligand
FC- Flow cytometry
FCS- Fetal calf serum
FGF-Fibroblast growth factor
FGFR- Fibroblast growth factor receptor
GDF- Growth/Differentiation factors
hECC- Human embryonic carcinoma cells
HEF- Human embryonic fibroblasts
hESC -Human embryonic stem cells
HSC- Hemapoietic stem cells
IAP- Inhibitor of apoptosis
IC- Immunocytochemistry
ICM - Inner cell mass
IGF- Insulin growth factor
iPS- Induced pluripotent stem
IVF- *In vitro* fertilization
KSR- Knockout serum replacement
Leptom- Leptomycin B
LIF- Leukemia inhibitory factor
LIFR- LIF receptor
L-Glut- L-glutamine
MEF- Mouse embryonic fibroblasts
MeOH- Methanol
mESC- Mouse embryonic stem cells
mRNA- Messenger RNA

miRNA- Micro RNA
NEAA- Non-essential amino acid
NGF- Nerve growth factor
ON- Overnight
Q-PCR- Quantitative PCR
PAGE - Polyacrylamide gel electrophoresis
PBS- Phosphate Buffered Saline
PBST- PBS-Tween
PCD- Programmed cell death
PCR- polymerase chain reaction
PDGF- Platelet derived growth factor
Pen/Strep- Penicillin/Streptomycin
PFA- Paraformaldehyde
PFT-mu- Pifithrin-μ
PI- Propidium iodide
RA- Retinoic acid
RE- Responsive elements
RISC- RNA-induced silencing complex
RNA- Ribonucleic acid
RNAi- RNA interference
RNP- Ribonucleoprotein complex
RT- Room temperature
RT-PCR- reverse-transcriptase polymerase chain reaction
SDS- Sodium dodecyl sulfate
siRNA- Small interfering RNA
shRNA- short hairpin RNA
SCID- Severe combined immunodeficiency
SNP- single nucleotide polymorphism
SR- Serum Replacement
TGCT- Testicular germ cell tumours
TNFα- Tumour necrosis factor alpha
TRK- Tropomyosin-related kinases
TUNEL- Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
WB- Western blotting

LIST OF PUBLICATIONS

Article

Grandela C, Pera MF and Wolvetang EJ. ***p53 is required for etoposide induced apoptosis of human embryonic stem cells.*** Stem Cell Research. *In press,* 2007.

Review article

Grandela, C., and Wolvetang, E. (2007). ***hESC Adaptation, Selection and Stability.*** *Stem Cell Rev* **3,** 183-91.

ABSTRACT

Human embryonic stem cells (hESC) display pluripotency and unlimited self-renewal while retaining a normal karyotype, suggesting they possess mechanisms to minimize the consequences of DNA damage. The molecular mechanisms controlling DNA-damage induced apoptosis of hESC are poorly understood. This study investigates the role of p53 in DNA damaged-induced apoptosis and cell differentiation. hESC are extremely sensitive to spontaneous apoptosis, as well as gamma irradiation-, hydrogen peroxide- and etoposide-induced apoptosis. Indeed, early passage hESC appear to be far more sensitive to etoposide-induced apoptosis than adapted hESC and cancer cell lines. The data further show that undifferentiated hESC that express Oct4 are much more sensitive to etoposide-induced apoptosis than their more differentiated progeny. It is shown that p53 is constitutively expressed at high levels in the cytoplasm of hESC. Etoposide treatment results in rapid and extensive induction of apoptosis and leads to a further increase in p53 and PUMA expression as well as Bax processing. p53 both translocates to the nucleus and associates with the mitochondria, accompanied by co-localisation of Bax with Mcl1. hESC stably transduced with p53 shRNA, display 80 % reduction of endogenous p53 and exhibit an 80 % reduction in etoposide-induced apoptosis accompanied by constitutive downregulation of Bax and an attenuated upregulation of PUMA. Furthermore, gene profile analysis of untreated GFP and p53 shRNA transduced hESC shows that p53 controls a set of genes under normal conditions. Around 70 % of these genes contain p53 responsive elements. No significant differences were observed between cell cycle entry of GFP and p53 shRNA transduced cell lines or distribution of phases of cell cycle and the karyotypes of both cell lines were found to be normal. p53 had a small but reproducible effect in inhibiting spontaneous differentiation. Preliminary studies to address the role of p53 in differentiation of hESC revealed a slight resistance of p53 shRNA transduced cell lines to BMP4-induced differentiation and the opposite was observed in relation to Activin A treatment. Furthermore, these cells formed teratomas (with representative tissues of all three germ layers) when implanted beneath the testis capsule of SCID mice that exhibited a higher proportion of immature neural tissue compared to control cells, suggesting that these cells are more prone to undergo differentiation to the neural lineage, or mature more slowly. This study demonstrates that p53 plays a role in differentiation, is required for etoposide-induced apoptosis of hESC and reveals, at least in part, the molecular mechanism of DNA damage-induced apoptosis in hESC.

RESUMO

As células estaminais embrionárias humanas (hESC) possuem características de pluripotência e capacidade ilimitada de auto-renovação, mantendo ao mesmo tempo um cariótipo normal, o que sugere que estas possuem mecanismos que minimizam as consequências de danos no ADN. Os mecanismos moleculares que controlam a apoptose das hESC induzida por danos no ADN são pouco compreendidos. Este estudo investiga o papel do p53 na apoptose induzida por agentes que causam danos no ADN e na diferenciação celular. As hESC são extremamente sensíveis à apoptose espontânea, como também à induzida por radiação gama, peróxido de hidrogénio e etopósido. De facto, as hESC com poucas passagens são muito mais sensíveis à apoptose induzida por etopósido do que hESC adaptadas e linhas celulares cancerígenas. Os dados deste estudo também demostram que as hESC indiferenciadas que expressam Oct4 são mais sensíveis à apoptose induzida pelo etopósido do que a sua progenia diferenciada. O p53 é constitutivamente expresso em níveis elevados no citoplasma das hESC. O tratamento com etopósido leva a uma rápida indução da apoptose e aumento da expressão do p53 e do PUMA, bem como ao processamento do Bax. O p53 transloca para o núcleo e associa-se com a mitocôndria e isto é acompanhado com a co-localização do Bax com o Mcl1. As hESC estavelmente expressam shRNA desenhado para diminuir a expressão do p53, possuem uma reducção em 80 % do p53 endógeno e exibem também uma reducção na apoptose induzida pelo etoposide, acompanhada por uma diminuição nos níveis do Bax e por uma atenuação no aumento dos níveis do PUMA. A análise do perfil da expressão de genes de hESC que expressam shRNA para reduzir a expressão do p53 e GFP mostra que o p53 controla um conjunto de genes em condições normais. Cerca de 70 % destes genes contêm nos seus promotores locais de ligação para o p53. Não foram observadas diferenças significativas na distribuição das fases do ciclo celular das hESC que expressam shRNA para reduzir a expressão do p53 e GFP e os cariótipos destas células são normais. O p53 tem um pequeno, mas reprodutivél efeito na inibição da differenciação espontânea. Estudos preliminares para investigar o papel do p53 na diferenciação das hESC revelaram uma pequena resistência das células que expressam shRNA para diminuir os níveis do p53 à diferenciação induzida pelo BMP4, sendo o oposto observado em relação ao tratamento com Activina A. Estas células formam teratomas (com tecidos representativos das três camadas germinativas) quando implantadas na cápsula de murganhos immunodeprimidos SCID que exibem uma maior proporção de tecido neural imaturo quando comparadas com teratomas formados pelas células controlo, o que sugere que elas preferencialmente se diferenciam numa linhagem neural ou que o seu processo de maturação é mais lento. Este estudo demonstra que nas hESC, o p53 desempenha um papel na diferenciação celular, é necessário para a apoptose induzida pelo etopósido e revela em parte o mecanismo molecular que controla a apoptose induzida por danos no ADN das hESC.

CHAPTER 1

Introduction

1.1- HUMAN EMBRYONIC STEM CELLS

*The excitement and controversy surrounding the potential role of human embryonic stem cells in transplantation therapy have often overshadowed their potentially more important use as a basic research tool for understanding the development and function of human tissue (*Xu *et al,* 2002*).*

Human embryonic stem cell lines (hESC) are established from the inner cell mass (ICM) of the blastocyst and differentiate into all cell lineages of the body (Figure 1.1). They were first derived in 1998 by James Thomson's group in the University of Wisconsin-Madison, USA (Thomson *et al*., 1998) and have enormous potential as a source of cells for cell replacement therapies and as a model for early human development (Hoffman and Carpenter, 2005). In fact, hESC represent a useful experimental system that provides access to stages of the human life cycle that were previously inaccessible to experimentation (Pera and Trounson, 2004). Conversely, better understanding of stem cell basic biology will help to improve maintenance and differentiation protocols for manipulating hESC, thus enabling the dissection of some developmental diseases and advancement of more effective treatments. Moreover, because hESC and their derivatives are diploid, they are more attractive than the transformed cell lines for drug screening and disease modeling.

Two important features of embryonic stem cells (ESC) are their self-renewal capacity (they can be maintained and expanded in culture) and pluripotency (they can differentiate to give rise to mesoderm, endoderm and ectoderm as well as germ cells). Unlike human embryonic carcinoma cell lines (hECC, the transformed counterpart of hESC), hESC can conserve a normal karyotype if cultured under appropriate conditions.

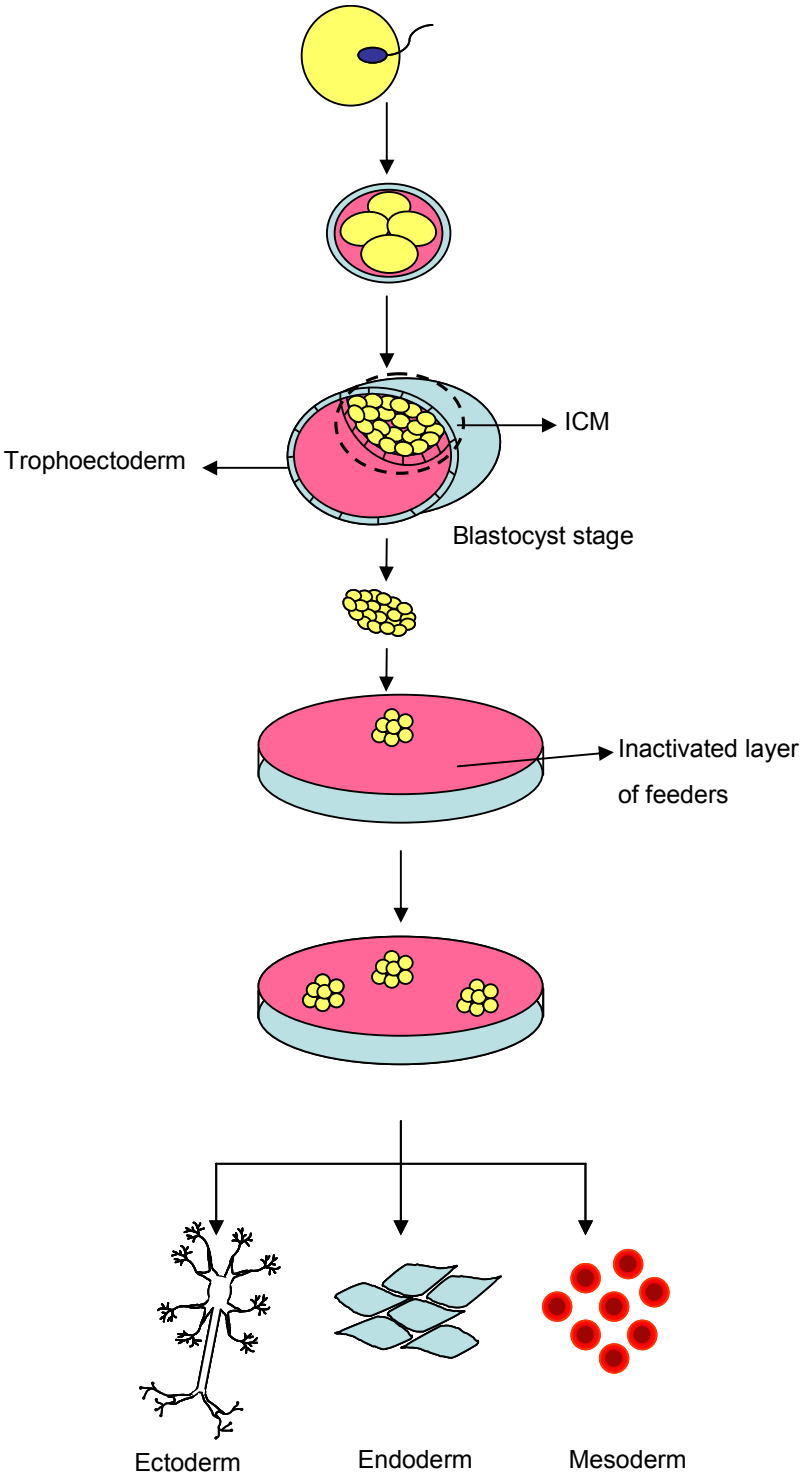

FIGURE 1.1- Schematic derivation of hESC from ICM of a human blastocyst.
hESC are derived from pre-implantation stage embryos. In most cases, these are donated to research after being deemed surplus to clinical requirements. After being removed from blastocyst, the ICM is placed upon an inactivate layer of feeder cells. A hESC line is formed by serial passaging of these cells. These cells can be maintained in the undifferentiated state (self-renewing) for long periods of time or can be differentiated into representatives of three germ layers (ectoderm, endoderm and mesoderm).

The mouse embryonic stem cells (mESC) were the first mammalian ESC derived and this was achieved by Evans, Kaufman and Martin in 1981 (Evans and Kaufman, 1981; Martin, 1981). Therefore, most of our understanding of the molecular mechanisms governing self-renewal of ESC, and requirements for growth and lineage commitment in the early mammalian embryo, are based on the mouse model. However, extensive studies on both mouse and human ESC have shown substantial differences, such as the dependence of mESC on leukemia inhibitory factor (LIF) to maintain their undifferentiated state (described latter in this chapter), and this may be explained by significant divergence between mouse and human development and/or to the fact that these cells are isolated/derived at different time points of development (Pera and Trounson, 2004).

1.1.1- Criteria to define an embryonic stem cell

According to Pera *et al* (Pera *et al*., 2000) and considering mESC as standard, there are several criteria that can be used to define an ESC: 1) it originates from a pluripotent cell population, 2) it is stably diploid and karyotypically normal *in vitro*, 3) it can be cultured indefinitely in its primitive state, 4) it can differentiate into tissues of the three embryonic germ layers: endoderm, mesoderm and ectoderm *in vitro* or teratomas and 5) it can give rise to any cell in the body when injected into a host blastocyst. All these criteria have been confirmed by experimentation for mESC and mouse embryonic germ cells. Due to ethical reasons, it is not possible to validate the last criterion in hESC (Pera *et al*., 2000).

1.1.2- Stem cell markers

Pluripotent cells are characterized by distinctive cellular markers and properties that relate to their uncommitted state. These markers were first identified in hECC and in the early embryo and are particularly useful to follow stem cell populations and for isolation purposes.

In order to characterize hESC a number of cell surface markers are currently used, including glycolipids and glycoproteins, such as SSEA3, SSEA4, Tra-1-60, Tra-1-81, GCTM2 and CD9. Despite their identification, the function of most of these markers is not completely understood. CD9 is a tetraspanin transmembrane protein and has probably a role in the organization of integrins and other receptors at the cell surface and has been implicated in mESC maintenance (Oka *et al*., 2002) and its expression is induced by STAT3 activation.

Some common ESC surface markers and their presence in both human and mouse cells are shown in table 1.1.

TABLE 1.1- Cell-surface markers for pluripotent stem cells (Laslett *et al*., 2003)

	mECC and mESC	hECC	hESC	Human ICM
SSEA1	+	-	-	-
SSEA2	-	+	+	+
SSEA3	-	+	+	+
TRA-1-60	-	+	+	+
TRA-1-81	-	+	+	+
GCTM-2	-	+	+	?
TG343	?	+	+	?
CD9	+	+	+	?

Not only is it important to verify the presence of these markers but also to quantify their expression, because it is becoming more evident, especially in the case of hESC, that even an apparently undifferentiated population of stem cells is constituted of different subpopulations when assessed by a combination of immunological and transcriptional analysis (Laslett *et al*., 2007). Downregulation of genes associated with undifferentiated state occurs in a coordinated fashion and during the early stages of stem cell differentiation these genes are co-expressed with lineage specific transcription factors in a continuum. The study by Laslett and colleagues (2007) has important practical implications for culture (for example by identifying new stem cell markers and growth factors that can be used to optimize culture conditions) and directed differentiation hESC (for example by defining the stages at which cells obtain lineage commitment). It can also help in the interpretation of studies of hESC self-renewal and commitment because if the population is heterogeneous when a given growth factor is added most likely a subset of cells will respond differently.

Also, certain transcription factors are well known to play a role in stem cell maintenance. Oct3/4 or Oct4, which belongs to POU family of transcription factors, is expressed by pluripotent stem cells *in vitro* as well as in the ICM of embryo, and is downregulated upon differentiation (Nichols *et al*., 1998; Niwa *et al*., 2000). Other important transcription factors are Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003; Hart *et al*., 2004) and Sox2 (Avilion *et al*., 2003). These transcription factors play crucial role in early development and self-renewal of embryonic stem cells and collaborate to form regulatory circuitry consisting of autoregulatory and feedforward loops (Boyer *et al*., 2005). Oct4 and Sox2 can form a protein complex and suppress the expression of Cdx2, which is involved in the differentiation to trophectoderm, while Nanog can suppress GATA6, involved in the differentiation to primitive endoderm (reviewed by Boyer *et al*., 2006; Niwa, 2007). Perhaps undifferentiated growth is a function of repression of lineage specific differentiation.

Three independent studies have reported reprogramming of mouse fibroblasts to a pluripotent state by ectopic expression of the transcription factors Oct4, Sox2, Klf4 and c-myc (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007) which were selected for Nanog expression. These cells were called induced pluripotent stem (iPS) cells and are very similar to mESC regarding morphology, deoxyribonucleic acid (DNA) methylation, gene expression and chromatin state. iPs cells were shown to contribute to the germline when introduced in mouse blastocysts since they were able to produce viable chimaeras. Unfortunately, Okita and colleagues have reported that about 20% of mice have developed tumours due to reactivation of c-myc transgene (Okita *et al*.,

2007). Recently, iPs cells were generated from human adult dermal fibroblasts with the same 4 factors mentioned above and from human fetal fibroblasts with Oct4, Sox2, Nanog, and LIN28 , opening the possibility of generating patient and disease-specific pluripotent stem cells (Takahashi *et al*., 2007; Yu *et al*., 2007).

1.1.3- Culture methods for hESC propagation: advantages and limitations

Two culture methods are widely used to propagate hESC (Figure 1.2): the original technique used to culture the cells, referred to herein as the standard method and the serum-free method. The standard method of culturing hESC involves weekly mechanical passaging of morphologically undifferentiated parts of a hESC colony onto full density ($6x10^4$ cell/cm^2) mouse or human fibroblast feeder (MEF and HEF, respectively) layers mitotically inactivated in Dulbeco's modified Eagle's medium (DMEM) containing 20 % fetal calf serum (FCS) or human serum (Reubinoff *et al*., 2000; Richards *et al*., 2002; Thomson *et al*., 1998). This protocol is demanding in terms of time, money and effort, but has proven to be a safe and reliable method for the long term propagation of hESC. This is highlighted by the fact that hESC thus cultured have rarely been reported to develop genetic abnormalities during culture. However, the major drawback of this method is the low number of hESC available for experimentation. Some laboratories therefore opt to maintain hESC stock cultures using this standard method and regularly establish fresh bulk cultures from these stocks for experimentation (Amit *et al*., 2000). The serum-free method of culturing hESC is performed according to a protocol developed by Amit and colleagues which involves passaging of enzymatically (for example collagenase and trypsin) or non-enzymatically (cell dissociation solution, EDTA, etc) dissociated clumps of hESC on 1/3 density feeders ($2x10^4$ cell/cm^2) in DMEM/F12 supplemented with 20% Knockout serum replacement (KSR) from Invitrogen, and supplemented with fibroblast growth factor 2 (FGF-2). Despite the fact that KSR contains fewer components than FCS, its composition is still undefined and it contains a large amount of Albumax, a lipid-rich preparation of bovine serum albumin (BSA). This method allows for dramatic expansion of hESC. However, a number of reports have indicated that this is accompanied by an increase in genetic instability, methylation changes and mitochondrial mutations, abnormalities also seen in cancer and hECC (Draper *et al*., 2004; Mitalipova *et al*., 2005; Brimble *et al*., 2004; Allegrucci *et al*., 2007). Allegrucci and colleagues examined the DNA methylation profiles of more than 2000 genomic loci by restriction landmark genome scanning and showed that the culture conditions can induce DNA methylation instability in the hESC epigenome (Allegrucci *et al*., 2007). The unstable loci have been previously associated with a tumour phenotype. They also showed that the adaptation of the cell lines to serum-free conditions result in additional epigenetic instability (Allegrucci *et al*., 2007). This study highlights the importance of using appropriate techniques to analyze epigenetic changes and evaluate their consequences on hESC experimental utility and biosafety, as well the need for optimization of derivation and culture protocols of hESC in order to minimize culture-induced instability.

In addition to optimized culture methods that prevent the occurrence of genetic instability, novel technologies to purge abnormal hESC from large scale hESC cultures will have to be developed.

Xu *et al* (Xu *et al*., 2001) introduced a feeder free culture system for the first time for hESC culture. This consists of plating the cells on Matrigel, with MEF conditioned KSR medium supplemented with FGF-2 (Amit *et al*., 2004) (Figure 1.2). Matrigel™ Basement Membrane Matrix (BD Biosciences) is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. It contains collagen IV, laminin, heparin sulphate proteolycan and entactin, as well as several growth factors such as FGF-2, insulin-like growth factor-1, nerve growth factor (NGF), transformed growth factor β1 (TGFβ1), epidermal growth factor and platelet-derived growth factor (PDGF). Similarly, hESC can be cultured on Laminin (purified from EHS mouse tumours or from human placenta) (Xu *et al*., 2001) or Fibronectin (extracted from mouse, bovine or human plasma and human foreskin fibroblasts) (Amit *et al*., 2004).

One of the biggest goals of hESC research is to culture the cells in the absence of animal products in order to minimize the risk of contamination with animal pathogens. Another goal is the development of a chemically defined media that could be used to test factors that modulate self-renewal and differentiation, because serum can contain antagonist of those factors. Ludwig and colleagues have reported the development of a feeder-independent hESC culture composed of protein components uniquely derived from recombinant sources or purified from human material which they called TeSR1 (Ludwig *et al*., 2006b). This is a DMEM/F12 based medium supplemented with human serum albumin, FGF-2, LiCl, g-aminobutyric acid (GABA), pipecolic acid and TGFβ. They also derived two new cell lines in this defined media, but they developed chromosomal abnormalities. Because this media was highly costly, the same group have developed a modified version, the mTeSR1, which includes animal sources of protein (bovine serum albumin and Matrigel) and cloned zebrafish FGF-2 (Ludwig *et al*., 2006a).

There are also studies currently being conducted that aim to identify components that support hESC self-renewal, especially the ones secreted by MEF, which will be described later.

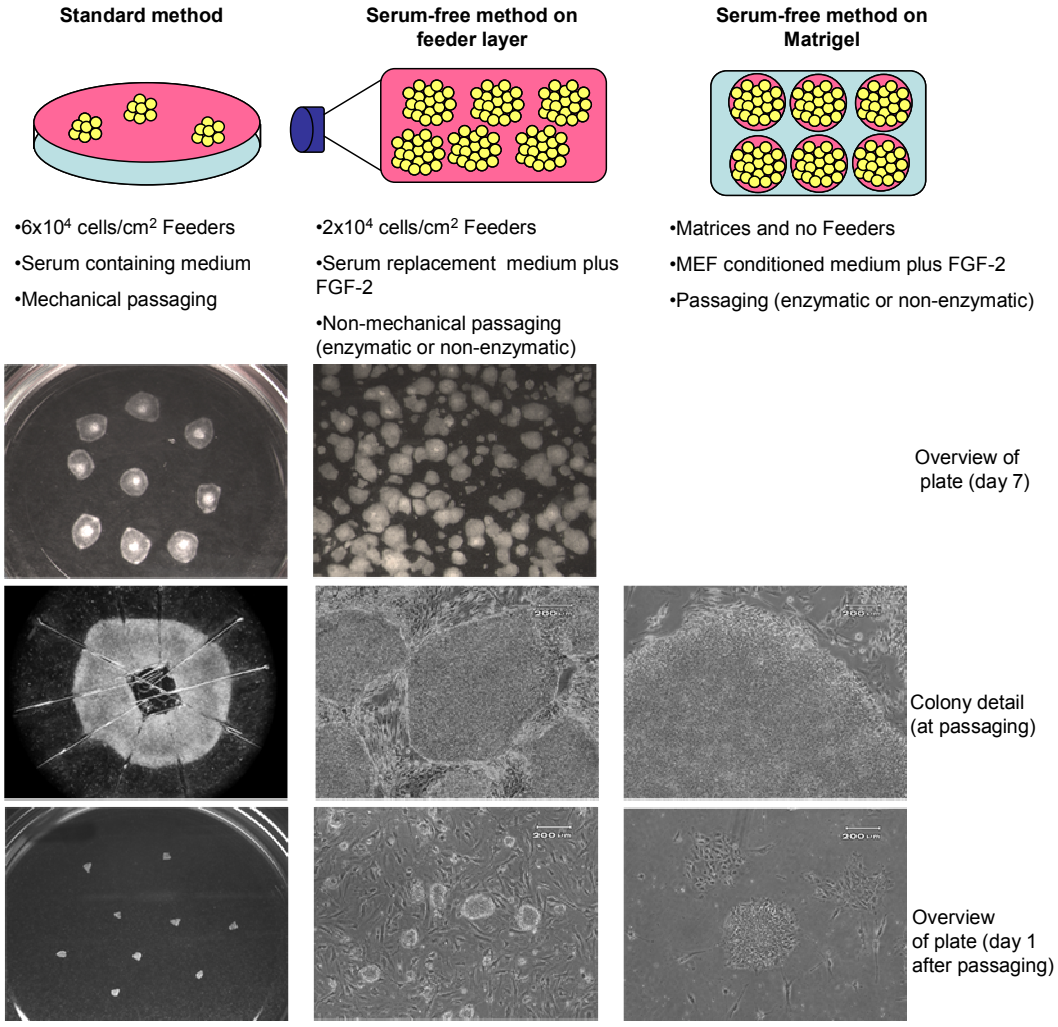

FIGURE 1.2- Different methods of culturing of hESC (adapted from Grandela and Wolvetang, 2007).

1.1.4- Maintenance factors of self-renewal and pluripotency

In order to have a continuous source of hESC for research and therapeutic purposes, it is essential to maintain them for extended periods in their undifferentiated state. ESC self-renewal can be maintained *in vitro* by culturing cells in the presence of specific factors.

1.1.4.1- LIF

LIF, a cytokine that belongs to the IL-6 family, is sufficient to maintain mESC without the presence of feeders or serum containing medium. LIF acts via heterodimerization of two members of class I cytokines, the low affinity LIF receptor (LIFR) and the signal transducer gp130 (Davis *et al*., 1993; Gearing *et al*., 1992) and ultimately activates the Jak/STAT pathway that is sufficient for self-renewal in these cells (Boeuf *et al*., 1997; Matsuda *et al*., 1999; Nakamura *et al*., 1998; Niwa *et al*., 1998). Although the LIFR and gp130 are present in hESC, and there is activation of STAT members in response to recombinant human LIF or mouse LIF (Daheron *et al*., 2004; Humphrey *et al*., 2004), this is not sufficient to maintain hESC in an undifferentiated state.

1.1.4.2- FGF-2

FGF-2, also known as basic fibroblast growth factor (bFGF), is a member of a family of over 20 polypeptide growth factors (Ornitz and Itoh, 2001) FGFs are found in different species ranging from nematodes to humans. The recombinant FGF-2 is a 16.5-kDa peptide with 144 amino acids (Okada-Ban *et al.*, 2000). The biologic activity of FGF-2 is mediated by four related transmembrane tyrosine kinase receptors: FGFR-1, -2, -3, and -4, and possibly other transmembrane receptors (Powers *et al.*, 2000; Khurana and Simons, 2003). Ligand binding results in homodimerization of these receptors, trans-phosphorylation and signal transduction. Numerous splice variants of multiple genes generate a wide diversity of FGFRs. FGF-2 has a pleiotropic effect on different types of cells and its cellular response can be affected by type of receptor binding, rate of intracellular growth factor uptake and interacting molecules inside the cell (Goldfarb, 2001). FGF-2 can act as a survival factor by blocking apoptosis (Fisher, 1997; Stachowiak *et al.*, 1997), it also stimulates angiogenesis (Slavin, 1995; Bikfalvi, 1995) and cell proliferation *via* activation of the Ras/Raf-MAPK pathway by enabling adaptor proteins Grb2, Shc, and Nck to participate in signal transduction cascades (Klein and Schneider, 1997). It can also promote tumor progression (Bikfalvi, 1995; Vacca *et al.*, 2001). FGF-2 was been used in hESC culture (Xu *et al.,* 2001; Amit *et al.,* 2004) in serum- and feeder-free culture methods because it inhibits hESC differentiation. Two studies were published that show that higher doses of FGF-2 (100 and 40 ng/ml) override the requirement for MEF feeders or its conditioned medium on maintenance of hESC and this effect is mediated by the inhibition of BMP signaling (Xu *et al.*, 2005a; Xu *et al.*, 2005b; Levenstein *et al.*, 2006).

Bendall and colleagues have found that under feeder-free culture conditions, the hESC organize themselves in order to create a niche in which derived hESC derived fibroblast-like cells are dependent on FGF-2 and in response to this factor express IGF-1 reported to promote self-renewal of undifferentiated hESC (Bendall *et al.*, 2007).

1.1.4.3- Transforming growth factor β (TGFβ) pathway

TGFβ superfamily members play an important role in a wide range of cellular processes including tissue differentiation, morphogenesis, proliferation, migration and apoptosis in embryonic development, as well in adult homeostasis and response to disease and injury (Massague, 1990). The TGFβ superfamily of ligands include TGFβ, Activin, Nodal, Bone morphogenetic proteins (BMP) and Growth/Differentiation factors (GDF), among others. All of these have been associated with ESC (Valdimarsdottir and Mummery, 2005). These ligands bind to heteromeric complex of serine/threonine kinase receptors called TGFβ type I and type II receptors. The type I receptor acts downstream of the type II receptor and transduces the signal through the phosphorylation of Smads (Massague, 2000; Piek *et al.*, 1999), wich play theirs role in the nucleus acting as transcription factors or target genes.

It has been shown that maintenance of hESC in an undifferentiated state requires the action of the TGFβ/Nodal/Activin branch of the TGFβ superfamily (Amit *et al.*, 2004; Ludwig *et al.*, 2006b; Vallier *et al.*, 2005; Vallier *et al.*, 2004). It has been suggested that TGFβ1 prevents differentiation

along the primitive endoderm lineage (Poon *et al*., 2006). On the other hand, BMP signaling needs to be repressed to prevent differentiation into to primitive endoderm or along the trophoblast lineage (Pera *et al*., 2004; Xu *et al*., 2002c). While TGF, Activin, and Nodal bind to a set of receptors that stimulates the activation of SMAD 2/3, BMP4 binds to different receptors and activates the SMAD 1/5/8 signaling (Valdimarsdottir and Mummery, 2005).

Culture medium supplemented with Activin A can maintain hESC in the undifferentiated state for over 20 passages without the need for feeder layers or conditioned medium from MEF (Beattie *et al., 2005*; Xiao *et al.*, 2006). The bone morphogenetic protein antagonist, Noggin, in combination with FGF-2, was shown to maintain the pluripotency of hESC in the absence of feeder layers (Wang *et al*., 2005). These three growth factors are all expressed in MEF, suggesting that they may be important factors secreted by MEF for maintaining undifferentiated hESC.

1.1.4.4- MEF secreted factors

A great deal of research is focusing on the identification of factors that are secreted by MEF. Conditioned medium from MEF is sufficient for the maintenance of hESC on Matrigel. This suggests that secreted factors produced by MEFs can activate the molecular programs required for maintaining hESC in an undifferentiated state. The identification of these factors, signaling pathways and downstream target genes is fundamental for the future of hESC research, especially because the ultimate goal is to produce chemically defined and xeno-free media to culture these cells. A genome-wide expression profiling performed by Greber and colleagues revealed that FGF-2 acts on MEF to regulates the expression of key members of the TGFβ pathway (upregulates *Inhba*, *Tgfb1*, *Grem1*, and downregulates *Bmp4*) that are most likely to influence hESC self-renewal (Greber *et al*., 2007). They also showed that FGF-2 has similar effect on hESC, suggesting that TGFβ ligands act in an autocrine manner.

1.1.4.5- Other factors

Another study claimed that activation of the canonical Wnt pathway is sufficient to maintain self-renewal of both hESC and mESC and this was achieved by using a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), 6-bromoindirubin-3'-oxime (BIO) (Sato *et al*., 2004). However, data reported by others (Dravid *et al*., 2005) and in our laboratory (Kathryn Davidson, personal communication) do not support the findings claimed by Sato and colleagues. Wnts seem to promote proliferation and inhibition of apoptosis of hESC rather than promoting self-renewal.

Pebay and colleagues (Pebay *et al*., 2005) have also reported a system for the maintenance of stem cells. They have shown that sphingosine-1-phosphate and PDGF, two known mitogens components of serum, can maintain hESC self-renewal in a serum-free culture medium but still in the presence of feeder layers. They suggested that this is mediated by activation of the extracellular signal-regulated kinases (ERKs).

In summary, different pathways seem to be required for ESC maintenance, proliferation and repression of differentiation.

1.1.5- Strategies to improve clonal survival

hESC are very difficult to clone and frequently die or differentiate when dissociated to single cells or small clumps. This limits the application of the most useful genetic techniques. In order to solve this problem some groups are developing strategies to improve clonal survival.

In 2006, Peter Donovan's group reported that hESC express receptors of tropomyosin-related kinases (TRK) family, which are mediators of antiapoptotic signals. They further showed that three TRK ligands, brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4, mediate hESC survival trough the phosphatidylinositol-3-kinase pathway (Pyle *et al*., 2006) for 15-20 passages. More recently, has been reported that Y-27632, an inhibitor of a selective Rho-associated kinase also known as ROCK inhibitor, when supplemented to a serum-free media to increases cloning efficiency of hES cells from 1% to 27% by inhibiting dissociation-induced apoptosis (Watanabe *et al*., 2007).

1.1.6- Cell cycle of hESC

When in culture the majority of mammalian cells undergo a limited number of cell divisions until they reach a state of irreversible proliferation, called replicative senescence (Hayflick and Moorhead, 1961). The number of division that the cells undergo before they senescence is called the Hayflick limit and this is dependent on the cell type or species. This limit suggests that there is something like a mitotic clock regulating the number of divisions. Loss of genomic and mitochondrial genomic integrity, epigenetic alterations, oxidative damage, progressive loss of DNA repair ability and erosion of telomeres have been implicated in senescence. hESC (Thomson *et al*., 1998), as well as transformed cells, have bypassed senescence and appear to be immortal in culture. ESC also display constitutive replication, which is a common of aspect of early embryo development in many species. This may reflect the requirement of establishing sufficient cell numbers to initiate gastrulation (Burdon *et al*., 2002).

The cell cycle of hESC still has the four cell cycle phases G1, S, G2, and M, however the duration of G1 is dramatically shortened (Becker *et al*., 2006). By measuring Bromodeoxyuridine (BrdU) incorporation and subsequent fluorescence-activated cell sorting (FACS) analysis this study was able to show that 65% of asynchronously growing human ES cells are in S phase. The duration of the S is approximately 8 h, G2 approximately 4 h, and M phases approximately 1 h are similar in ES and somatic cells, but G1 phase has a duration of only 2.5-3 h that is significantly shorter than what is commonly observed for somatic cells (Becker *et al*., 2006).

In the case of mESC, Burdon and colleagues have suggested that G1 control pathways that operate in other types of cell are reduced or absent in ESC and that the uncoupling from G1 regulation might be involved in sustaining the undifferentiated state cells possibly by constraining the temporal opportunity for both chromatin remodeling and the establishment of heritable transcription programs (Burdon *et al*., 2002).

1.1.7- Epigenetics

Epigenetic modifications, such as autosomal imprinting and X-chromosome inactivation, are crucial for proper development and cell fate determination. A number of genetic diseases and cancers have been associated with loss of imprinting.

Additionally, epigenetic mechanisms contribute to the repression of inappropriate developmental programs in time and space while ensuring heritability of existing or newly acquired phenotypic states. Several studies have been done to evaluate the epigenetic status of mESC and hESC, including the assessment of the abundance of modified histones and enzymes responsible for modifications, Polycomb group (PcG) protein binding patterns, replication timing and chromatin accessibility (reviewed by Spivakov and Fisher, 2007).

Determining how these epigenetic features relate to the transcriptional signatures of ESC, and whether they are also important in other types of stem cell, is a key challenge for the future (Spivakov and Fisher, 2007).

Rugg-Gunn PJ and colleagues (Rugg-Gunn *et al*., 2005) examined the allele-specific expression of six imprinted genes and the methylation profiles of three imprinting control regions. They identified generally monoallelic gene expression and normal methylation patterns. During prolonged passage, one cell line became biallelic with respect to H19, but without loss of the gametic methylation imprint. The authors of this study claimed a significant degree of epigenetic stability in hESC (Rugg-Gunn PJ *et al*., 2005).

As mentioned before, a more recent study has reported DNA methylation instability in hESC epigenome induced by culture conditions (Allegrucci *et al*., 2007) and that the unstable loci have been previously been associated with tumour phenotype.

1.1.8- hESC signature and gene expression profiling

In order to define the gene expression signature of undifferentiated hESC, to compare this signature between different stem cell lines and to shed more light on the common regulatory networks that are operational in ESC, a large number of high throughput studies have been performed on these cells. Serial analysis of gene expression, massively parallel signature sequencing, transcriptional profile analysis by microarrays and proteomic approaches are the most commonly carried out.

Many gene expression profiling studies have been performed on hESC. These microarray studies have revealed that hESC are enriched for genes that encode for certain transcription factors (for example, Oct4, Nanog, Sox2, FoxD3, Rex1, SRY), DNA modifying enzymes (for example, TERF1, CHK2, DNMT3), surface markers (for example, connexin43), growth/differentiation factors (FGF-2, CER1, GDF3), receptors (FGFR1, FGFR2, TDGF1, FZD5, FZD), and genes involved in the control of the cell cycle, apoptosis, and DNA repair, among others with known and unknown function (Abeyta *et al*., 2004; Bhattacharya *et al*., 2005; Bhattacharya *et al*., 2004; Ginis *et al*., 2004; Rao *et al.,* 2004; Richards *et al*., 2004).

Ginis and colleagues (Ginis *et al.,* 2004) compared gene expression profiles of hESC and mESC by immunocytochemistry, reverse-transcriptase polymerase chain reaction (RT-PCR), and

membrane-based focused cDNA array analysis. Many genes were found to be commonly expressed in ESC of these two species. Genes that showed differences in their expression were vimentin, beta-III tubulin, alpha-fetoprotein, eomesodermin, HEB, ARNT, and FoxD3 as well as the LIFR complex LIFR/gp130. Differences in genes related to cell cycle regulation, control of apoptosis, and cytokine expression were also found. The profile of gene expression observed in H1 cells was similar to that of two other hESC lines tested (line I-6 and clonal line-H9.2) and to feeder-free subclones of H1, H7, and H9. This indicates that differences between hESC and mESC in terms of pluripotency marker expression were species-specific and not due to differences in culture conditions.

One of the limitations/problems of most of microarray studies performed on hESC is the fact that hESC are analyzed as if they were a homogenous population. Because such populations include both undifferentiated and differentiated cells, microarray analysis will reflect this heterogeneity. Enver and colleagues developed the first microarray analysis using Affymetrix analysis on hESC sorted by flow cytometry based on presence or absence of the cell surface glycoprotein SSEA3 (Enver *et al*., 2005). The found that 468 genes were uniquely expressed in normal SSEA3+ and proposed a cellular differentiation hierarchy for HESC culture maintenance: normal SSEA3+ cells represent pluripotent stem cells while normal SSEA3- cells have exited this compartment, but retain multilineage differentiation potential. As mentioned before Laslett and colleagues also took heterogeneity in account and went further in dividing hESC into more subpopulations based on their immunoprofile (GCTM-2 and CD9 immunostaining) and performed their transcriptional analysis on a Compugen platform (Laslett *et al*., 2007). They divided the population in 4 subpopulation expressing different levels of antigens mentioned above (P7, expresses the highest level of stem cells antigens, P6, P5 and P4, negative cells for cell surface markers) and showed that these subpopulations exhibit different gene expression profiles. Comparing this study to the first mentioned, the greatest overlap observed was between SSEA3+ versus SSEA3- and P7 versus P4.

1.1.9- International Stem Cell Initiative: an attempt to compare different hESC cell lines

The International Stem Cell Initiative is an international consortium that is comparing the properties of 75 hESC from laboratories around the world (Andrews *et al*., 2005).

The characterization studies included flow cytometry analysis of cell surface markers, reverse transcriptase polymerase chain reaction (RT-PCR) of genes characteristic of hESC and their early differentiation derivatives, as well as to investigate the change on gene expression during embryonic body formation, a simple differentiation protocol. The results of these collaborative studies were recently published. They reported that 59 hESC lines from 17 laboratories worldwide, despite diverse genotypes and different techniques used for derivation and maintenance, exhibited similar expression patterns for several markers (such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM2, GCT343, CD9, Thy1, tissue-nonspecific alkaline phosphatase, class 1 HLA, Nanog, Oct4, TDGF1, DNMT3B, GABRB3 and GDF3). However, the lines showed differences in expression of several lineage markers and although several imprinted genes showed generally

similar allele specific expression patterns, some gene-dependent variation was observed. In addition, some female lines expressed readily detectable levels of XIST while others did not. Fortunately, the study did not detect contamination of the lines with mycoplasma, bacteria or cytopathic viruses.

1.1.10- *In vitro* Differentiation of hESC

There are many studies demonstrating that hESC can differentiate into multiple cell lineages (Table 1.2). These protocols involve either spontaneous differentiation, mainly through the formation of embryoid-bodies (EB), directed differentiation using growth factors and/or extracellular matrices, or even co-culture with other cell types. hESC have been shown to be able differentiate into cell types of ectodermal, endodermal, mesodermal and extraembryonic lineages. EB formation was the first method used to induce differentiation in ECC (Martin and Evans, 1975) and mimics the process of formation of germ layers from the ICM. Other protocols explore the role of known morphogens implicated in embryonic development, such as BMP4, Activin (at higher concentrations than the reported to promote maintenance of hESC), Retinoic acid (RA) and Wnt.

TABLE 1.2- Published differentiation of hESC (Adapted from Hoffman and Carpenter, 2005)

Cell lineage	Cell type	Cell line	Note	Reference
General differentiation				
	Endoderm, ectoderm and mesoderm derivatives	H9.1	Formation of EBs followed by treatment with growth factor and analysis of expression of mRNA	(Schuldiner *et al.*, 2000)
		H9	Expression of several markers by PCR and immunostaining	(Itskovitz-Eldor *et al.*, 2000)
		H13	Gene expression profile by microarray during differentiation	(Dvash *et al.*, 2004)
Ectoderm				
	Neurons	H9	RA and NGF neuronal differentiation	(Schuldiner *et al.*, 2001)
	Neurons and glia	H1,H7,H9	Differentiation into enriched populations of functional neurons and neural progenitors	(Carpenter *et al.*, 2001)
	Neurons and glia	H1,H9, H9.2	Differentiation into neural progenitors, neurons and glia. Transplantation into neonatal rats	(Zhang *et al.*, 2001)
	Neurons and glia	HES-1	Differentiation into neural progenitors, neurons and glia. Transplantation into neonatal rats	(Reubinoff *et al.*, 2001)
	Neurons	BG01, BG02	Differentiation into neural precursors and neurons	(Schulz *et al.*, 2003)
	Neurons	HES-1	Differentiation into dopaminergic neurons Transplantation into Parkinsonian rats, resulting in functional improvement	(Ben-Hur *et al.*, 2004)
	Neural precursors	HES-2, HES-3	Differentiation into neural precursors	(Pera *et al.*, 2004)
	Neurons	H1, H9, HES-3	Differentiation into midbrain dopaminergic neurons	(Perrier *et al.*, 2004)
	Neurons	BG01, BG03	Differentiation into dopaminergic neurons	(Schulz *et al.*, 2004)
	Neurons	BG01	Differentiation into dopaminergic neurons	(Zeng *et al.*, 2004)
	Neurons	MB03	Differentiation into dopaminergic neurons	(Park *et al.*, 2004)
	Neurons	H1,H9	Differentiation into motoneurons neurons	(Li *et al.*, 2005)
	Oligodendrocytes	H7	Differentiation into enriched populations of oligodendrocytes and myelination after transplantation	(Nistor *et al.*, 2005)
Endoderm				
	Insulin positive cells	H9	Direct differentiation	(Assady *et al.*, 2001)
	Insulin-producing cells	H9, H9.2, H13, I6	Direct differentiation	(Segev *et al.*, 2004)
	Hepatocyte-like cells	H1, H9	Direct differentiation	(Rambhatla *et al.*, 2003)
	Hepatocyte-like cells	N/A	Direct differentiation	(Lavon *et al.*, 2004)
Mesoderm				
	Cardiomyocytes	H9.2	Evaluation of structural and functional properties	(Kehat *et al.*, 2001)
	Cardiomyocytes	H9.2	High resolution electrophysiology	(Kehat *et al.*, 2002)
	Cardiomyocytes	H1,H7,H9	Differentiation into enriched populations of functional cardiomyocytes	(Xu *et al.*, 2002a)
	Cardiomyocytes	HES-2	Differentiation into cardiomyocytes and their characterization	(Mummery *et al.*, 2002), (Kehat *et al.*, 2003)
	Cardiomyocytes	H1,H7,H9, H14 14	Evaluation of electrophysiological and pharmacological properties	(He *et al.*, 2003)
	Cardiomyocytes	HES-2	Differentiation into cardiomyocytes by co-culture with END-2 cells	(Mummery *et al.*, 2003)

Cardiomyocytes	H9.2	Evaluation of ultrastructural and proliferative characteristics	(Snir *et al.*, 2003)
Cardiomyocytes	H9.2	Evaluation of electrophysiological and pharmacological properties	(Satin *et al.*, 2004)
Cardiomyocytes	H1	Demonstration of functional integration after transplantation into guinea pig model	(Xue *et al.*, 2005)
Hematopoietic colony-forming cells	H1, H1.1, H9.2	Differentiation of multipotent hematopoietic precursors	(Kaufman *et al.*, 2001)
Hematopoietic progenitor cells	H1, H9	Differentiation of multipotent $CD45^+$ hematopoietic precursors	(Chadwick *et al.*, 2003)
Hematopoietic progenitor cells	H1, H9	Differentiation of multipotent $CD34^+$ hematopoietic precursors	(Vodyanik *et al.*, 2005)
Hemangioblasts	H1, H7, H9, MA01, MA03, MA40 and MA09)	Differentiation into multiple hematopoietic lineages and endothelial cells. Transplantation into rats and mice injured models, resulting in vascular repair	(Lu *et al.*, 2007)
Leucocytes	H1	Differentiation into antige-presenting leucocytes	(Zhan *et al.*, 2004)
Endothelial cells	H9	Isolation and characterization of hESC-derived endothelial cells	(Levenberg *et al.*, 2002)
Endothelial	H1, H9	Identification of primitive endothelial-like cells that generate endothelial and hematopoietic cells	(Wang *et al.*, 2004)
Endothelial	H9.2	Evaluation of endothelial markers during spontaneous differentiation	(Gerecht-Nir *et al.*, 2005)
Vasculogenesis	H9.2, H13	Vasculogenesis using alginate scaffolding	(Gerecht-Nir *et al.*, 2004b)
Vasculogenesis	H9.2, H13, I6	Vasculogenesis in teratomas	(Gerecht-Nir *et al.*, 2004a)
Extraembryonic			
Trophoblast	H1, H7, H9, H14	Differentiation into trophoblasts	(Xu *et al.*, 2002c)
Trophoblast	H1	Identification of trophoblast cells in EB	(Gerami-Naini *et al.*, 2004)
Germ cells			
Germ cells	HSF-1, HSF-6, H9	Spontaneous differentiation of stem cells	(Clark *et al.*, 2004)

As seen from studies above, hESC have been vigorously investigated as a source for cell replacement therapies, and it is apparent that they can differentiate into a wide range of specific cell types. In order to deliver on their promise, large amounts of high quality hESC will be needed that are genetically stable, free of animal products and manipulated to evade the immune system through either somatic cell nuclear transfer or other technologies. A deeper understanding of the molecular mechanisms that control the genetic stability of hESC is required.

1.2- GENETIC STABILITY: HESC AS A MODEL

Arguably the ES/EC cell pair, and the phenomenon of culture adaptation of ES cells, provides a rare, if not the only, circumstance in which one can readily access the normal counterpart of a tumour stem cell and observe progressive changes as the 'normal' stem cell converts to a malignant state (Baker *et al*., 2007).

Although the original reports on hESC indicated that they can maintain a normal diploid karyotype during long term propagation *in vitro*, there is evidence that this depends on culture conditions. Different laboratories have reported that hESC cultured for long periods of time tend to gain chromosomal abnormalities, resembling the ones observed in hECC (reviewed by Grandela and Wolvetang, 2007). The most frequently observed karyotypic abnormalities are gain of chromosome 17q, 12 and the X chromosome (Brimble *et al*., 2004; Draper *et al*., 2004; Mitalipova *et al*., 2005), while there also seems to be a high proportion of partial or total chromosome 1 duplication. It has been postulated that increased dosage of those chromosomes might give the cells some sort of selective advantage.

Draper and colleagues (Draper *et al*., 2004) were the first to report gain of chromosome 17q and chromosome 12 in H7 and H14 cell lines cultured with KSR (Invitrogen/GIBCO) instead of FCS and passaged with an enzymatic method (Collagenase IV). They performed interphase fluorescence in situ hybridization (FISH) and showed that after 22 passages trisomy 17 was observed in 76% of the cells and after an additional 17 passages, in 95% of the cells, again indicating that such cells possess enhanced survival or proliferation properties. Buzzard and colleagues (Buzzard *et al*., 2004) cultured six NIH-registered hESC lines (hES1-6) between 34 and 140 passages using mechanical method of passaging cells rather than enzymatic or chemical methods of cell dissociation. They reported just one karyotype abnormality was detected in an early passage hES5, demonstrating that hESC are not necessarily predisposed to karyotypic abnormalities even grown for extended time. Another group studied three cell lines (BG01, BG02 and BG03) and showed that karyotypic changes also give rise to differences in expression of genes, especially those involved in pluripotency (Mitalipova *et al*., 2005). They reported that bulk passaging (nonenzymatic- Cell Dissociation Buffer- or enzymatic- colagenase, trypsin) methods can compromise the genetic stability of hESC, in contrast to manual methods.

Because classical G-band karyotyping can only give a rough idea of chromosomal stability, techniques with higher resolution should be used to detect smaller chromosomal and gene alterations/mutations. Other studies have attempted to overcome that problem by using techniques, like SNP, to track down these alterations on hESC, especially those that are cultured for long periods of time. Maitra and colleagues (Maitra *et al*., 2005) compared nine hESC lines (BG01, BG02, BG03, H1, H7, H9, HES-2, HES-3, SA001 and SA002) derived and cultured in different laboratories at both early and late passage, and found the former contain one or more genomic alterations commonly observed in human cancers, mitochondrial DNA sequence mutations and gene promoter methylation. Enver *et al* (2005) investigated the adaptation process by comparing early passage (normal) and late passage (adapted) sublines of H7. The karyotype

of early passage cells was normal, whereas the adapted showed an extra copy of chromosome 1 and chromosome 17q amplification. A slightly higher proportion of SSEA-3+ cells were found in the adapted cultures as compared to the normal ones (83 and 64%, respectively). This group also showed X-inactivation in adapted cells suggesting that epigenetic changes are also associated with adaptation. Thus the mechanism of adaptation may be similar to the early events involved in oncogenesis and tumour progression. Baker and colleagues (Baker *et al*., 2007) have made an attempt to link culture adaptation and malignancy. In agreement with other laboratories they also observed a nonrandom gain of certain regions in the three mainly affected chromosomes in hESC (12, 17 and X). They hypothesized that these chromosomes may contain candidate genes that could be important for the observed culture adaptation and/or play a role in stem cell maintenance *in vitro* and cell tumorogenesis *in vivo*. One of these candidate genes present in terminal half of q arm of chromosome 17 is BIRC5, also known as Survivin. Survivin can act as an antiapoptotic gene and also plays a role in chromosome segregation and is expressed in a large number of tumour types such as neuroblastomas and associated with highest-risk tumours and poor survival outcome. Gain of chromosome 12 was also frequently observed in both hESC and testicular germ cell tumours (TGCT). Nanog, a pluripotency gene, is located on 12p13 and overexpression of this gene promotes self-renewal and prevents differentiation and thus providing hESC with an anti-differentiation and proliferative advantage in culture. Another amplified region on chromosome 12, 12p11.2-12 that is also frequently amplifies in TGCT harbours the oncogene K-RAS and SOX5, a gene involved in cell fate determination. Reduced apoptosis has been observed in malignant cells with amplification of this genomic region (Roelofs *et al.,* 2000), and it is possible that amplification of this region may confer a similar survival advantage to hESC. Multiple studies have found that hESC acquire additional copies of chromosome X and often TGCT have more than one X chromosome. Enver *et al* further identified ELK1, A-RAF, the androgen receptor and its interacting protein NONO, as potential candidate genes on X that could be involved in hESC adaptation. The non random gain of chromosomes 12, 17 and X observed in different hESC lines in different laboratories around the world lends support to the hypothesis that there may be genes on these chromosomes that confer a growth or anti-differentiation advantage to hESC in culture. Generating small clumps or single cells from hESC colonies in general leads to apoptosis and differentiation. The fact these abnormalities occur more frequently in bulk cultured cells suggests that the seeding of small clumps of hESC puts selective pressure on hESC that favors hESC with such genetic abnormalities. In this context increased expression of genes that inhibit apoptosis and differentiation of hESC, such as CD30, a biomarker for transformed human pluripotent stem cells, (Herszfeld *et al*., 2006), prior to overt genetic abnormalities may increase the likelihood of acquiring further abnormalities.

Because of their pre-existing capacity for self-renewal and unlimited replication, stem cells are also appealing candidates as the “cell of origin” for cancer (Beachy *et al*., 2004; Reya *et al*., 2001). Investigating the adaptation process by which cells go from normal to transformed can give insights to mechanisms responsible for the development of cancers. However, it is still to be addressed experimentally if a mutation or other genetic or epigenetic change that gives a

selective advantage to undifferentiated hESC will have an effect on the behavior of their differentiated progeny (Baker *et al*., 2007).

If one wants to limit the appearance of abnormal hESC within the culture it is crucial to investigate the response of hESC to genomic insults or DNA damage. In response to DNA damage various mechanisms can be activated: DNA repair, transcriptional response, DNA damage checkpoints with cell cycle arrest, or if the damage is irreparable, apoptosis. This study will focus mainly on apoptosis.

1.3- APOPTOSIS

Programmed cell death is critical for development and homeostasis in metazoans
(Vaux and Korsmeyer, 1999).

The phenomenon of apoptosis was first described by Carl Vogt in 1842, and its name, introduced by Kerr, Wyllie and Currie in 1972, is derived from the ancient Greek meaning the ‘‘falling off of petals from a flower’’ or ‘‘shedding of leaves from a tree in autumn’’. This term was used to describe a common type of programmed cell death that was observed in various tissues and cell types. The dying cells shared many morphological features, that were distinct from the features observed in cells undergoing necrotic cell death, and this could be a result of common and conserved endogenous cell death programme. Apoptosis is a form of caspase-mediated cell death with particular morphological features and usually does not lead to inflammation (Fink and Cookson, 2005). Unlike apoptosis, necrosis denotes a form of cell death usually accompanied by inflammation (Savill, 1997), is not energy dependent, does not involve caspase activity or gene regulation.Table 1.3 shows differences these two processes.

TABLE 1.3- Comparison between apoptosis and necrosis (Adapted from Academic Pathology, Queen's University Belfast, 2003)

Apoptosis	Necrosis
Physiological or pathological	Always pathological
Energy dependent	Energy independent
Cell shrinkage	Cell swelling
Membrane integrity maintained	Membrane integrity lost
Characteristic nuclear changes	Nuclei lost
Apoptotic bodies form	Do not form
DNA cleavage	No DNA cleavage
Evolutionarily conserved	Not conserved
Dead cells ingested by neighbouring cells	Dead cells ingested by neutrophils and macrophages

Apoptosis should not be used synonymously with programmed cell death (PCD), which can occur via apoptosis, but is rather used to identify a specific morphology of cell death. The term PCD refers to time- and position-programmed cell death during development of an organism. However, apoptosis can be induced by anti-cancer drugs, toxins and other types of stresses (Lawen, 2003). One of the main reasons for the interest in this process is due to its involvement in many diseases, such as neurodegenerative and autoimmune diseases (if it occurs too much) and cancer (if it occurs too little).

1.3.1- Apoptosis in development and homeostasis

During animal development, there are numerous structures or specific populations of cells that are formed that are later removed by apoptosis. This allows greater flexibility because primordial structures can be adapted for different functions at various stages of life or in different sexes (Meier *et al*., 2000). A classical example of this is the development of the Müllerian duct (that gives rise to the uterus and oviduct in females) that is removed in males, and the Wolffian duct (that gives rise to the male reproductive organs) that is deleted in females. Other examples are

cavitation, neural tube and kidney formation, mammary gland development and the separation of the digits.

In the adult a balance between the number of cells that divide and die must be kept relatively constant in order to maintain homeostasis. Furthermore, cells need to be replaced when they are malfunctioning or eliminated if they can potentially become tumorigenic.

During early human development a high degree of genetic abnormality occurs, since the majority of first trimester miscarriages are attributed to chromosomal defects (Metcalfe *et al*., 2004). Preimplantation embryos undergo apoptosis at the blastocyst stage, primarily in the ICM. As many as 7-8% of these cells undergo apoptosis at any one time (Hardy *et al*., 1989) and coupled with the observation that aneuploid cells can be present in the human ICM (Evsikov and Verlinsky, 1998), this emphasizes the potential role of apoptosis in eliminating genomic damage from foetal development. Dead ICM cells usually display typical morphological features of apoptosis and are adequately phagocytozed (Fabian *et al*., 2005). Furthermore, the incidence of apoptosis in *in vitro* produced embryos can be increased in suboptimal culture conditions, resulting for example from inappropriate composition of culture medium or inappropriate culture density of embryos. Although apoptosis may be necessary to eliminate genetic damage, it must also be tightly regulated in order to prevent inappropriate loss of normal cells.

1.3.2- Apoptosis in ESC

DNA damage response and cell-cycle regulation differ markedly between mESC and somatic cells (Hong and Stambrook, 2004). Spontaneous mutation frequency in mouse somatic cells is approximately 100-fold higher than in mESC cells. mESC cells also lack a G1 checkpoint as mentioned before and are hypersensitive to ionizing radiation and other DNA-damaging agents (Hong and Stambrook, 2004). These characteristics facilitate apoptosis and the removal of cells with a mutational burden from the population.

Sumi and colleagues using an inducible system to promote the sustained activation of proto-oncogene c-myc found that this results in apoptosis and differentiation into extraembryonic lineages of hESC (Sumi *et al*., 2007). These effects were transcription dependent, but blocking caspase activity or knocking-down p53 were not sufficient to prevent induction of differentiation markers by c-myc activation.

It is anticipated that a detailed understanding of apoptosis pathways in hESCs will be useful for future cell replacement therapies using hESCs since it may allow for the selective elimination of unwanted cell lineages prior or subsequent to lineage specific differentiation. An example of the usefulness of such information apoptosis was demonstrated by Bieberich and colleagues (2004) in mice, who showed that ceramide-induced apoptosis eliminates residual pluripotent ESC, prevents teratoma formation, and enriches the embryoid body-derived cells for cells that undergo neural differentiation after transplantation (Bieberich *et al*., 2004).

During transplantation of ESC derivatives, most of the cells die, restricting the contribution of the graft to recovery in injury or disease models. A study performed by Wei and colleagues (Wei *et al*., 2005) circumvented this limitation by overexpressing the anti-apoptotic gene *Bcl2* in mESC. The

cells were then differentiated with RA down to neural lineages and transplanted into the post-infract brain of adult rats. After transplantation, ESC derived cells with the *Bcl2* gene showed increased expression markers for neural cells, and the survival of these cells and functional recovery of ischaemic rats that received them was also increased. There was no formation of tumours, possibly because overexpression of Bcl2 did not completely prevent apoptosis in the host brain.

Another problem is the low survival of hESC that are frozen using conventional slow-cooling cryopreservation protocols. They die predominantly by apoptosis rather than necrosis (Heng *et al*., 2006). A study used the caspase inhibitor zVAD-fmk to enhance the survival rate of these cells after thawing 2007 from 9.9% to 18.7% and this was not accompanied by a significant enhancement in spontaneous differentiation of hESC (Heng *et al*., 2007).

There is little published work on apoptotic signaling pathways in hESC and no information on the molecular mechanism of DNA damage-induced apoptosis in these cells prior to the body of work presented in this thesis.

1.3.3- Major molecular regulators of apoptosis

1.3.3.1- Bcl2 family

The Bcl2 family of proteins are the central regulators of caspase activation, and its anti- and pro-apoptotic members are key arbiters of the life-or-death decision (Cory and Adams, 2002). The first member to be identified was Bcl2 at the chromosomal breakpoint of t(14;18) bearing human follicular B cell lymphoma (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985). Later it was shown that this proto-oncogene, instead of promoting cell proliferation, inhibits apoptosis following physiological and pathological stimuli (McDonnell *et al.*, 1989; Vaux *et al.*, 1988).

In mammals, the Bcl2 family has at least 20 members which share at least one conserved Bcl2 homology (BH) domain. The anti-apoptotic members are Bcl-xL, Bcl-w, A1 and Mcl1, and these possess four conserved Bcl2 homology domains, BH1 through BH4. The pro-apoptotic proteins are divided in two categories: multidomain or BH1–3, containing BH1, BH2, and BH3 regions (Bax, Bak) and BH3-only proteins (PUMA, Noxa, Bnip3, Bik, Bim, Bad, Bmf, and Hrk) (Kuwana *et al*., 2005). Both types of pro-apoptotic proteins are required to initiate apoptosis. Some groups have classified the BH3 only proteins in 2 groups: the direct activators and derepressors. The direct activator BH3 peptides (like Bid and Bim) can directly activate Bax and sequester anti-apoptotic family members, were the only ones to induce direct cytochrome c release from the mitochondria. The derepressor BH3 peptides, which cannot activate Bax directly, just have the ability to sequester anti-apoptotic family members (Kuwana *et al*., 2005).

Similar to their role in the blastocyst stage of the embryo, members of Bcl2 family are essential for the survival of both adult and embryonic stem cells. In hematopoietic stem cells (HSC), growth factors including stem cell factor increased transcription of the Mcl1 gene and Mcl1 was required to increase survival of purified bone marrow progenitors (Opferman *et al*., 2005). In fact, Mcl1 is

the most abundantly and consistently expressed Bcl2 family member detected in the preimplantation embryo (Jurisicova and Acton, 2004) and is the only pro-survival Bcl2 family member whose disruption results in preimplantation embryonic lethality (Rinkenberger *et al*., 2000). Although Bcl2 is not usually express in mESC, LIF and Bcl2 overexpression are sufficient to expand these cells *in vitro* under serum- and feeder-free conditions (Yamane *et al*., 2005). The antiapoptotic effect of Bcl2 allows cells to survive and, because no serum or feeders are present, it is possible to assess the effect of single growth factors, cytokines, and small molecules on the proliferation and differentiation of ESC. The authors of this study also suggest that Bcl2 possible blocks ERK signaling pathway, which in mESC leads to differentiation.

1.3.3.2- Caspases

Caspases are a family of evolutionarily conserved cysteinyl proteases that are involved both in apoptosis and inflammation through aspartate-specific cleavage of a wide range of cellular substrates (Lamkanfi *et al*., 2007). During the apoptotic process, caspases are associated with cellular dismantling, whereas in inflammation they mediate the proteolytic activation of inflammatory cytokines. In mammals, at least 15 members of the caspase family have been identified (Martinon and Tschopp, 2004). The first caspase identified was ICE, which proteolytically activates the proform of the cytokine interleukin (IL)-1β to its active form (Cerretti *et al*., 1992). A later study has shown that it can also activate IL-18 (Akita *et al*., 1997). Other studies, mainly through exhaustive functional analysis of conditional caspase-deficient mice or derived cells, have suggested a broader role for these proteases such has cell differentiation, proliferation and NF-κB activation (reviewed by Lamkanfi *et al*., 2007).

Caspases can be categorized in initiator and effector caspases and these have distinct structural domains. Initiator caspases have prodomains that interact with other molecules to aggregate caspases into homo- or heterophilic polymers required for caspase activation. Two such prodomains are the caspase activation and recruitment domain (CARD) and the death effector domain (DED) (Zheng *et al*., 2000). DEDs consist of six or seven anti-parallel α-helices that provide homotypic interaction required to recruit procaspases to receptor-adaptor protein complexes (eg, the death-inducing signaling complex, DISC, with Fas) (Kischkel *et al*., 1995). CARD has been observed to have a structure similar to DED, thereby allowing interaction with and subsequent activation of caspase proforms. On the other hand, effector caspases have short prodomains (they lack CARD and DED). Usually initiator caspases are of low abundance, but their function is amplified by the proteolytic activation of more abundant effector caspases that do not possess the long CARD or multiple DEDs. Upstream initiator caspases, such as caspase-8 and caspase-9, in turn proteolytically activate caspase-3, -6, and -7 (Slee *et al*., 2001). Initiator caspases may themselves function as effector caspases in the death process. For example, caspase-8 acts on members of the Bcl2 family.

1.3.4- Biochemistry of apoptosis

Apoptosis is associated with biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane. It involves a cascade of events that lead to the death of the cell and, ultimately, to the formation of apoptotic bodies that can be engulfed and degraded by neighboring cells avoiding inflammation.

1.3.4.1- Apoptotic pathways

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway (Figure 1.3). The extrinsic pathway is initiated through the stimulation of the trans-membrane death receptors, such as the tumour necrosis factor (TNF) and Fas receptors, located on the cell membrane. On the other hand, the initiation of the intrinsic pathway occurs through the release of signal factors by mitochondria within the cell.

1.3.4.1.1- The Extrinsic Pathway

The extrinsic pathway, or receptor-mediated pathway, is activated by ligands, which are released by other cells that bind to transmembrane death receptors on the target cell to induce apoptosis. This pathway is mainly involved in the inflammatory response and regulation of immune function. Typical death receptors are tumour-necrosis factor receptor (TNF-R) and Fas (also called Apo-1 or CD95), which are part of the TNF-R family and contain a cytosolic death domain (DD) (Wong *et al*., 1992; Csipo *et al*., 1998). In case of TNFα, its binding to TNF-R1 on the target cell will trigger multiple receptors to aggregate on the surface of the target cell (reviewed by Chen and Goeddel, 2002). The aggregation of these receptors in their cytoplasmic domains recruits an adaptor protein known as TNF receptor-associated death domain (TRADD) that by it self recruits other proteins such as Fas-associated death domain (FADD). FADD, in turn, recruits caspase-8 or caspase-10, initiator caspases, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3 to initiate degradation of cellular targets. Active caspase-8 can also cleave Bid protein to tBid, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway. A functional damage to selective components of the mitochondrial electron transport chain (particularly those that increase the production of oxygen radicals) and structural alterations in mitochondrial morphology has been shown to mediate TNF citotoxicity (Schulze-Osthoff *et al*., 1992). The mitochondria can be also source of second messenger molecules responsible for gene-inductive effects of TNF (Schulze-Osthoff *et al*., 1993).

1.3.4.1.2- The Intrinsic Pathway

The intrinsic pathway, also called the mitochondrial pathway, is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage (Rich *et al*., 2000), heat shock and cytotoxic drugs (Liu *et al*., 1996). When an apoptotic signal is received, proapoptotic proteins in the cytoplasm, such Bax and Bak, bind to the outer membrane of the mitochondria to signal the release of the internal content. Following the release cytochrome c from the

intramembrane space to the cytosol, it binds to the Apoptotic Protease Activating Factor-1 (Apaf-1) (Adrain *et al*., 2006; Adrain and Martin, 2001; Hill *et al*., 2003). This complex will catalyze the activation of pro-caspase-9, an initiator caspase, forming together a complex called the Apoptotosome (around 1 MDa), which in turn activates caspase-3, the effector caspase that initiates cleavage of cellular substrates. In addition to the release of cytochrome c, the apoptosis inducing factor (AIF) (Joza *et al*., 2001) and Smac/DIABLO (Du *et al*., 2000; Verhagen *et al*., 2000), a protein that inhibits the inhibitor of apoptosis (IAP), can also be released from the mitochondria.

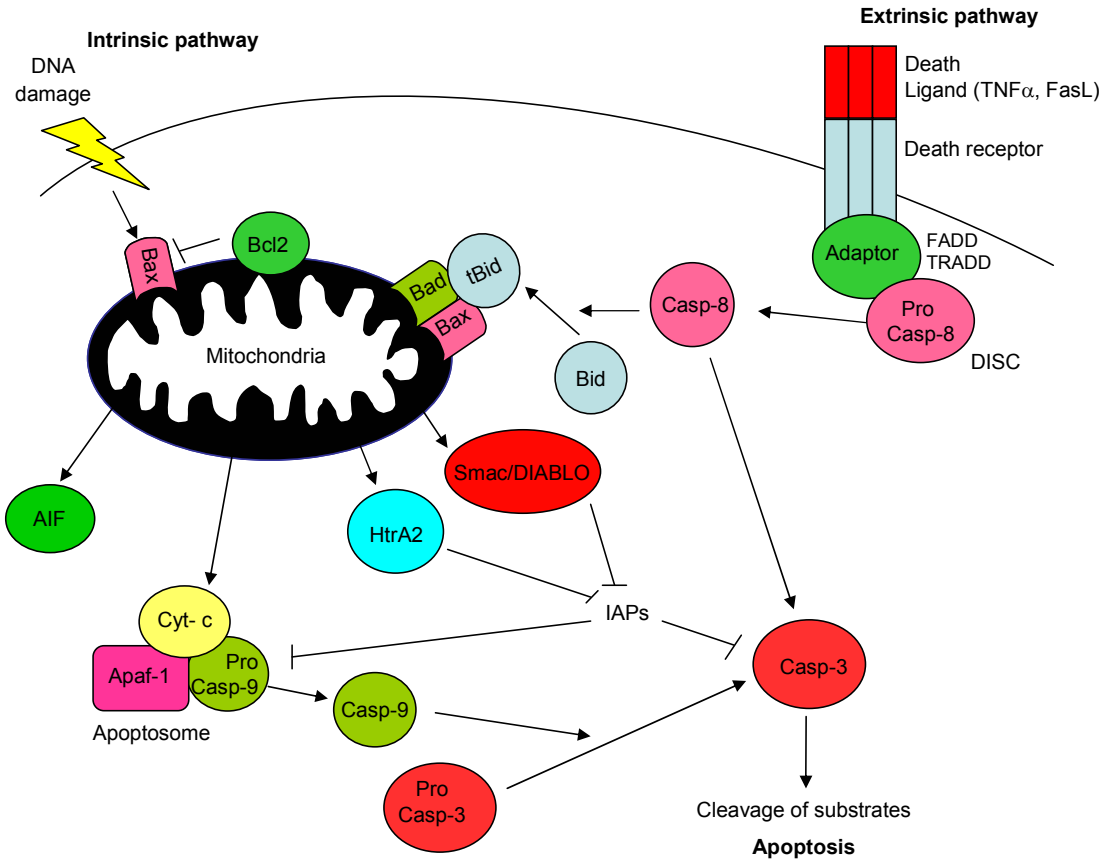

FIGURE 1.3- Schematic representation of intrinsic and extrinsic apoptotic pathways. See text for details.

One of the most important mediators of extrinsic or intrinsic signal and apoptotic responses that decide the future of the cell is the tumor suppressor p53. This study focuses mainly on p53 responses to DNA damage through the intrinsic pathway.

1.4- TUMOUR SUPPRESSOR P53

Although the p53 tumor suppressor was identified nearly three decades ago and plays a pivotal role in human cancer, its complexity continues to surprise the research community (Mills, 2005).

The tumour suppressor p53 is considered a critical regulator of genomic integrity because of its ability to trigger apoptosis and/or cell cycle arrest in response to genotoxic stress. Indeed, mutations of the *p53* gene occur in approximately 50% of human cancers (Hollstein *et al*., 1991; Hussain and Harris, 1998; Levine, 1997). p53 can be involved in maintaining genetic stability in several ways. p53 exerts control over both the G2/M and the G1 cell cycle checkpoints. p53 inactivation can synergize with oncogene activation leading to aneuploidy and chromosome imbalance. If the p53 pathway is altered in ESC and an oncogene gets activated, these cells may have a survival or growth advantage and can take over the culture. p53 can also affect genetic stability through its ability to regulate centrosome duplication via either transactivation dependent mechanisms involving p21, or transactivation independent mechanisms, by interacting directly with other centrosomal proteins at the centrosome. Indeed, Survivin, an IAP and a chromosomal passenger protein whose deregulation leads to aneuploidy, is negatively regulated by p53 (reviewed by Sah *et al*., 2006). As mentioned earlier, Survivin is also one of the candidate ESC transforming genes on the frequently duplicated chromosome 17.

The p53 protein consists of 393 amino acids and is commonly divided into three functional domains: the acidic amino-terminal domain (required for transcriptional activation), the central core sequence-specific DNA-binding domain (where most interactions between p53 and target proteins occur) and the carboxy-terminal end (contains the tetramerization domain, a nuclear export signal and nuclear localization signals). p53 can be post-translational modified in many ways on many residues (reviewed by Bode and Dong, 2004). p53 has a short half-life and is normally maintained at low levels levels in unstressed mammalian cells through continuous ubiquitylation by RING-finger ubiquitin E3 ligase Mdm2 (one of its downstream targets) and subsequent protesomal degradation. This creates an autoregulatory feedback loop to regulate expression and activity of this tumour suppressor.

1.4.1- p53 knockout mice

p53 knockout mice are viable and fertile but are prone to the spontaneous development of a variety of neoplasms (Donehower *et al*., 1992). At first glance the viability of these animals indicates that p53 function is not essential for embryonic development. However, a fraction of p53-deficient embryos in fact do not develop normally (Sah *et al*., 1995). These animals display defects in neural tube closure resulting in an overgrowth of neural tissue in the region of the mid-brain, a condition known as exencephaly. Additionally, a significant proportion of female p53-/- mice die during embryogenesis or in the period between birth and weaning, being subject to a spectrum of abnormalities (Armstrong *et al*., 1995). However, mice lacking Mdm2, a negative regulator of p53, die at an early embryonic stage, but can be rescued in a p53-null background

(Jones *et al*., 1995; Montes de Oca Luna *et al*., 1995). This implies that at an early embryonic stage p53 is functional and its functional activity has to be controlled to allow survival of embryos and subsequent development. Embryonic phenotypes of homozygotes deficient for either gene appear more obvious upon challenge. Lack of either of these genes is sufficient to rescue blastocysts from cell death induced by hyperglycaemia (Keim *et al*., 2001; Moley *et al*., 1998). Suppression of hyperglycaemia-induced cell death is dependent on both Bax and p53, and is accompanied by a decrease in the number of embryo resorptions, directly implicating cell death in postimplantation embryonic loss (Moley, 2001).

1.4.2- p53 in apoptosis

There are different apoptotic pathways controlled by p53. In response to cellular stress, p53 transactivates proapoptotic genes (PUMA, Noxa, Bax, p53 AIP1, Apaf-1 and PERP) activating the receptor-mediator (extrinsic) pathway, the mitochondrial (intrinsic) pathway and the ER pathway (Figure 1.4). p53 can also repress the transcription of relevant prosurvival genes (Bcl2 and IAPs). Bioinformatic studies predict that more than 4000 human genes contain putative p53 binding sites (Lu, 2005). The activity of p53 is controlled in different ways depending on the cell context either trough post-translational modifications (phosphorylation, sumoylation, acetylation) or through the activity of cofactors and inhibitors, like Mdm2 (Bourdon *et al*., 2003). Several in vivo studies reported that a fraction of induced p53 translocates to the mitochondria. A study published by Erster *et al* (Erster *et al*., 2004) showed that in sensitive organs, like the thymus, mitochondrial p53 accumulation *in vivo* occurs soon after a death stimulus, triggering a rapid first wave of apoptosis that is transcription independent and may precede a second slower wave that is transcription dependent. At the mitochondrion p53 interacts with Bcl2 family of regulators of mitochondrial permeability (Chipuk *et al*., 2004; Leu *et al*., 2004).

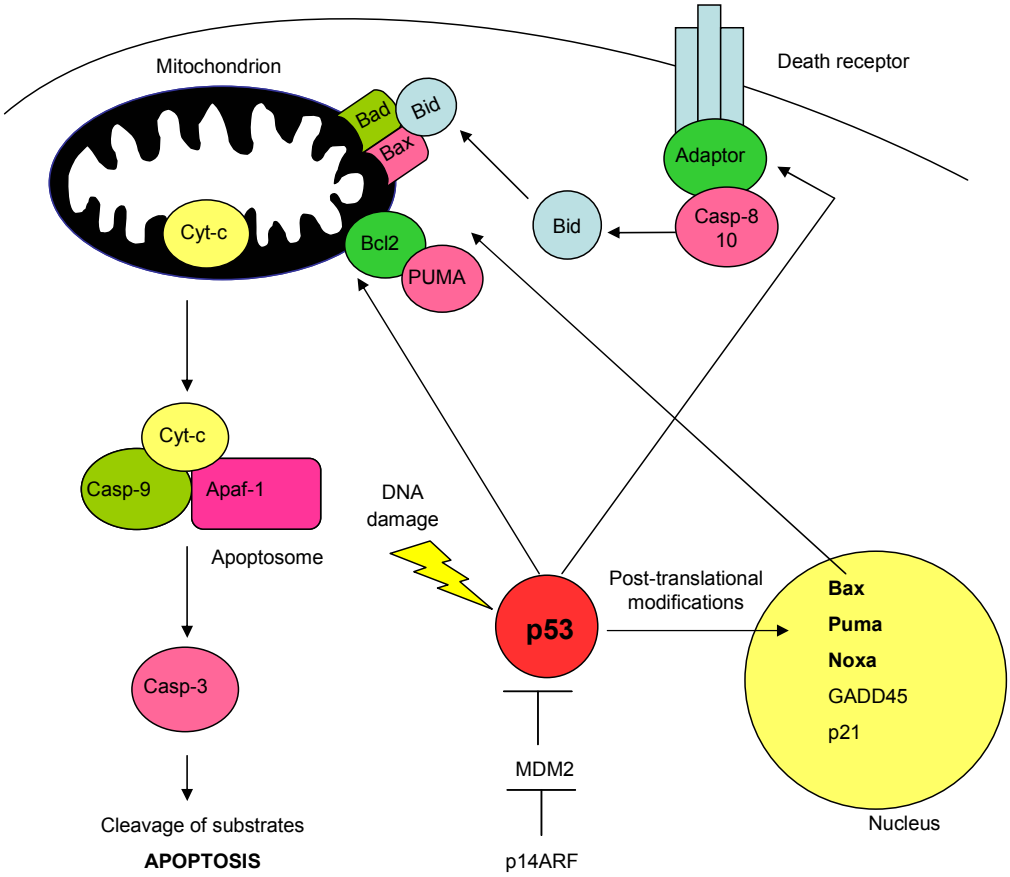

FIGURE 1.4- Schematic representation of apoptotic pathways controlled by p53.
DNA damage and other stress signals converge on p53 signaling by activation of protein kinases and/or acetyltransferases, which phosphorylate or acetylate p53, respectively. The inactivation of p53 by Mdm2 is counteracted by mechanisms involving post-translational modification of p53 or the Mdm2 inhibitor ARF. These posttranslational modifications generally result not only in stabilization, but also in activation of p53 in the nucleus, where p53 interacts with sequence-specific DNA binding sites in its target gene promoters. Through its activity as a sequence-specific transcriptional activator, p53 increases the expression of pro-apoptotic Bcl-2 family proteins. p53 can also have a direct apoptogenic role on the mitochondria, interacting with Bcl2 family of regulators of mitochondrial permeability. Released apoptogenic factors facilitate the activation of the effector caspases through the Apaf-1–caspase 9-apoptosome.

p53 can be activated pharmacologically by the topoisomerase II inhibitor etoposide. This is an antineoplastic drug that has been widely used to induced apoptosis in response to DNA damage (Karpinich *et al*., 2002). Topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription (Hande, 1998). Topoisomerase II prevents "knots" from forming in DNA by allowing the passage of an intact segment of the helical DNA through a transient double strand break (Burden and Osheroff, 1998). Topoisomerase II inhibitors such as etoposide stabilize the complex formed by topoisomerase II and the 5'-cleaved ends of the DNA, thus forming stable (nonrepairable) protein-linked DNA double strand breaks (Burden and Osheroff, 1998). Cells are apparently able to recognize such DNA damage and, in turn, to eliminate the injured cells by apoptosis. This is known to be p53-mediated via the mitochondrial death pathway.

Etoposide is extensively used to investigate the p53 mediated apoptosis of hESC all throughout this study.

1.4.3- p53 family and differentiation

p53 and its related family members, p63 and p73, have also been implicated in cellular differentiation. All three genes are involved in cell cycle arrest and apoptosis after DNA damage. Despite the fact that they share sequence homology with p53, enabling them to bind to p53 DNA-binding sites, to transactivate p53-responsive genes, and exhibit partial functional similarity, mouse knockout studies revealed an unanticipated functional diversity among them. p63 and p73 knockouts exhibit severe developmental defects but no increased cancer susceptibility, while p53 knockouts display high incidence of tumours and a less profound developmental phenotype. p63-deficient mice are born alive but their limbs are absent or truncated due failure in the formation of the apical ectodermal ridge, a structure required for limb outgrowth along the proximal-distal axis (Mills *et al*., 1999). Their skin lacks stratification and does not express differentiation markers. These mice do not have ectodermal structures that are produced through epidermal-mesenchymal interactions during embryonic development, like hair follicles, teeth and mammary glands. These observations indicate that p63 plays an essential role in the maintenance of progenitor-cell populations that are required to sustain epithelial development and morphogenesis. Indeed, p63 is considered to be an important keratinocyte stem cell marker (Pellegrini *et al*., 2001). P73-null mice exhibit hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, as well as defects in pheromone sensory pathways (Yang *et al*., 2000). Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer (Mills *et al*., 1999; Moll and Slade, 2004; Yang *et al*., 2000). There are also alternative splicing isoforms of all these genes that encode natural truncated forms involved in regulation of target gene expression (Benard *et al*., 2003; Bourdon *et al*., 2005; Dohn *et al*., 2001; Liu *et al*., 2004; Wu *et al*., 2003).

Several reports have shown that endogenous p53 levels and activities are modulated upon differentiation. It has been reported that p53 expression and activity was upregulated during maturation of human hematopoietic cells (Kastan *et al*., 1991) and differentiating epidermal keratinocytes (Weinberg *et al*., 1995). p53 has also been shown to enhance macrophage differentiation (Matas *et al*., 2004). Recently**,** Brynczka and colleagues (Brynczka *et al*., 2007) have studied the transcriptional role of p53 during NGF-induced differentiation of the PC12 line into neuron-like cells. They claimed that receptor-mediated p53 transcriptional activity is involved in cell differentiation and suggested a contributory role for p53 in neuronal development. Overexpression of p53 in some cell types induces differentiation, such as leukemic K562 cells, HL-60 cells, Friend virus transformed erythroleukemic cells or squamous carcinoma (Banerjee *et al*., 1995; Brenner *et al*., 1993; Ehinger *et al*., 1995; Feinstein *et al*., 1992; Soddu *et al*., 1994) or even in non- transformed cells of the myeloid and muscle lineages (Soddu *et al*., 1996). Although these results suggest that p53 has a regulatory role in differentiation, the possibility that the reported effects are due to a proliferation inhibition by p53 in some cell lines cannot be excluded.

1.4.4- Role of p53 in hESC

Transcriptional profiling analysis performed on ESC have shown a consistent expression of p53 and other proteins involved in apoptosis (Bhattacharya *et al*., 2005; Ginis *et al*., 2004; Ramalho-Santos *et al*., 2002). Array analysis of H1 cells indicated (Ginis *et al*., 2004) that ESC express p53, the negative regulator of p53 HDM2, the pro-apoptotic downstream target of p53 Bax, as well as the Bax related genes Bak, HRK and the Bcl2 family member Mcl1. Therefore the basic machinery for cytochrome c release and apoptosome activation is present.

The precise role of p53 in both mESC and hESC apoptosis remains unclear. While some argue that p53 is non-functional in undifferentiated mESC (Aladjem *et al*., 1998) others show that p53 does have a role to play in both UV-induced apoptosis (Chao *et al*., 2000; Xu *et al*., 2002b). Sabapathy and colleagues (Sabapathy *et al*., 1997) have also reported that undifferentiated mESC express high levels of functional p53 in a wild-type conformation, and upon differentiation p53 levels suffer a reduction and the protein undergoes a conformational change to a mutant form. Furthermore, its functional activity is lost upon differentiation and these cells become less sensitive to UV irradiation as compared with undifferentiated mESC. Both undifferentiated and differentiated cells derived from mESC lacking p53 display enhanced proliferation, and apoptosis accompanying differentiation is reduced. The authors of this study claimed that functional inactivation of p53 protects differentiating cells from apoptosis.

Interestingly, Lin and colleagues (Lin *et al*., 2005) have shown that in mESC p53 becomes activated in response to DNA damage and induces differentiation by suppressing Nanog expression. This may represent an alternative mechanism for p53 to maintain genetic stability in ESC.

Recently, a study has shown that hESC undergo p53 mediated-apoptosis induced by UV through a mitochondrial pathway (Qin *et al*., 2006). These authors further reported that relatively high rate of spontaneous apoptosis of hESC in culture (10-15 % daily) is controlled by p53. However, according to these authors, despite the accumulation of p53 and occupancy of this transcription factor on the promoter of gene targets, it fails to activate the transcription of its target genes. They further demonstrated that knocking down p53 expression also slows the differentiation rate of hESC after treatment with Activin. At present it remains to be established whether this spontaneous apoptosis is due to suboptimal culture conditions or is an intrinsic feature of hESC that is involved in eliminating cells with acquired DNA damage.

Because of present difficulty of gene targeting by homologous recombination in hESC to generate specific knock-out alleles, an alternative and more feasible way of investigating the role of p53 in hESC is the use of RNA interference (RNAi) to downregulate the expression of this tumour suppressor.

1.5- RNA INTERFERENCE: shRNA AND LENTIVIRUS

Just when scientists thought they had deciphered the roles played by the cell's leading actors, a familiar performer has turned up in a stunning variety of guises. RNA, long upstaged by its more glamorous sibling, DNA, is turning out to have star qualities of its own (Couzin, 2002).

The mechanism of RNAi was discovered in 1998. The combination of RNAi and ESC manipulation will be useful not also for advancement of basic research but also helping developing new therapies that are currently not possible due to technical limitations (reviewed by Heidersbach *et al.*, 2006).

RNAi was first described by Fire and colleagues (Fire *et al.*, 1998) and is a conserved mechanism of post-transcriptional gene silencing which acts through degradation of messenger RNA (mRNA) transcripts by the action of homologous short RNA species. This mechanism plays a role in different processes such as the response to viral infections and in development. In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNAi in *C. elegans*.

1.5.1- Mechanism of RNAi

Most of the understanding on the mechanism of RNAi comes from studies on plants, *C. elegans* and *Drosophila*.

The first observations in *C. elegans* showed that exposure to double stranded RNAs (dsRNAs, generally ranging from 500 to 1000 nucleotides) resulted in loss of corresponding mRNA. Promoter and intronic sequences failed to trigger silencing. In plants, it was also observed that exposure to dsRNAs resulting from transcription of viral RNA in the cytoplasm also triggered depletion of mRNA sequences without an apparent effect on the rate of transcription. This was suggestive that RNAi induced degradation of homologous mRNAs results in a measurable decrease in gene-specific expression and this was confirmed in subsequent studies (Hannon, 2002).

In mammalian cells, RNAi can be triggered by dsRNA or dsRNA domain-containing molecules that are processed by the endoribonucleases Drosha and Dicer (Figure 1.5). In the nucleus, Drosha processes endogenously expressed RNAs with dsRNA domains, mostly micro RNA (miRNA) precursors (Bartel, 2004). In the cytoplasm, these precursors are further processed by Dicer to yield miRNAs. Dicer is also responsible for the processing of other dsRNAs molecules from exogenous sources into small duplex RNAs, called small interfering RNAs (siRNAs) (Bernstein *et al.*, 2001; Elbashir *et al.*, 2001; Zhang *et al.*, 2004). Dicer-processed RNAs are unwound, and one strand of the duplex is integrated into a ribonucleoprotein complex (RNP) containing members of the Argonaute family of proteins (Hammond *et al.*, 2000; Hammond *et al.*, 2001; Nykanen *et al.*, 2001; Pham *et al.*, 2004). This RNP complex is known as RNA-induced silencing complex (RISC) in the case of siRNAs (Hammond *et al.*, 2000; Hammond *et al.*, 2001).

The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the complementary mRNA.

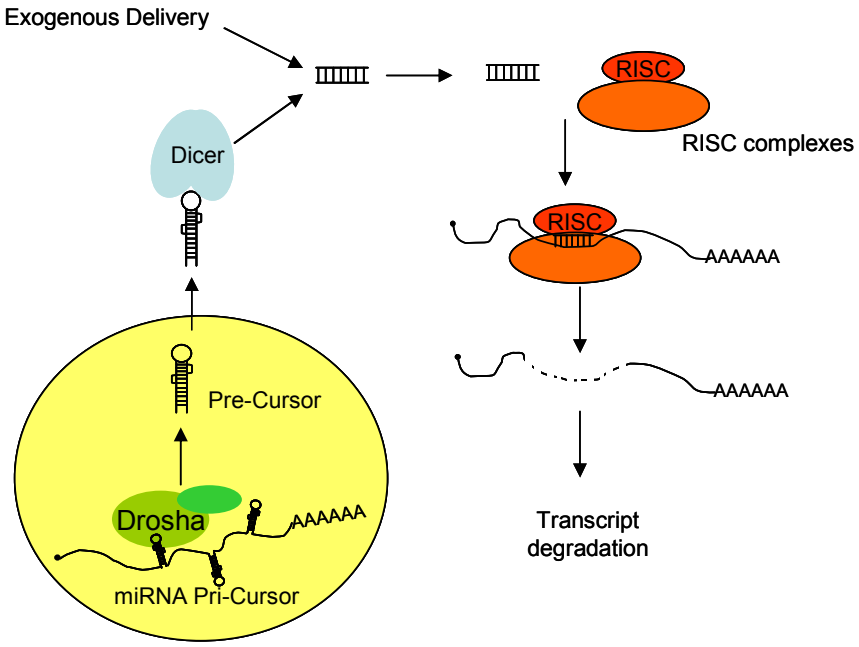

FIGURE 1.5- Mechanism of RNAi (adapted from Heidersbach *et al*., 2006).
In the nucleus, miRNA begin to be processed to from long primary miRNAs by Drosha. This enzyme cleaves the long pre-cursor into short nucleotide (pre-cursor) hairpins which are then transported to cytoplasm. In the cytoplasm, the enzyme Dicer recognizes the pre-cursor and cleaves it into a 19–21nucleotides RNA duplex. Exogenous delivered shRNAs are also recognized by Dicer. A single strand of the duplex originating from a Dicer-processed precursor or an exogenous siRNA is incorporated into the RISC. In case of perfect pairing between the siRNA and its target mRNA, RISC cleaves the target.

1.5.2- Tool to study gene function

The selective and robust effect of RNAi on gene expression (similar in what occurs in loss-of-function mutation), prompted the use of this methodology as a valuable research tool for *in vitro* and *in vivo* gene function analysis. Since RNAi often does not totally abolish the expression of the gene of interest, this technique is sometimes referred as a "knockdown", as opoposed to "knockout" procedures in which expression of a gene is entirely eliminated.

Some studies have report that in many types of mammalian cells exposure to long dsRNA leads a non-specific response directed by dsRNA dependent protein kinase (Kumar and Carmichael, 1998). This is mediated by the interferon pathway and will activate apoptotic cell death. This limitation was bypassed by the introduction, or expression, of shorter dsRNA species (less than 21 bp) into cells (Elbashir *et al*., 2001). This breakthrough made possible the use of RNAi-based tools for the large-scale screens in mammalian systems. Nevertheless, there are some mammalian cell types, like the mouse oocytes/zygotes, ESC and ECC, that do not induce the interferon pathway in response to long dsRNA (Billy *et al*., 2001; Wianny and Zernicka-Goetz, 2000; Yang *et al*., 2001).

RNAi is carried out by transfection of siRNAs and, more recently, by transcription of short hairpin RNAs (shRNAs) from expression vectors and retroviruses. The introduction of synthetic siRNA or expression of shRNA is very similar to what was described above for the processing of endogenous miRNA. However, certain cell types are resistant to transfection based approaches, both *in vitro* and *in vivo* (McCaffrey *et al*., 2002). To circumvent this limitation, lentiviral vectors were used to stably deliver shRNAs. The main advantages of lentiviruses are their high efficiency in delivering shRNA to target cells, their ability to infect both dividing and non-dividing cell, stem cells, zygotes, and their differentiated progeny (Rubinson *et al*., 2003) and their ability to escape transgene silencing that is observed with oncoretrovirus-based vectors in HSC and ESC (Zaehres and Daley, 2006). This permits a rapid and efficient analysis of gene function and can be applied in potential therapies. However, a recent study has reported that transgenes delivered lentiviral vectors can be suppressed in a promoter-dependent manner in hESC (Xia *et al*., 2007). This suppression is higher then that observed in mESC. Because silencing has not been observed in transient transfection of hESC with shRNA expression vectors, suppression of the transgene occurs right after transduction and integration. Therefore, caution should be taken in selection of optimal promoters when using lentiral vectors in hESC.

1.6- AIMS AND HYPOTHESIS

The general aims of this thesis are
I) the identification of apoptotic pathways in hESC.
II) the elucidation of the role of p53 in apoptosis of hESC.
III) the investigation of the role of p53 in differentiation of hESC.

Hypothesis: p53 is required for hESC apoptosis and has a role in cell differentiation.

Chapter 3 of this thesis describes the etoposide-induced apoptosis of hESC mediated by p53 using existing apoptosis assays, flow cytometric analysis, Western blotting and immunofluorescence. This chapter also describes a model of p53-mediated apoptosis in hESC.
Chapter 4 of this thesis analyses the phenotype of hES cell lines (HES-2 and HES-4) that have been genetically modified with stable lentiviral U6 driven shRNA vectors designed to downregulate p53 and describes the transcriptome analysis of GFP and p53 shRNA transduced hESC. This Chapter also addresses the role of p53 on spontaneous and induced differentiation, as well as teratoma formation.
In Chapter 5 the general significance of the results reported in this thesis and the future directions are presented.

CHAPTER 2

Material and Methods

This chapter has been divided into two sections dealing with cell culture and molecular techniques. Supplier and catalogue numbers are included. Culture medium composition, biological reagents, and specific working solutions are detailed within the methodology section in which they are used or referenced. General laboratory solutions and buffers are described in ***Appendix I***.

Section 2.1:

This section describes methods used in culture of mammalian cells. Methods adapted from published protocols or from manufacturer's guidelines are described in the text unless otherwise stated.

Section 2.2:

This section describes methods employed for molecular biology analysis. Commercially available kits or preparations were used according manufacturer's instructions and are described in the text.

Section 2.1- Cell Culture Methods

hESC lines HES-2, HES-3 and HES-4 were provided by this laboratory (Reubinoff *et al*., 2000). HES-2 and HES-3 are karyotypically female lines while HES-4 is of male karyotype. HT1080 is a human fibrosarcoma cell line, HepG2 is a human hepatocellular liver carcinoma cell line and CFPac-1 is a ductal pancreatic adenocarcinoma derived from a metastatic lesion in the liver of Caucasian male with cystic fibrosis. The 293FT cell line is a human embryonic kidney cell line, genetically modified and carries the pUC-derived plasmid (pCMVSPORT6TAg.neo.).

2.1.1- Commonly Used Tissue Culture Solutions, Reagents and Materials

General laboratory reagents utilized were of tissue culture grade and were supplied by Sigma-Aldrich and tissue culture grade plastic ware was supplied by Falcon (Becton Dickinson), unless otherwise stated. Polypropylene 1.7 ml microtubes (Axygen, Union City CA, USA) [Cat No: MCT175-C] for use in tissue culture were autoclaved in a glass beaker covered in aluminium foil or ultra wrap (Dräger Medical, Lübeck, Germany) and subsequently opened strictly in the hood. When possible, items utilized in tissue culture that were not purchased as tissue-culture grade (such as glass coverslips, slides, and forceps for example) were baked at 180°C for 2 hours to sterilize prior to use.

2.1.1.1- Growth factors

All growth factors were supplemented fresh from stock solutions into cell culture medium immediately prior to use.

Fibroblast Growth Factor-2 (FGF-2) human recombinant protein expressed in *E. coli* (R&D Systems, Minneapolis, USA) was obtained as 25 μg of lyophilized powder [Cat No: 233-FB] stored at -20°C. The powder was reconstituted as stock solution in 500 μl sterile PBS^- (Gibco) with 0.1% BSA fraction IV (Sigma-Aldrich), to produce a 50 ng/μl stock concentration of growth factor, stored at -20°C for up six month in 50 μl aliquots. When required, a vial of FGF-2 was thawed and added to experimental media without serum (SR culture media) at 4 ng/ml. The remainder of the contents was stored up to 2 weeks at 4°C. This reagent was used from 1 September 2004 to 9 November 2006 and from 9 August 2007 until 26 September 2007.

Recombinant Human Fibroblast Growth Factor- Basic- (Chemicon International) [Cat No: GF003] was obtained as 50 μg of lyophilized powder and stored at -20°C. The powder was reconstituted as stock solution in 1 ml sterile PBS- (Gibco) with 0.1% BSA fraction IV (Sigma-Aldrich), to produce a 50 μg/ml stock concentration of growth factor, stored at -20°C for up six month in 50 μl aliquots. When required, a vial of FGF-2 was thawed and added to experimental media without serum (SR culture media) at 4 ng/ml. The remainder of the contents was stored up to 2 weeks at 4°C. This reagent was used from 9 November 2006 till 9 August 2007.

2.1.1.2- Extracellular matrices

Gelatin, Type A from porcine skin (Sigma-Aldrich) [Cat No: G1890] 1% (w/v) stock solution was prepared by adding 5 g gelatin to 500 ml sterile distilled water (Gibco) [Cat No: 15230-204] in a 500 ml glass bottle, mixing well and autoclaving. The solution was kept at room temperature (RT) and diluted to a 0.1% gelatin working concentration when required.

Matrigel™ Basement Membrane Matrix (BD Biosciences, MA, USA) [Cat No: 354234] is a solubilized basement membrane preparation extracted from EHS mouse sarcoma, a tumour rich in extracellular matrix proteins. The matrix is stable for minimum of 3 months when stored at -20°C. Each lot is supplied at different concentration. Matrigel is diluted into cold DMEM/F12 (Gibco). The coating concentration is 0.0347 mg/cm^2 and the minimal time of 3 hours in the incubator at 37°C or overnight (ON). The coating mixture is then aspirated and the dish is pre-equilibrated with conditioned media from MEF (CM) before seeding cells.

2.1.1.3- Enzymes and dissociation solutions

Cell Dissociation Buffer enzyme free Hanks'-based (Gibco) [Cat No: 13150016] was stored at 4°C and used undiluted. Dissociation Buffers are membrane-filtered isotonic and enzyme-free aqueous formulations of salts and chelating agents in either Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution or Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline. They are used for gentle dissociation of mammalian cells from support substrates and each other in particular for studies requiring intact cell surface proteins such as ligand binding flow cytometry and immunohistochemistry.

Collagenase Type I (Worthington Biochemical Corporation, Lakewood, NJ USA) [Cat No: L5004196] powder was stored at 4°C. A 4 mg/ml solution was prepared fresh as needed by gently dissolving 0.04 g of powder in 10 ml at RT DMEM/F12 (Gibco) [Cat No: 11320-033]. The powder was allowed to dissolve for 5 min and the solution was filtered sterilized using a pre-wet 0.22 μm filter and stored at 4°C.

Dispase (Gibco) [Cat No: 17105-041] was stored at 4°C. A 10 mg/ml solution was prepared as needed by dissolving 0.1 g (1 vial) of the powder into 10 ml of 37°C pre-warmed hESC serum medium. The powder was allowed to dissolve for 5 min and pipetted up and down a couple of times. Dispase was allowed to dissolve for further 10-20 min at RT and filtered sterilized using a pre-wet 0.22 μm filtered, then stored at 4°C.

TrypLE Express Stable Trypsin Replacement Enzyme (Gibco) [Cat No: 12604-013] was used neat and stored at 4°C.

Trypsin-EDTA (0.25% Trypsin, EDTA 1X liquid) (Gibco) [Cat No: 25200-056] containing 2.5 g/L Trypsin and 0.38 g/L EDTA•4Na in Hanks' Balanced Salt Solution was divided into 2 ml aliquots and stored at -20°C. To prepare a 0.05% (w/v) Trypsin/EDTA working solution it was diluted with

8 ml of PBS^- (Gibco). The remaining volume of 0.05% working solution was stored at 4°C and used or discarded within one week.

2.1.1.4- Miscellaneous Culture Components, Solutions and Materials

Bovine Serum Albumin (BSA) (Sigma-Aldrich) [Cat No: A8806] was used in reconstitution of FGF-2. To prepare a solution containing BSA, powder was weighed, allowed to dissolve undisturbed in the solvent for 10-15 minutes, and filtered through a 0.22 μm syringe filter unit.

Capillaries, glass (Harvard Apparatus, Holliston MA, USA) [Catalog number 30-0039] were pulled over a low Bunsen burner flame to generate finely edged tips for cutting hESC colonies.

Dimethylsulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) [Cat No: D2650] was used at a final concentration of 10% (v/v) in freezing medium for cryostorage of cell lines in liquid nitrogen.

Distilled water (Gibco) [Cat No: 15230-162]

Fetal calf serum (FCS) used for MEF and hESC culture was batch tested and supportive batches were ordered in bulk to provide reagent for up to several years. Different types of FCS were used for specific tissue applications: for MEF culture JRH Biosciences FCS [Cat No: 12003-500N] and CSL Biosciences FCS (Parkville VIC, Australia) [Cat No: 09702301] while for hESC culture Hyclone FCS (Logan UT, USA) [Cat No: SH30070-03] and JRH Biosciences FCS (Brooklyn VIC, Australia), [Cat No: 12203-100M].

Fugene 6 Transfection Reagent (Roche Applied Science) [Cat No: 11 814 443 001] was obtained in 1 ml volume and stored at 4°C.

Geneticin 50 mg/ml (Gibco) [Cat No: 10131-035], also known as G418 sulfate, is an aminoglycoside used as a selective antibiotic for genetically modified cell lines which contain the neomycin resistance gene. Working concentration in cell culture media was 500 μg/ml.

Pasteur pipettes, glass disposable (Chase Scientific Glass, Rockwood, Tennessee) [No. 93, 9 inch length, flint glass] were packed into metal canisters and baked at 180°C for 2 hours to sterilize. Glass Pasteur pipettes were attached to a vacuum tap with tubing and used to aspirate media from tissue culture flasks.

Mitomycin C (Sigma-Aldrich, Steinheim, Germany) [Cat No: M0503] was received as 2 mg lyophilized powder and stored at 4°C. A 0.5 mg/ml stock solution was made by adding 4 ml of sterile distilled water (Gibco) [Cat No: 15230-204] into each Mitomycin C vial and allowed to dissolve completely. The solution was filter sterilized using a pre-wet 0.22 μm filter into a 15 ml Falcon tube. The tube was protected from light with aluminum foil and stored at 4°C. The working solution was used at 10 μg/ml in MEF culture media immediately before adding to MEF.

Phosphate Buffered Saline (Na^+/Mg^{+2} free) (PBS-) (Gibco) [Cat No: 15230-162]

Phosphate Buffered Saline (Na^+/Mg^{+2}) (PBS+) (Gibco) [Cat No: 15230-162]

Trypan blue dye (Sigma-Aldrich, Steinheim, Germany) [Cat No: T8154] was diluted 1:2 in PBS or medium to examine cell viability when counting on a hemocytometer.

2.1.1.5- Medium solutions

Media for cell culture was prepared in disposable tissue culture grade plastic ware (Millipore, Billerica MA, USA). All media was either sterile filtered through a 0.22 μm GP Express PLUS membrane filter unit (Millipore) [Cat No: SCGPT02RE and SC00B05RE] or sterility tested for a minimum of 2 days before use. The media was light protected with aluminum foil, stored at 4°C and pre-warmed to 37°C prior to addition to live cells.

The following components were ordered in large volumes, aliquoted under sterile conditions, and stored at the temperature recommended by the manufacturer: FCS, KSR, non-essential amino acid (NEAA) solution, L-glutamine (L-Glut) solution, and Penicillin/Streptomycin (Pen/Strep) solution.

Serum based hESC Culture Medium (500 ml)

DMEM high glucose (Gibco) [Cat No: 11960-044]	382 ml
FCS (for hESC Culture)	100 ml (20%)
NEAA solution (10 mM) (Gibco) [Cat No: 11140-050]	5 ml (0.1 mM)
L-Glut solution (200 mM) (Gibco) [Cat No: 25030-081]	5 ml (2 mM)
Pen/Strept solution (Gibco) [Cat No: 15070-063]	2.5 ml (Pen: 25U/ml; Strep: 25ug/ml)
β-Mercaptoethanol (55Mm) (Gibco) [Cat No: 21985-023]	0.9 ml (0.1 mM)
Insulin-Transferrin-Selenium-A (Gibco) [Cat No: 51300]	5 ml (Insulin: 10mg/L; Transferrin: 5.5mg/L; Na-Selenite: 6.7ug/L; Na-Pyruvate: 110mg/L)

hESC Serum Replacement (SR) Culture Medium (500ml)

DMEM/F12 (Gibco) [Cat No: 11320-033]	389.1 ml
KSR (Gibco) [Cat No: 10828-028]	100 ml (20%)
NEAA solution (10 mM) (Gibco) [Cat No: 11140-050]	5 ml (0.1 mM)
L-Glut solution (200mM) (Gibco) [Cat No: 25030-081]	5 ml (2 mM)
β-Mercaptoethanol (55 mM) (Gibco) [Cat No: 21985-023]	0.9 ml (0.1 mM)

*FGF-2 was supplemented fresh into the media volume used each day at final concentration of 4 ng/ml (stock at 50 μg/ml).

MEF Feeder Cell Culture Medium (500 ml)

DMEM (Gibco) [Cat No: 1190-044]	442.5 ml
FCS (for MEF culture)	50 ml (10%)
L-Glut solution (200 mM) (Gibco) [Cat No: 25030-081]	5 ml (2.0 mM)
Pen/Strept solution (Gibco) [Cat No: 15070-063]	2.5 ml (Pen: 25U/ml; Strep: 25ug/ml)

Conditioned medium (CM) from MEF

Serum replacement culture medium plus 4 μg/ml FGF-2 as added to a T75 of MEF at full density ($6x10^4$ cells/cm^2) and collected 24 h after. The CM medium was filtered sterilized using a 0.22 μm filter and FGF-2 (4 ng/ml) was added before use with Matrigel cultures. SR medium was replaced on the flask of MEF feeders each day to continue producing conditioned medium.

Complete medium for 293 FT and HT1080

DMEM high glucose (Gibco) [Cat No: 11960-044]	415 ml
FCS (JRH Biosciences) [Cat No: 12003-500N]	50 ml (10%)
NEAA solution (Gibco) [Cat No: 11140-050]	5 ml (0.1 mM)
L-Glut solution (200 mM) (Gibco) [Cat No: 25030-081]	15 ml (6 mM)
Sodium Pyruvate (Gibco) (100 Mm) [Cat No: 11360070]	5 ml (1mM)
Pen/Strept solution (Gibco) [Cat No: 15070-063]	5 ml

* Geneticin® (Gibco) [Cat No: 10131-035] was added fresh at final concentration of 500 μg/ml (Stock at 50 mg/ml)

Complete medium for CFPac-1 (500 ml)

Iscove's modified Dulbecco's medium	440 ml
FCS (JRH Biosciences) [Cat No: 12003-500N]	50 ml (10%)
L-Glut solution (200 mM) (Gibco) [Cat No: 25030-081]	10 ml (4 mM)
1.5 g/L sodium bicarbonate	

Complete medium for HepG2 (500 ml)

Dulbecco's modified Eagle's medium and Ham's F12 medium	
FCS (JRH Biosciences) [Cat No: 12003-500N]	50 ml (10%)
L-Glut solution (200 mM) (Gibco) [Cat No: 25030-081]	7.5 ml (2.5 mM)
HEPES (1M) (Gibco) [Cat No: 15630-080]	7.5 ml (15 mM)
Sodium pyruvate (100 mM) (Gibco) [Cat No: 11360070]	2.5 ml (0.5 m
Geneticin® (500 μg/ml) (Gibco) [Cat No: 10131-035]	4 ml (0.4 mg/ml)
1200 mg/L sodium bicarbonate	

Freezing Medium for hESC serum-free culture (10 ml)

FCS (for hESC culture)	9 ml (90%)
DMSO (Sigma-Aldrich) [Cat No: D2650]	1 ml (10%)

Freezing Medium for 293FT and HT1080, HepG2 and CFPac-1 (10 ml)

Complete medium	9 ml
DMSO (Sigma-Aldrich) [Cat No: D2650]	1 ml (10%)

2.1.2- hESC and other cell lines Culture Methods

Cell cultures were maintained at 37°C with 5% CO_2 in a humidified Steri-Cycle CO_2 incubator (Thermo Electron Corporation, Marietta OH, USA). Tissue culture procedures were performed in air handling class II biological safety cabinets (DCE-BRT Environmental Ltd., Leicester, UK and Bioair Instruments Aura 2000 MAC, Laf Technologies Pty Ltd & Bio Cabinets Australia Pty Ltd, Ringwood, VIC Australia) or an HWS-series laminar flow workstation (Clyde-Apac, Woodville, SA Australia). These will be referred as “hoods”. Standard aseptic techniques were rigidly followed in culturing cell lines. 70% ethanol in deionized water was used to disinfect surfaces and equipment as needed. Cultured cells were examined by phase-contrast microscopy using an Olympus IX71 inverted microscope with attached Olympus DP12 digital camera system (Olympus Corporation, Tokyo, Japan). Routine centrifugation of cells was performed in a Sigma 2-4 centrifuge (Sigma-

Aldrich Laborzentrifugen, Harz, Germany) or Sigma 2-5 centrifuge. Hoods were exposed to UV lamps after use for a minimum of 20 minutes. Pipettes (Gilson, Middleton WI, USA), pipet-aids (Falcon Express Pipet-aid) (Beckton Dickinson, Franklin Lakes NJ, USA) and other equipment used for tissue culture methods were used generally only in the laboratory dedicated for tissue culture to minimize the risk of contamination.

2.1.2.1- Gelatinizing Tissue Culture Plates

Sufficient volume of 0.1% Gelatin was added to cover culture surface area and incubated at RT for minimum of 1 hour. Then gelatin was aspirated and cells were plated.

2.1.2.2- Expansion and culture of MEF feeder support cells

The derivation, culture and preparation of MEF feeder cells for hESC propagation were routinely carried out by laboratory technical staff Linh Nguyen, Karen Koh, Irene Tellis, Pegah Jamshidi, Tracey Lomas, Lisa Kass and Sheetal Saini.

2.1.2.2.1- Subculturing MEF feeder cells

1. MEF were subcultured when flasks reach 80-90% confluency. The T-175 flasks containing MEF were washed twice with 10-15 ml PBS^- (Gibco) and trypsinized by incubating the cells for 5 minutes at 37°C.
2. The flasks were tapped firmly against an open palm until cells detach from flask wall. 10 ml MEF medium were added to stop the action of the trypsin. The cells were dissociated into a single cell suspension by mixing up and down in a serological pipette, and then transferred to a 50 ml Falcon tube. The flask was washed with another 10 ml MEF media and added to tube and then centrifuged for 2 minutes at 500 x g.
3. The supernatant was aspirated and the pellet was gently flicked to disperse cell pellet. The cells were resuspended in 10-20ml MEF media and split evenly among several T175 at a ratio of 1:3-1:6. Generally, MEF at early passage (P0, P1, or P2) could be split higher at 1:5 or 1:6 (from an 80-90% confluent flask). Later passage MEF (P3, P4) of the same confluency were split 1:3 or 1:4. MEF were expanded to a maximum of four passages (P4) before mitomycin C treatment for hESC co-culture.

2.1.2.2.2- Mitomycin C inactivation of MEF feeder cells

1. Mitomycin C (MC) (200 mg) (Sigma-Aldrich) was added to 200 ml FCS/DMEM and the solution was sterilized through a 0.2 μm cellulose acetate filter (Millipore). This solution can be stored at 4°C and used to mitotically inactivate MEF cells for 4 weeks.
2. MC-FCS/DMEM was warmed to 37°C and enough added to MEF cell containing flasks to cover the cells with a thin film of medium. The treated flasks were placed in an incubator at 37°C for 2-3 hours.
3. While the MEF feeder cells were being inactivated, designated tissue culture vessels for MEF were coated with 0.1% (v/v) gelatine solution for at least 1 hour.
4. At the end of inactivation process, the MC-FCS/DMEM medium was aspirated and the MEF cells washed 3 times with 10 ml PBS^-. A volume of 5ml of trypsin/EDTA solution per T-75

flask (1.5 ml per T-25 flask) was added to the cells and incubated at 37°C for 5 min. The cells were tapped gently for easier dislodgment.

5. The trypsin/EDTA was inactivated with 10 ml of FCS/DMEM and cells were gently resuspended into a single cell suspension using a 5 ml pipette tip using the medium speed on a Falcon Express Pipet-aid (Beckton Dickinson, Franklin Lakes NJ, USA).
6. The cell suspension was transferred to a 15 ml Falcon conical tube and pelleted for 2 min at 500 x g. The cell pellet was then resuspended in a 10 ml of fresh FCS/DMEM medium and the cell number/ml counted using a haemocytometer.
7. Gelatin was removed from gelatin-coated plastic tissue culture flasks and a PBS^- wash was applied to remove excess gelatine solution. The appropriate volume of mitomycin C treated MEF was added to tissue culture flask ($6x10^4$ cells/cm^2 for conventional serum grown hESC, $2x10^4$ cells/cm^2 for SR/FGF-2 grown hESC).
8. Plating of hESC on feeder cell layers was possible a minimum of 3 hours after MEF seeding, although typically MEF cells wee used after an ON attachment period. The feeders were used for hESC cultures within one week of mitotic inactivation.

2.1.2.3- Expansion and culture of hESC

The hESC lines were cultured using both conventional mechanical excision of the colony for growth in the presence of serum medium and mouse embryonic fibroblast feeder cells MEF ($6x10^4$ cells/cm^2) (Reubinoff *et al*., 2000; Thomson *et al*., 1998). Alternatively cells were cultured using SR supplemented medium with FGF-2 on one third of the conventional mouse embryonic fibroblast feeder cell line density ($2x10^4$ cells/cm^2) (Amit *et al*., 2000) passaged with Colagenase I or cells were plated on Matrigel (BD Biosciences) with MEF-conditioned serum replacement medium as described by (Xu *et al*., 2001). hESC lines were used only up to passage 18 when cultured as per Amit *et al* (2000) or Xu *et al* (2001). hESC colonies were routinely transferred on day 7. All hESC lines used for this work were proven to be mycoplasma-free, of normal karyotype, and capable of forming teratomas representative of all three germ layers.

2.1.2.3.1- Propagation of hESC in serum containing medium

Mechanical dissection of hESC colonies was performed using a Leica MZ6 stereomicroscope (Leica Microsystems, Wetzlar, Germany) with a microscope warm stage (LEC Instruments, Scoresby, VIC Australia; model LEC916) set up in a laminar flow workstation. The standard tissue culture vessel for hESC propagation in serum containing medium is a polystyrene 61x15 mm centre-well organ culture plate (Beckton Dickinson, Franklin Lakes NJ, USA).

1. Glass capillaries (Clark Electromagnetics Industries) were prepared by pulling over a low Bunsen burner flame, generating finely edged tips for cutting selected areas of colonies. Although glass capillaries generally delivered better results, a 27-30 gauge syringe needle (Beckton Dickinson, Franklin Lakes NJ, USA) was also be used for colony cutting.
2. Day 7 hESC serum cultures were examined under an inverted microscope and plates harbouring undifferentiated colonies were selected for transfer. The plates were washed twice with 1 ml PBS^+ per dish.

3. A pulled glass capillary was placed into a micropipette holder and used to make cuts to undifferentiated colony areas (alternatively a 27.5 gauge needle can be used). This work was conducted under a Leica MZ6 dissection microscope (3.2 X magnification) on a heating stage inside the hood Colonies were typically cut into square pieces approximately 0.5 mm^2 and proportions of the colonies that were overtly differentiated were avoided.
4. The media from each plate was aspirated and colonies washed with 1 ml PBS^+ per culture dish. A volume of 1 ml of Dispase II (10 mg/ml) dissolved in hESC serum medium was applied to each dish for 5 min at 37°C in a CO_2 incubator. Dispase dislodges the cut colony pieces from the culture dish.
5. The colony pieces were picked up using a 20 μl pipette (Gilson) and transferred to a dish filled with 1ml PBS^+. Another wash was conducted to remove Dispase by transferring then pieces into a second dish containing 1 ml PBS^+.
6. The washed pieces were carefully placed on a newly prepared MEF feeder cell coated plate (at $6x10^4$ cell/cm^2 density) filled with fresh hESC serum medium. Approximately 6-9 colony pieces were seeded per dish equidistant from each other. The dishes were placed carefully at 37°C in a CO_2 incubator, making sure that pieces did not move and clump together.
7. Media changes were conducted every second day until the next transfer event by aspirating old serum medium and adding a new pre-warmed 1 ml aliquot per organ culture dish.

2.1.2.3.2- Establishing serum-free cultures (bulk cultures)

1. Serum hESC medium was aspirated from 4-6 organ culture dishes bearing undifferentiated hESC colonies grown for 7 days.
2. The culture dishes were washed with 1 ml sterile PBS^+ solution. A volume of 1 ml of Dispase (10 mg/ml) dissolved in hESC serum medium was applied to each dish for 5 min at 37°C in a CO_2 incubator.
3. The hESC colonies were dislodged and aspirated using a 200 μl pipette (Gilson) into a 500 μl volume of transfer medium [30% (v/v) FCS-hESC medium, 70% (v/v) SR medium with FGF-2] in a sterile polypropylene 1.7 ml microtube.
4. Using a 200 μl pipette (Gilson) the colonies were triturated into a fine cell clumps. 500 μl of cell clumps was then injected using a 1000 μl pipette (Gilson) into a T-25 tissue culture flask containing 4.5 ml of transfer medium. The flask was labelled accordingly: the name of the hESC line and the existing serum grown passage number + 1 SR/FGF-2 passage number, for example HES4 p32+1. The T-25 tissue culture flask was placed at 37°C in a CO_2 incubator, agitated repeatedly to prevent hESC clumps sticking together and left ON.
5. The next day, transfer medium was aspirated along with free floating cell debris and 7 ml of SR hESC medium with FGF-2 (4 ng/ml) was added to a T-25 flask. The flask was examined for attached cell clumps. The media was changed daily Monday-Friday and once over the weekend. The cells were routinely monitored under a microscope for the extent of differentiation in culture and scored as poor, average or good. Poor = overt differentiation, Average = some differentiated areas, Good = minimal differentiation

2.1.2.3.3- Subculturing hESC in serum-free conditions

Bulk cultures were routinely split with Collagenase type I on Day 7 or when hESC culture reached approximately 80-90% confluency. Flasks with fresh MEF (plated at $2x10^4/cm^2$ density) with SR media plus 4 ng/ml FGF-2 were pre-equilibrated in the morning.

1. Collagenase type I (4 mg/ml) (w/v) (Worthington) was dissolved in unsupplemented DMEM/F12 media at RT. The enzyme solution was filtered through a 0.22 μm filter (Millipore), ensuring sterility.
2. Existing culture medium was aspirated and the flasks washed with 5 ml of PBS^-. The wash solution was removed and 1-2 ml of 4 mg/ml Collagenase type I was added to a T25 flask and incubated at 37°C for 15-20 min.
3. The flask was tapped until MEF were released leaving only hESC colonies slightly curled on flask surface.
4. Then hESC colonies were flushed from flask surface with 5ml of SR media (without FGF-2) using a 5 ml pipette and a cell suspension was collect into a 15 ml Falcon tube. The larger cell clumps were dissociated by pipetting up and down several times.
5. Cell suspension was filtered using a 70 μm BD Falcon Cell Strainer [Cat. No: 352350] to avoid the carrying over of larger clumps of differentiated hESC.
6. The cells were spun for 2 min at 500 x g and the supernatant aspirated. The cells were resuspend with 2-3 ml of SR media plus 4 ng/ml FGF-2. The split ratio depends on a number of factors such as flask confluency and cell health, morphology and growth rate patterns. Such characteristics may vary from week to week and can be observed over several passages.

2.1.2.3.4- Freezing hESC in serum-free conditions

1. hESC colonies were harvested from tissue culture flasks, triturated and purified according to the protocol on section 2.1.2.2.3. Cell material from confluent T-25 tissue culture flask was used for the freezing procedure.
2. hESC clumps were resuspended again in 5 ml SR medium with FGF-2 and centrifuged at 500 x g for 2 min. The medium was aspirated and the cell pellet gentle flicked to ensure dispersal of cell clumps.
3. 1 ml of ice-cold Freezing medium [90% DMEM/FCS medium, 10% DMSO (v/v)] was added, gentle resuspending the cell clumps using a sterile plastic 2 ml pipette.
4. The aliquots were pipetted into 1ml cryovials (Falcon). The vials were closed tightly and the lids wrapped in parafilm followed by immediate transfer to a Nalgene 1°C isopropanol freezing container. The container was placed at -80°C ON.
5. The next day, the cryovials were removed from -80°C and placed into liquid nitrogen for long term storage.

2.1.2.3.5- Thawing hESC grown in SR with FGF-2

1. The designated hESC containing cryovial was removed from liquid nitrogen and its cap loosened to allow trapped nitrogen gas to escape.

2. The cell content was thawed by immersing the bottom half of cryovial in 37°C water bath and swirling the tube repeatedly. Care was taken not to immerse the whole tube in the water bath as this can compromise sterility.
3. Immediately after thawing, the content was transferred into a 15 ml centrifuge tube (Falcon) and 10 ml of SR medium at RT was added drop by drop to the tube and immediately centrifuged at 500 x g for 2 min.
4. The DMSO containing supernatant was removed and the cell pellet resuspended in 5 ml of fresh SR medium with FGF-2. This cell washing step was repeated once again.
5. The tube was centrifuged and the 5 ml of wash medium aspirated. The pellet was resuspended in 5 ml of pre-warmed SR medium with FGF-2 and transferred into a T-25 flask containing fresh MEF feeders. The flasks were placed at 37°C in a CO_2 incubator, agitated again repeatedly to prevent hESC clumps sticking together and left to attach and resume growth.

2.1.2.3.6- Culturing hESC in MEF-free conditions on Matrigel

Serum-free cultures of hESC on low density MEF were split onto Matrigel-coated vessels for short term (1 week maximum) experiments. hESC were not serially subcultured under MEF-free conditions. The same protocol was followed as in section 2.1.2.3.5 for subculture of hESC in serum-free conditions, except that collagenase-treated hESC clumps were seeded onto Matrigel-coated vessels with MEF CM instead of onto flasks containing MEF. Approximately 30,000 hESC per cm^2 were seeded on flasks coated with Matrigel (with coating concentration of 0.0347mg/cm^2) and pre-equilibrated with MEF conditioned media supplemented with 4 ng/ml FGF-2. Media was changed daily.

2.1.2.4- Growth and Maintenance of 293FT cell line

Cell line 293 FT was purchased from Invitrogen (Cat No: R700-07) and was used for the production of lentiviral stocks.

2.1.2.4.1- Thawing Cells

1. A vial of frozen cells (supplied in a vial containing $3x10^6$ cells/ml) removed from liquid nitrogen and thawed quickly in a 37°C water bath.
2. Just before the cells were completely thawed, the outside of the vial was decontaminated with 70% ethanol, and the cells transferred to a sterile 15 ml tube containing PBS. Briefly the cells were centrifuged at 500 x g for 2 min and the cells resuspended in 2 ml complete medium without Geneticin®.
3. The cells were transferred to a T-75 cm2 flask containing 10 ml of complete medium without Geneticin®.
4. The flask was incubated overnight at 37°C to allow the cells to attach to the bottom of the flask.
5. The next day, the medium was aspirated off and replaced with fresh, complete medium containing 500 µg/ml Geneticin®.

6. The cells were incubated and checked daily until 80-90% confluent.

2.1.2.4.2- Subculturing Cells

1. All medium was removed from the flask and the cells were washed once with 10 ml PBS to remove excess medium and serum. Serum contains inhibitors of trypsin.
2. 2 ml of trypsin/EDTA solution were added to the monolayer and incubated 3 minutes at room temperature until cells detached. The cells were checked under a microscope to confirm detachment.
3. 8 ml complete medium containing Geneticin ® were added and the cell suspension was transferred to a 15 ml sterile, conical tube.
4. The number of viable cells was determined using the trypan blue exclusion method.
5. The cells were seeded at $3x10^6$ cells/cm^2 density diluted in pre-warmed complete medium containing 500 µg/ml Geneticin®. The flask was incubated at 37°C.
6. The cells were maintained as adherent monolayer cultures in complete medium containing 500 µg/ml Geneticin®.

2.1.2.5- Differentiation of hESC Activin A and BMP4

Activin A and BMP4 were used to differentiate hESC.

1. Briefly, hESC were seeded on Matrigel with CM from MEF.
2. When cells reached about 70% confluency, Activin A (20 ng/ml and 40 ng/ml) was added to cells in serum-free medium published by Ng *et al*. (2005) and BMP4 (25 ng/ml and 50 ng/ml) was added to cells in SR medium.
3. The media was changed every two days and these factors were added at each change.
4. After 7 days of exposure to Activin A and BMP4, the cells were harvested and total mRNA was isolated (described in section 2.2.9 of this Chapter).

2.1.2.6- Teratoma formation

The ability of hESC to form teratomas is the best test of pluripotency presently available. The cells are implanted for testing beneath the testis capsule of SCID mice between 5-6 weeks of age. The first part of the procedure, carried out to exteriorise the testis, is similar to the procedure used in vasectomy. The examination of teratomas was carried out by Professor Stephen Fox from the Peter MacCallum Cancer Centre (Melbourne, Australia).

1. The cells were prepared about 15-20 min before surgery. Between 2 to 3 organ culture dishes (8 hESC colonies/dish) were needed to inject into 2 mice. Tumours can be formed using 8 hESC colonies (containing about 50,000 cells in total) per mouse.
2. The cells were washed twice with PBS^+. Using a pulled glass pipette a cut was made around colonies which were harvested Dispase.
3. The colonies were collected and washed twice. The colonies were then transferred into a microcentrifuge tube and broken up by pipetting up and down with a 200 microliter Gilson pipettor.

4. The cells were centrifuged and resuspended in 50 μl of hESC injection buffer (DMEM, 25 Mm HEPES, 10% FCS) and kept on ice.
5. Using aseptic technique within a laminar flow hood, the animal give strain sex and age of mice was anesthetised with avertin.
6. The abdomen was swabbed with alcohol, and a small longitudinal incision was cut through the skin and abdominal wall just bellow the level of origin of the hindlimb. The animal was transferred to the stage of a dissecting microscope.
7. The scrotum of the mouse was squeezed to bring the testes into the abdominal cavity. A piece of fat was visible inside the incision towards the caudal part of the mouse and just a bit lateral to the midline and this tissue was drawn outside the abdomen with a forceps and the vas deferens and testis followed.
8. A small artery clamp was used to gently put traction on the testis, which was fully outside the body. A 26 gauge needle was used to make a small hole in the testis capsule in a region where there are no major blood vessels.
9. The cell inoculum was injected using a pulled glass pipette, approximately 10 μl/testicle. The cells were inserted into the testis about halfway to avoid escaping of cells.
10. After the testis was returned well inside the abdominal cavity, the procedure was repeated with the other testis. Then the internal and external incisions were closed separately with 6-0 silk.
11. The mice were monitored until they awakened from the anaesthetic, and checked again the following day.
12. The animals were monitored weekly beginning at around four weeks for tumour development. Clinical examination was carried out by bringing the testis down into the scrotum and palpating it. Lesions become apparent by about five weeks as swellings. The mice were humanely euthanized, and the tumours were removed, fixed in formalin and sent for routine histological processing.
13. Sections of teratomas were stained with hematoxylin and eosin (H&E) and examined under a bright field microscope.

2.1.2.7- Karyotyping analysis

hESC lines were sent to karyotyping analysis to Cytogenetics Labotory, Monash Medical Centre, Clayton, Victoria, Australia.

Section 2.2- Molecular Biology Methods and Materials

2.2.1- Commonly Used Molecular Biology Reagents and Equipment

General laboratory reagents utilised were of molecular biology grade and were supplied by Sigma-Aldrich, Merk or Invitrogen unless otherwise stated. Composition of commonly used molecular biology reagents and commercial suppliers is described with their methodology in this chapter. Wherever possible, composition of reagents supplied in kits or reaction mixes have been detailed in the text.

Equipment used in specific laboratory procedures is outlined in the relevant sections of the text. The *Eppendorf 5417R* and *5415D* microcentrifuges (Eppendorf) were routinely used for centrifugation of 0.5-1.7ml tubes. For procedures involving larger volumes or temperature controlled conditions, a *Multifuge 3 S-R* centrifuge (Heraeus, Germany) was utilised. Bacterial plates were incubated in a Memmert incubator Beschickung LM 100-800 (Schawabach/Germany).

2.2.2- Imunological Material and Methods

2.2.2.1- Mounting materials

Vectashield ® Mounting Medium (Vector Laboratories, CA, USA) [Cat No: H-1000]. The mounting medium was applied neat as a thin film of solution over slides.

Glass coverslips 22x60 mm (Menzel-Glaser, Mannheim, Germany) [Deckglaser # 1] and (HD Scientific Supplies Pty Ltd).

Blue Glass 12 well slides (IGN Industries, Victoria, Australia)

Nail polish, clear (L'Oreal, Paris, France)

2.2.2.2- Nuclear Dyes

4',6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich, Steinheim, Germany) [Cat No: D9542]. The stock solution was diluted 1:2000 in ddH_2O to a final working concentration of 10 μg/ml. DAPI was applied to cell DNA on slide preparations by covering the slide surface with 0.5 ml working solution and incubating for 2 min at RT.

Propidium iodide (PI) (Sigma-Aldrich, Steinheim, Germany) [Cat No: 70335] PI was used to label cell viability (0.5 μg/ml).

2.2.2.3- Primary antibodies

TABLE 2.1- Primary antibodies names, source, dilution used and application

Reagent	Source	Dilution	Application
CD9 mouse monoclonal IgG_{2a} (Clone TG30) anti-human antibody	Hybridoma supernant prepared in the laboratory	Applied neat	IC, FL
GCTM-2 mouse monoclonal IgM anti-human antibody	Hybridoma supernant prepared in the laboratory	Applied neat	IC, FL
Mitochondrial marker mouse monoclonal IgG1 (clone 1E6) anti-human antibody	Hybridoma supernant prepared in the laboratory	Applied neat	IC
Mouse Monoclonal IgG_{2b} anti-human Oct3/4 (C-10) antibody	(Santa Cruz Biotechology, CA, USA) [Cat No: sc-5279]	1:40 1:200	IC, FL WB
Mouse Monoclonal IgG_{2a} anti-human p53 (DO-1) antibody	(Santa Cruz Biotechnology, CA, USA) [Cat No: sc-126]	1:50 1:1000	IC WB
Mouse Monoclonal IgG1 p53 (PAb 1801) antibody	(Abcam) [Cat No: ab28]	1:100	IC
Mouse Monoclonal IgG_1 Mcl1 (RC13) antibody	(Abcam) [Cat No: ab3184]	1:50 1:1000	IC WB
Mouse IgG_1 anti-human/rat/mouse Hdm2 (clone SMP14), ascites fluid	(Sigma-Aldrich) [Cat No: M4308]	1:50 1:1000	IC WB
Rabbit polyclonal IgG anti-human Hdmx antibody	(Abcam) [Cat No: ab16058]	1:50 1:1000	IC WB
Rabbit polyclonal IgG anti-human Bax antibody	(DAKO) [Cat No: A3533]	1:50 1:1000	IC WB
Rabbit polyclonal anti-human/mouse/rat Cleaved Caspase-3(Asp175) antibody	(Cell Signaling) [Cat No: 9661]	1:50 1:1000	IC WB
Mouse Monoclonal IgG_1 anti-human/canine/rat/mouse β-Tubulin (clone SAP.4G5), ascites Fluid	(Sigma-Aldrich) [Cat No: T7816]	1:5000	WB
Mouse Monoclonal IgG_1 anti-human Lamin B Antibody	(Calbiochem) [Cat No: NA12]	1:1000	WB
Mouse Monoclonal IgG_1 anti-human/canine/rat/mouse GAPDH (Clone 6C5) antibody	(Ambion) [Cat No: 4300]	1:4000	WB
Mouse Monoclonal IgG_1 anti-human/canine/rat/mouse Cytochrome c antibody	(BD Pharmingen) [Cat No: 556433]	1:200	WB
Rabbit polyclonal IgG anti-human PUMA antibody	(Calbiochem) [Cat No: PC686]	1:1000	WB
Mouse Monoclonal IgG_1 anti-human Noxa antibody	(Abcam) [Cat No: ab13654]	1:1000	WB

Rabbit polyclonal IgG anti-human Bcl2 (ΔC21) antibody	(Santa Cruz Biotechnology) [Cat No: sc-783]	1:50 1:1000	IC WB
Mouse Monoclonal IgG_1 Nanog antibody	eBioscience [Cat No: 14-5769]	1:1000	WB
Mouse Monoclonal IgG_1 p21 antibody	(BD Pharmingen) [Cat No: 554228]	1:1000	WB

Abbreviations: FC = flow cytometry, IC = immunocytochemistry, WB = western blotting

2.2.2.4- Isotype controls

TABLE 2.2- Isotype controls names, source, dilution used and application

Reagent	Source	Dilution	Application
Purified mouse IgG_1	BD Pharmingen [Cat No: 553447]	*	FL
Purified mouse IgG_1	DAKO [Cat No: X 0931]	*	IC
Purified mouse IgG_{2a}	BD Pharmingen [Cat No: 553447]	*	FL
Purified mouse IgG2a	DAKO [Cat No: X 0943]	*	IC
Purified mouse IgG2b	BD Pharmingen [Cat No: 559530]	*	FC
Purified mouse IgG2b	DAKO [Cat No. X 0944]	*	IC
Purified mouse IgM	BD Pharmingen [Cat No. 553472]	*	FC

* The dilution of isotype-matched negative control antibodies is the same as dilution of primary antibody for each experimental application.

2.2.2.5- Secondary antibodies

TABLE 2.3- Secondary antibodies names, source, dilution used and application

Reagent	Source	Dilution	Application
Alexa Fluor ® 488 conjugated goat anti-mouse IgG_1	Molecular Probes, Eugene, USA [Cat No: A21121]	1:500	IC, FL
Alexa Fluor ® 488 conjugated goat anti-mouse IgG_{2a}	Molecular Probes, Eugene, USA [Cat No: A21131]	1:500	IC, FL
Alexa Fluor ® 488 conjugated goat anti-mouse IgG_{2b}	Molecular Probes, Eugene, USA [Cat No: A21141]	1:500	IC
Alexa Fluor ® 488 conjugated goat anti-mouse IgM	Molecular Probes, Eugene, USA [Cat No: A21042]	1:500	IC
Alexa Fluor ® 568 conjugated goat anti-mouse IgG_1	Molecular Probes, Eugene, USA [Cat No: A21124]	1:500	IC
Alexa Fluor ® 568 conjugated goat anti-mouse IgG_{2b}	Molecular Probes, Eugene, USA [Cat No: A21144]	1:500	IC
Alexa Fluor ® 568 conjugated goat anti-Rabbit (H+L)	Molecular Probes, Eugene, USA [Cat No: A11011]	1:500	IC
Alexa Fluor ® 647 conjugated goat anti-mouse IgG_{2b}	Molecular Probes, Eugene, USA [Cat No: A21242]	1:2000	FL
Alexa Fluor ® 647 conjugated goat anti-mouse IgM	Molecular Probes, Eugene, USA [Cat No: A21238]	1:2000	FL
HRP conjugated polyclonal rabbit anti-mouse Ig-HRP	(Dako Cytomation, Glostrup, Denmark) [Cat No: P0260]	1:1000	WB
HRP conjugated goat anti-mouse Ig-HRP	(Jackson Immuno Research Laboratories, PA, USA) [Cat No: P0161]	1:1000	WB
HRP conjugated polyclonal goat anti-rabbit IgG	(Dako Cytomation, Glostrup, Denmark) [Cat No: P0448]	1:1000	WB

Abbreviations: FC = flow cytometry, IC = immunocytochemistry, WB = western blotting.

2.2.2.6- Western blotting solutions

Skim milk powder (Coles Supermarket, Clayton, Victoria, Australia) was prepared fresh at 5% (w/v) in TBS-T (see *Appendix I* for solution preparation) for blocking of western blots.

30% Acrylamide/Bis Solution (Bio-Rad) [Cat No. 161-0156] is obtained as a 30% Solution of Acrylamide/Bis at a 29:1 w/w ratio and stored at 4˚C in dark.

Restore Western Blot Stripping Buffer (Pierce) [Catalog number 21059] was stored at RT and applied neat for removal of primary and secondary antibody probes from PVDF and nitrocellulose blots to enable re-probing.

2.2.3- Indirect immunofluorescence microscopy methods

Fluorescent immunostained samples were analyzed and photographed using an Olympus BX51 microscope and ULH100HG epifluorescence system with DP70 camera. For confocal microscopy, a Leica TCS NT laser scanning confocal microscope attached to an inverted Leica DMIRB/E fluorescence was used.

2.2.3.1- Fixation and permeabilization of cells

Various cell lines were fixed onto slides or coverslips.

2.2.3.1.1- Ethanol (EtOH) or Methanol (MeOH) fixation protocol

1. The cells were washed gently cells twice with cold PBS¯, taking care not to flush cells off the surface of the slide or coverslip.
2. Ice-cold EtOH or MeOH was added and incubated 5 minutes on ice.
3. EtOH or MeOH were aspirated, and the preparations were air dried and either stained or wrapped in plates in parafilm and store at -20˚C until needed.

2.2.3.1.2- Paraformaldehyde (PFA) fixation protocol

1. Medium was aspirated from the slides/coverslips and these were rinsed well with ice cold PBS.
2. 4% PFA (see *Appendix I* for solution preparation) solution was added to the slides and left for 15 minutes at RT°C.
3. PFA solution was aspirated from the Plate or slide chamber and slides were washed with PBS.
4. Immediately following fixation, the samples were processed, beginning with the permeabilization step.

2.2.3.1.3- Permeabilization (for PFA-fixed specimens)

PFA-fixed cells to be used for immunostaining were first permeabilized to allow the antibody access across the cell and nuclear membranes.

1. A PBS solution containing 0.1% Triton X-100 was incubated over the cells for 5 min on ice.
2. Samples were washes twice with PBS before antibody labeling.

2.2.3.2- Antibody labelling and examination of cells

1. The majority of staining was performed on 8-well chamber slides or coverslips. In this last case coverslips were put on top of inverted microtube lids that served as a platform.
2. A blocking solution (usually 10% SR medium) was added and incubated with the cells at RT for 15 minutes.
3. Primary antibody was diluted and placed on slides according to manufacturer's directions. In some cases a range of antibody dilutions was performed to determine an optimal concentration for staining. Coverslips required 100 μl solution while chamber slides required 50 μl per well.
4. Coverslips or slides were incubated for 2 hour at RT or at 4˚C ON.
5. Slides were washed three times with PBS and similarly incubated with the appropriate secondary antibody at RT 30 minutes or at 4˚C ON.
6. Slides were washed three times with PBS as previously and subsequently counterstained with DAPI (1 μg/ml).
7. Cells were mounted under a glass coverslip (Menzel Glaser) with Vectashield mounting solution (Vector Labs) to minimise bleaching of fluorescence, sealed with nail polish and analyzed and photographed an Olympus BX51 microscope and ULH100HG reflected fluorescence system with DP70 camera or on the Leica confocal system.
8. The slides were archived at 4˚C and protected from light.

2.2.4- Flow cytometry methods

Indirect immuno-labeled hESC were analyzed with CXP software (Beckman Coulter) using a Cytomics FC 500 series flow cytometry system (Beckman Coulter) or a BD FACSVantage SE flow cytometer with FACSDiva option (Beckton Dickinson Biosicences). Fluorescence-activated cell sorting (FACS) and *In situ* Cell proliferation assay were carried out on the FACSDiva instrument with the assistance of Andrew Fryga and Darren Ellemor.

2.2.4.1- Apoptosis and flow cytometry analysis

Etoposide (Sigma-Aldrich) [Cat No: E1383], gamma-irradiation, human TNF alpha (Sigma-Aldrich) [Cat No: T0157], serum removal and hydrogen peroxide were used as apoptosis-inducing agents. Cyclic pifithrin-α (cPFT, 25 μM, (Sigma-Aldrich) [Cat No: P4236], pifithrin-μ (PFT-mu, 10 μM) (Calbiochem) [Cat No:506155] and Bongkrekic acid (BA, 50 μM, Sigma-Aldrich) [Cat No: B6179] were added 1 h before the treatment with etoposide. zVAD-fmk (25 μM, Sigma-Aldrich) [Cat No: V116], Cycloheximide (40, 10 and 4 μM, Sigma-Aldrich) [Cat No: C7698], Leptomycin B (5 ng/ml, Sigma-Aldrich) [Cat No: L2913] and Cyclosporine A (10 μM, Sigma-Aldrich) [Cat No: 30024] were added 30 min before the treatment with etoposide.

2.2.4.1.1- Annexin V staining

Annexin-V-Fluos binds in a Ca^{2+} -dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine, which during early stages of apoptosis translocates from the inner part of the plasma membrane to the outer layer becoming exposed at the external surface of the cell (Vermes *et al*., 1995). Annexin-V-FLUOS Staining Kit (Roche Applied Science) [Cat No: 11858777001] was used for detection and quantification of apoptosis.

1. hESC cells were cultured for 5 days on Matrigel with MEF conditioned medium supplemented with FGF-2 and treated exposed to hydrogen peroxide or DMEM/F12 alone for 22 h.
2. After treatments, floating cells were collected and adherent cells were harvested with Cell Dissociation Solution (Gibco) (2ml/T25). These cells were pooled and pelleted by centrifugation for 2 min at 500 x g.
3. The cells were washed with PBS^- and resuspended at $1x10^6$ cells/ml.
4. The cells were centrifuged for 2 min at 500 x g and the cell pellet was resuspended in 100 μl of Annexin-V-FLUOS labelling solution containing PI.
5. The suspension was incubated for 20 min at RT.

2.2.4.1.2- OCT4-Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) detection assay

This flow cytometry immunoassay quantifies DNA strand breaks by using terminal deoxynucleotidyl Tranferase (TdT), which catalyses polymerization of labelled nucleotides to free 3'-OH DNA ends. *In Situ* Cell Death Detection Kit, Fluorescein from (Roche Applied Science) [Cat No: 11684795910] was used for detection and quantification of apoptosis. Simultaneously, cells can be characterized further for expression of the marker Oct4.

1. hESC cells were cultured for 5 days Matrigel with MEF conditioned medium supplemented with FGF-2. To test the sensitivity of hESC to apoptosis-inducing agents, cells were treated with Etoposide (Sigma-Aldrich) and gamma-irradiation. The cells were analysed for apoptosis at different doses of agent and different time points. cPFT (25 μM, Sigma-Aldrich), PFT-mu (10 μM, Sigma-Aldrich) and BA (50 μM, Sigma-Aldrich) were added 1 h before the treatment with etoposide. zVAD-fmk (25 μM, Sigma-Aldrich) and Cycloheximide (4 μM, Sigma-Aldrich) were added 30 min before the treatment with etoposide.
2. After treatments, floating cells were collected and adherent cells were harvested with Cell Dissociation Solution (Gibco) (0.5 ml/well of 6-well plate or 2ml/T-25). These cells were pooled and pelleted by centrifugation.
3. The cells were washed with PBS^- and resuspended at $1x10^6$ cells/ml.
4. Cells were divided in 1 ml aliquots and resuspended in 500 μl of freshly prepared Fixation solution [PFA 2% in PBS^- (pH 7.4)]. The cells were incubated at RT for 1h. (Note: before adding PFA cells were in a small volume of PBS to avoid clump formation). After fixation, the cells were centrifuged at 375 x g for 2 min and the fixation solution was gently aspirated.

5. The cell pellet was resuspended in 200 μl of Permeabilization solution [Triton X-100 0.1% in PBS^-] for 2 min on ice. The cells were centrifuged at 375 x g for 2 min and the permeabilization solution was aspirated.
6. The cells were washed twice with PBS^-.
7. Using a P200 micropipette, 45 μl of Label Solution ® (Roche Applied Science) [Cat No: 11684795910] containing fluorescein conjugated nucleotides was then mixed with 5 μl of Enzyme Solution ® containing terminal deoxynucleotidyl transferase from calf thymus, recombinant in *E. coli*, in storage buffer (Roche Applied Science). The cell pellet was resuspended in this reaction mixture and the cells incubated for 1h at 37 °C.
8. The pellet was briefly resuspended in 200 μl of PBS, the cells were centrifuged at 375 x g for 2 min and the wash solution was aspirated.
9. The cells were resuspended in 200 μl of SR medium with 1:40 of monoclonal antibody Oct3/4 C-10 (Santa Cruz) and incubated on ice for 1h. Purified mouse IgG_{2b} (BD Pharmingen) was used as isotype control at same concentration as Oct3/4 antibody. The cells were pelleted for 2 min at 375 x g and washed with 500 μl.
10. After pelleting, cells were resuspended in 200 μl of SR with 1:1000 of appropriate secondary antibody Alexa Fluor anti-mouse IgG_{2b} 647 (Molecular Probes- Invitrogen) and incubated on ice for 1h.
11. Cells were again washed with in PBS, resuspended in 500 μl PBS and samples were then ready for flow cytometry analysis. Cells were gated initially using forward and right angle light scatter and AF488 and AF647 fluorescence signals were collected. Data were analyzed and present using CXP Analysis Software (Beckman Coulter).

2.2.4.1.3- Measurement of mitochondrial membrane potential

MitoProbe™JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) assay kit for flow cytometry (Molecular Probes, Invitrogen) [Cat No: M34152] as used to measure mitochondrial membrane potential and as performed accordingly to manufacturer instructions.

1. Cell Dissociation Solution (Gibco) was used to harvest cells and then these were washed with PBS.
2. Cells ($1x10^6$ cells) were incubated with 1 μM JC-1 (reconstituted in DMSO) diluted in KSR medium for 30 min at 37 ºC in the dark.
3. Cells were washed with PBS, resuspended in PBS and analyzed by flow cytometry (Cytometrics FC 500 System, Beckman Coulter).
4. As a positive control, cells were incubated with 100 mM of CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a mitochondrial membrane-potential disrupter, for 5 min prior the addition of JC-1.

2.2.4.1.4- Cell staining for viability

LIVE/DEAD® Fixable Dead cell stain kit (green fluorescence, Molecular Probes, Invitrogen) [Cat No: L23101] was used to distinguish apoptosis from necrosis and the assay was performed accordingly to manufacturer instructions.

1. Cells were harvested with Cell Dissociation Solution (Gibco) and washed with PBS.
2. Cells ($1x10^6$ cells) were incubated with 1 μl of fluorescence reactive dye (reconstituted in DMSO) diluted in SR medium for 30 min on ice in the dark.
3. Cells were washed with PBS, resuspended in PBS, fixed in 4% PFA solution and analyzed by flow cytometry (Cytometrics FC 500 System, Beckman Coulter).

2.2.4.1.5- Cell proliferation assay

In Situ Cell Proliferation Kit, FLUOS (Roche Applied Science) [Cat. No: 11810740001] was used to measure cell proliferation (incorporation of BrdU) and the assay was performed according to the manufacturer's instructions.

1. BrdU labeling was performed by diluting BrdU labeling reagent 1:1000 in the culture medium for 2h in the incubator at 37 °C and 5% CO_2.
2. Culture medium was aspirated and the flasks washed with 5 ml of PBS^-. The wash solution was removed and TrypLE Express Stable Trypsin Replacement Enzyme (Gibco) was applied to form a thin film over a confluent T-25 flask (1 ml) or a well of a 6-well plate (Matrigel cultures- 0.5 ml).
3. BrdU labelled cells were washed in PBS.
4. The cells were resuspended in 250 μl of TG30 monoclonal antibody supernant containing mouse anti CD9 (1:1) and incubated on ice for 30 min. Purified mouse IgG_{2a} (BD Pharmingen) was used as an isotype control (1:125). Cells were pelleted for 2min at 375 x g and washed with 500 μl of PBS.
5. After pelleting, cells were resuspended in 200 μl of SR with 1:1000 of secondary antibody Alexa Fluor anti-mouse IgG_{2a} (Molecular Probes- Invitrogen) and incubated on ice for 30 min. Then cells were washed twice with PBS.
6. The cells were fixed with fixative solution (70% Ethanol in 50 mM Glycine buffer, pH 2.0) and then cells were washed twice with PBS.
7. The cells were incubated in denaturation solution (4 M HCl) and then cells were washed twice with PBS and the pH was measured to make sure that was above 6.5.
8. To block, cells were incubated with incubation buffer for 10 min at RT.
9. The cells were resuspended in 50 μl of anti-BrdU-FLUOS antibody working solution (1:5 anti-BrdU-FLUOS in incubation buffer) for 45 min at 37°C and then washed twice with PBS.
10. Before analysis on the flow cytometer, cells were resuspended in 500 μl PBS^- containing 5 μg/ml of PI.
11. Data were analyzed and present using CXP Analysis Software (Beckman Coultier).

2.2.4.1.6- Preparation of cells for FACS

1. Culture medium was aspirated and the flasks washed with 5 ml of PBS^-. The wash solution was removed and Cell Dissociation Buffer solution (Gibco) was applied to form a thin film over a confluent T-25 flask (1 ml) or a well of a 6-well plate (Matrigel cultures- 0.5 ml). The cells were treated for 5 min in an incubator at 37 °C and 5% CO_2.

2. The Cell Dissociation Buffer solution was aspirated from flasks and hESC colonies were sloughed off by gently and repeatedly spraying the entire culture flask or well with SR medium at RT using a 5 ml sterile pipette attached to a Drummond Scientific Co. express pipet-aid on the high speed setting (Becton Dickinson).
3. The wash medium (5 ml) was aspirated, transferred to a sterile 15 ml Falcon tube and centrifuged 2 min at 375 x g. The medium as aspirated and the cells were washed with SR medium.
4. The cells were resuspended in 500 μl of monoclonal antibody GCTM-2 and TG30 supernant (TG30) (1:1) and incubated on ice for 30 min. Purified mouse IgG_{2a} and IgM (BD Pharmingen, Cat # 559530) were used as isotype controls (1:125). Cells were pelleted for 2min at 375 x g and washed with 500 μl of PBS.
5. After pelleting, cells were resuspended in 200 μl of SR with 1:1000 of appropriate secondary antibodies Alexa Fluor anti-mouse IgG_{2a} and anti-mouse IgM 647 (Molecular Probes-Invitrogen) and incubated on ice for 30 min.
6. PI (1:1000 from a 1 mg/ml stock solution) was added to determine cell viability.
7. Cells were again washed with in PBS^-, resuspended in 500 μl PBS^- and samples were then ready for flow cytometry analysis. Cells were gated initially using forward and right angle light scatter and AF488 and AF647 fluorescence signals were collected. Data were analyzed and present using CXP Analysis Software (Beckman Coultier).
8. Double positive cells were sorted and colleted to a tube containing SR medium.
9. Samples were then processed for RNA extraction.

2.2.5- SDS-PAGE and Western Blotting

Sodium dodecyl sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by western blotting was used to determine the size and presence of proteins expressed in various cell lines.

2.2.5.1- Preparation of samples

1. Laemmli buffer was prepared (see *Appendix I* for solution preparation).
2. Cells were harvested with Cell dissociation buffer (Gibco) and washed once with 1ml PBS (Gibco).
3. The PBS was aspirated the carefully and 250 μl of Laemmli buffer was added per sample (per well of 6 well plate).
4. Samples were then transferred to tubes and these were covered with boiling caps and boiled for 10 minutes on a heating block.
5. Samples were stored at -20˚C for later use.

2.2.5.2- Preparation of SDS-PAGE Gels

SDS-PAGE gels were prepared in two stages: a resolving gel and a stacking gel. The resolving gel was made with 30% acrylamide/bis solution (Bio-Rad) [Cat No. 161-0156] at a range of 8 to 15% final concentration of acrylamide depending on protein size (Table 2.4). Stacking gel was made up at acrylamide concentration of 4% (Table 2.5).

TABLE 2.4- Solutions for preparing resolving gels for SDS-PAGE

Resolving gel	8%	10%	12%	15%
	10 ml	10 ml	10 ml	10 ml
MQ H_2O	4.6	4.0	3.3	2.3
1.5 M Tris pH 8.8	2.5	2.5	2.5	2.5
30% acrylamide/bis	2.7	3.33	4.0	5.0
10% SDS	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1
TEMED	0.006	0.004	0.004	0.004

TABLE 2.5- Solutions for preparing stacking gels for SDS-PAGE

Stacking gel	4%
	4 ml
	2.7
0.5 M Tris pH 6.8	0.67
30% acrylamide/bis	0.5
10% SDS	0.04
TEMED	0.04
10% APS	0.004

1. To prepare 10 ml of 12% resolving gel the following reagents were combined: 4ml of 30% acrylamide solution, 2.5ml of 1.5M Tris HCl (pH 8.8), 100µl SDS (10% stock) and ddH_2O to 10ml. 4 µl of the polymerizing agent TEMED (Bio-Rad) [Cat No: 161-0801] was added along with 100µl of freshly made 10% Ammonium Persulfate (APS) (Sigma-Aldrich) [Cat No: A3678]. The solution was mixed and immediately poured into the gel cast before being overlaid with 1-2ml isopropanol. The resolving gel was allowed to set at RT for approximately 20 min.
2. The isopropanol was poured off the resolving gel and allowed to evaporate off before the stacking gel was cast. 4% stacking gel is composed of 1 ml of 30% acrylamide solution, 1 ml of 0.5M Tris HCl (pH 6.8), 60 µl SDS (10% stock) and ddH_2O to 6 ml. The polymerizing agents were added as for the resolving gel before casting.

2.2.5.3- Electrophoresis of SDS-PAGE gels

1. The samples were denatured by boiling at 100°C for 2 min.
2. The samples were briefly centrifuged at 10,000 x g for 1- 2 min before loading (10-22 µl of sample per lane) in duplicate. A protein size standard ladder, Kaleidoscope™ Prestained Standards (Bio-Rad) [Cat No: 161-0324] was run on each gel to allow determination of sample molecular weight.
3. Protein samples were electrophoresed at 100 V in 1x Running Buffer [25 mM Tris, 192 mM Glycine, 0.05% SDS] for 1.5-2 h until the dye front reached the end of the gel.
4. Gels were carefully removed from the casting plates and prepared for transfer.

2.2.5.4- SDS-PAGE Semi-Dry Transfer

Gels were placed into a *Semi-phor* semi-dry transfer apparatus (Hoefer Scientific Instruments) (Amersham Biosciences) and transfer of proteins was conducted according to manufacturer's instructions.

1. A PVDF transfer membrane (Amersham Pharmacia Biotech) [Cat No: RPN303F] previously washed 5 min in MeOH for 5 min, transferred to MilliQ H_2O for 5 min and finally incubated in running buffer for 30 min.
2. The gel was removed and assembled with transfer membrane as recommended by the manufacturer of the apparatus (2x blotting paper, membrane, gel, 2x blotting paper, cover).
3. A 100 mA current, approximately 10-15V, was applied for 1 h at RT. When finished, the apparatus was dismantled.

2.2.5.5- Immunohybridization and detection of proteins

1. A blocking agent, 5% skim milk in PBS-Tween 0.01% (PBS-T), was incubated with the membranes for 1 h or overnight at 4˚C to prevent non-specific hybridization.
2. The membranes were incubated with a primary antibody specifically directed to the protein of interest diluted in 5% skim milk in PBS-T for 1 hour at RT or overnight at 4˚C on the rocker. A heat sealer was used to make a bag for each blot to be probed. For most full sized blots, 2.5 ml solution was applied to cover the blot.
3. Membranes were washed 3x 5 min with PBS-T.
4. HRP labeled secondary antibody was diluted in 5% skim milk in PBS-T for 1 hour at RT on rocker.
5. Membranes were washed 2x 5 min with PBS-T and 1x5 min with PBS.

2.2.5.6- Detection of proteins

The proteins were then detected using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) [Cat No: RPN2132] or ECL Western Blotting Substrate (Pierce) [Cat No: 32106]. Bio-Rad Gel ChemiDoc™ XRS System was used to acquire the images.

1. Once detection reagents were warmed to RT, the two detection solutions were mixed according to the manufacturer's instructions. The final volume of detection reagent required is 0.1 ml/ cm^2 (for normal sized blot approximately 1-2ml). In case of ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, the solutions A and B were mixed in a ration of 40:1. When ECL Western Blotting Substrate (Pierce) was used equal volumes of solutions 1 and 2 were mixed.
2. Excess wash buffer was drained from washed membranes and these were placed on the black tray within the gel-doc system.
3. The detection reagents were pipetted on top of membrane covering it surface. The tray was placed into the gel-doc system and 5 minutes were allowed for occurrence of the substrate reaction before proceeding with imaging.
4. Blots were exposed for different intervals of time, depending on primary antibody used, and images were acquired using Gel ChemiDoc™ XRS System (Bio-Rad). Lastly, software based densitometric analysis (Gel-Pro Analyser version 3.1, MediaCybernetics) was performed on cross-reactive bands and the β-tubulin loading controls.

2.2.5.7- Stripping and re-probing membrane

1. Blots were washes with PBS-T and were incubated with Restore PLUS Western Blot Stripping Buffer (Pierce) [Cat No: 21059] for 15 minutes at RT on a rocker.
2. The membranes were blocked with 5% skim milk in PBS-T for 1 hour at RT or ON at 4˚C on the rocker and then probed with the next antibody according to the protocol described in section 2.2.7.3.

2.2.6- Cell subfractionation

Cell subfractionation was performed to obtain cytosolic fraction to determine release of cytochrome c from the mitochondria.

1. hESC were washed with PBS and permeabilized with 20 μg/ml digitonin (Sigma-Aldrich) [Cat No: D141] for 10 min at 37^{o}C.
2. The cytosolic/soluble fraction was collected with a P1000 micropipette to a 1.7ml tube and boiled in 3x Laemmli sample buffer.
3. Cells were washed twice with PBS and boiled in Laemmli sample buffer (non-soluble fraction).
4. Samples were then processed and analysed through SDS-PAGE and Western blotting.

2.2.7- DNA laddering protocol

DNA laddering is a technique used to identify DNA cleavage that occurs during apoptosis.

1. After treatment with etoposide for different time points, cells hESC and apoptotic bodies were collected then pelleted and the supernant was discarded.
2. The cells were resuspended on ice in 20 μl of lysis buffer (50 mM tris-HCl (pH 8.0), 0.5% SDS, 0.5 mg/ml proteinase K added fresh) and pipetted to ensure complete lysis
3. The lysate was incubated at 55^{o}C for 60 min.
4. The cell debris was pelleted and then 5 μl of 1 mg/ml RNase A was added to the supernatant. The suspension was incubated at 55^{o}C for another 60 min.
5. The sample was spun briefly to pellet any further cell debris and the supernatant was collected.
6. The lysate was heated to 70^{o}C for a few minutes and mixed with 10 μl of loading buffer with 1% low melting point agarose (Scientifix) [Cat No: 9040A].
7. The samples were run on a 2% agarose gel (Quantum Scientific) [Cat No: 200-0011] containing 0.5 μg/ml ethidium bromide and run at 40V for 2h using a Bio-Rad power pack until appropriate separation of bands had occurred.
8. The DNA laddering was visualized and photographed under UV light in a Gel ChemiDoc^{TM} XRS System (Bio-Rad).

2.2.8- Lentiviral-mediated delivery of shRNA

HES-2 cells and HES-4 were transduced with lentivirus carrying short hairpin RNA (shRNA) designed to knockdown p53 or GFP using the BLOCK-it^{TM} Lentiviral RNAi Expression System (Invitrogen) [Cat No: K4944-00], as described by the manufacturer. The p53 and GFP shRNA sequences used were the following:

p53A top strand

5'-CACGCGCACAGAGGAAGAGAATCTCGAAAGATTCTCTTCCTCTGTGTGCGC-3' and

p53A bottom strand

5'-AAAAGCGCACAGAGGAAGAGAATCTTTTCGAGATTCTCTCTTCCTCTGTGCGC-3'.

p53B top strand

5'-CACCGTCTGTGACTTGCACGTACCGAAGTACGTGCAAGTCACAGAC-3' and

p53B bottom strand

5'-AAAAGTCTGTGACTTGCACGTACTTCGGTACGTGCAAGTCACAGAC-3'.

GFP top strand

5'-CACCGAAGAAGTCGTGCTGCTTCATCGAAATGAAGCAGCACGACTTCTTC-3' and

GFP bottom strand

5'-AAAAGAAGAAGTCGTGCTGCTTCATTTCGATGAAGCAGCACGACTTCTTC-3'.

Cells were plated on Matrigel and infected with lentivirus. Apoptosis assays and Western blotting analysis were performed as described above.

2.2.8.1- Producing lentivirus in 293FT cell line

TABLE 2.6- Optimized transfection conditions for production of lentiviral stocks in 293FT cells

Conditions	Amount
Tissue culture plate size	T75
Number of 293FT cells to transfect	6 x 10^6 cells
Amount of ViraPower™ Packaging Mix	11.5 µg
Amount of pLenti6/BLOCK-iT™-DEST expression plasmid	4 µg
Amount of Fugene 6 Transfection Reagent	36 µl

1. Fugene 6 Transfection Reagent was pipetted directly into 800 µl of DMEM High glucose without allowing contact with the walls of the plastic tube and incubated 5 min at RT.
2. Then 4 µg of pLenti6/BLOCK-iT™-DEST expression plasmid and 9 µg of ViraPower™ Packaging Mix were added to Fugene 6 Transfection Reagent and the complex was mixed and incubated for 20 min at RT.
3. The complex was added to the cells in a drop-wise manner. The flasks were swirled to ensure distribution over the entire plate surface. Antibiotics were omitted from the culture medium of 293FT cells during production of virus.
4. The next day the media containing the DNA-Fugene 6 complexes was removed and replaced with complete media containing sodium pyruvate.
5. Virus-containing supernatants were harvested 48-72 hours posttransfection by removing medium to a 15 ml sterile, capped, conical tube and filtered sterilizing it using a 0.45 µM filter.
6. The viral supernatants were pipetted into cryovials in 1 ml aliquots and stored at -80°C.

2.2.8.2- Titering Lentiviral Stock

2.2.8.2.1- Determining Antibiotic Sensitivity

1. HT1080 cells and HES cells were plated at approximately 25% confluence. Set of 6 plates were prepared. The cells were allowed to adhere ON.

2. The next day, the culture medium was substituted with medium containing varying concentrations of Blasticidin (0, 2, 4, 6, 8, 10 µg/ml Blasticidin) (Invitrogen) [Cat. No: R210-01].
3. The selective media was replenished every 3-4 days, and the percentage of surviving cells was observed.
4. The appropriate concentration of Blasticidin that kills the cells within 10-14 days after addition of antibiotic was determined.

2.2.8.2.2- Transduction and Titering Procedure

1. The day before transduction (Day 1), the HT1080 cells were trypsinized, counted and plated in 2 x 10^5 cells per well a 6-well plate such that they were 30-50% confluent at the time of transduction. The cells were incubated at 37°C ON.
2. On the day of transduction (Day 2), the lentiviral stock was thawed and 10-fold serial dilutions ranging from 10^{-2} to 10^{-6} were preapared. For each dilution, the lentiviral construct was diluted into complete culture medium to a final volume of 2 ml.
3. The culture medium was removed from the cells. Each dilution was mixed gently by inversion and added to one well of cells.
4. Polybrene® (Sigma-Aldrich) [Cat No: 10768-9] was added to each well to a final concentration of 6 µg/ml. The plate was swirled gently to distribute the added solutions and incubated at 37°C ON.
5. The following day (Day 3), the media containing virus was removed and replaced with 2 ml of complete culture medium.
6. The following day (Day 4), the medium was removed and replaced with complete culture medium containing the appropriate amount of Blasticidin (10 µg/ml) to select for stably transduced cells.
7. Medium was replaced with fresh medium containing Blasticidin every 3-4 days.
8. After 10-12 days of selection (day 14-16), no live cells were seen in the mock well and discrete Blasticidin-resistant colonies appeared in one or more of the dilution wells. The medium was removed and the cells washed twice with PBS.
9. Crystal violet solution was added (1 ml for 6-well dish) and incubated for 10 minutes at RT.
10. The crystal violet stain was removed and the cells washed with twice with PBS.
11. The blue-stained colonies were counted and the titer of the lentiviral stock was determined.

2.2.8.3- Transducing hESC with lentivirus

1. On the day of transduction, the lentiviral stock was thawed.
2. The culture medium was removed from the HES-2 and HES-4 cells on Matrigel.
3. To each well was added Polybrene® was added to a final concentration of 6 µg/ml.
4. An appropriate lentiviral stock dilution was added. As control no viruses was added to a well. The plate was swirled gently to mix and incubated at 37°C ON.
5. On the following day, the media containing virus was removed and replaced with 2 ml of culture medium.

6. The following day, the medium was removed and replaced with complete culture medium containing the appropriate amount of Blasticidin (2.5-7.5 μg/ml) to select for stably transduced cells.
7. The medium was replaced with fresh medium containing Blasticidin every day.
8. After 10-12 days of selection (day 14-16), the absence of live cells in the mock well was confirmed The cells were harvested and subcultured with serum-free medium on $2x10^4$ cell/cm^2 density of MEF.

2.2.9- Total RNA isolation

RNA isolation was conducted with an RNeasy Mini Kit (Qiagen) [Cat No: 74104], according to manufacturer's instructions. Buffer RPE was prepared by adding 4 volumes of EtOH (RNase-free), as indicated on the bottle, to obtain a working solution and Buffer RLT was made by adding 10 μl β-ME per 1ml RLT immediately prior to use.

1. Cells were pelleted in a microtube by centrifuging at 375 x g for 2 minutes and then washed in PBS.
2. The cell pellet was resuspended in 350 μl Buffer RLT +β-ME and mixed by pipetting.
3. The sample was homogenized by passing the lysate through a 21-gauge needle (with 1ml syringe) 5-6 times or using a Quiashredder columns (Qiagen) [Cat No: 79654].
4. 350 μl (1 volume) of 70% EtOH (RNase-free) were added to the homogenized lysate and mixed well by pipetting.
5. 700 μl sample were applied, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube (supplied with the kit). The lid was closed and centrifuged for 15 seconds at 10,000 x g. The flow-through was discarded
6. 350 μl Buffer RW1 were pipetted into the RNeasy mini column and centrifuged for 15 seconds at 10,000 x g. The flow-through was discarded.
7. DNAse treatment was performed using RNase-free DNase Set (Qiagen) [Cat. No: 79254]. 70 μl of buffer RDD was added to a 10 μl aliquot of DNase I and mixed gently by tapping. DNase I incubation mix (80 μl) was pipetted directly onto the spin column membrane, incubate at RT for 15 min.
8. 350 μl Buffer RW1 were pipetted into the RNeasy mini column at the end the flow-through and collection tube were discarded.
9. The RNeasy column was transferred to a new round bottom collection tube. 500 μl of Buffer RPE were pipetted onto the column, the lid was closed and centrifuged for 15 seconds at 10,000 x g. The flow-through was discarded.
10. Another 500 μl Buffer RPE were added to the column, the lid was closed and centrifuged for 2 minutes at 10,000 x g to dry the RNeasy silica-gel membrane.
11. To eliminate any possible Buffer RPE carryover, the collection tube was discarded and placed the RNeasy column placed in a new collection micro tube. Tubes were centrifuged for 1 minute at 10,000 x g.

12. To elute, the RNeasy column were transferred to a new 1.5 ml snap cap collection tube (supplied). 30-50 µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was closed and centrifuged for 1 minute at 10,000 x g to elute.
13. RNA concentration and purity were determined using a ND-1000 spectrophotometer (Nanodrop®, Wilmington, DE, USA) using the software ND-1000 v3.2.1 (Nanodrop®, Wilmington, DE, USA) and total RNA was stored at -80˚C until needed.

2.2.10- Gene expression profile analysis

The samples were sorted by flow cytometry for the presence of the ESC markers CD9 and GCTM-2. Two separate hESC lines were used (HES-2 and HES-4) and there were 3 replicates of each. Expression profiling was performed using Sentrix Human-6 V2 Expression BeadChip arrays from Illumina®. RNA was harvested from HES-2 and HES-4, as described above, using RNeasy (Qiagen). The aim was to compare hESC which have been transduced with shRNA for GFP to the ones transduced with p53 shRNA. Samples were submitted to the SRC Microarray facility with collaboration with Sean Grimmond and Gabriel Kolle from the University of Queensland, Australia.

1. In brief, all RNA samples were assessed for integrity using the Agilent Bioanalyzer 2100 and obtained RNA integrity numbers (RIN scores) above 8.5 as evaluated using the Expert 2100 software version B.02.02.
2. For each sample, amplification was performed on 200 ng of total RNA using the Illumina TotalPrep RNA Amplification kit (Ambion) [Cat No: IL1791] with a 14 hour *in vitro* transcription reaction period.
3. Amplified cRNA (1500 ng per array) was hybridized to Human-6.v2 BeadChip arrays according to the manufacturer's guidelines and detected with Fluorolink Streptavidin-Cy3 (GE Healthcare Biosciences).
4. BeadChip arrays were scanned using the Illumina BeadStation Array Scanner and image intensity values extracted. The raw intensity values obtained for the scanned array images were compiled using the BeadStudio v2.3.41 software from Illumina®.

2.2.10.1- Normalisation of Array data

1. Normalisation of data was performed in R utilising the BeadExplorer and Affy packages. R is a language and environment for statistical computing and graphics (BioConductor: http://bioconductor.org).
2. Raw data from BeadStudio were imported into R using BeadExplorer.
3. The Affy package was used to background subtract the raw data using Robust MultiChip Analysis (RMA) (Irizarry *et al*., 2003).
4. The dataset was normalized per chip by Quantile normalization (Bolstad *et al*., 2003). This data was exported into GeneSpring GX v7.3.1 (Agilent) for visualization.

2.2.10.2- Generation of significant gene lists

1. Differential expression analysis was performed using the limma package from BioConductor in R.
2. For each pairwise comparison, data was fitted to the linear model using lmFit and significance scores for each probe were calculated using the Empirical Bayes method (Smyth, 2004).
3. B-statistical scores were defined for the comparison between GFP and p53 shRNA.
4. Adjustments to the linear fitting model were made for pairwise effects of the different hESC lines.
5. Significant gene lists for each comparison were defined as a B-statistical score of greater than 0 (Greater than 50% chance of true differential expression).

2.2.10.3- Ingenuity Analysis

Significantly differentially expressed genes between the GFP versus p53 shRNA transduced cell lines comparison were uploaded into Ingenuity Pathways Analysis 5.0 (Ingenuity® Systems) on the basis of Genbank Accession Number.

2.2.11- Reverse transcription reaction

A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) [Cat No: 4368814] was used to make cDNA from total RNA.

1. 2x Reverse Trancriptase master mix was prepared combining the reagents in table 2.7 on ice.

TABLE 2.7- Reagents for 2x reverse transcriptase master mix

Component	Volume (μl)
10X RT Buffer	2.0
25X Dntp	0.8
10X Random primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free water	3.2
Total per reaction	10

2. 10 μl of 2x reverse transcriptase master mix were pipetted into an individual tube.
3. 10 μl of RNA sample (1 μg of RNA) were added to each tube and mixed by pipetting up and down. Tubes were briefly centrifuged.
4. The samples were run using the program thermal cycling conditions indicated on Table 2.8.
5. Samples were then diluted 200 times for further analysis.

TABLE 2.8- Thermal cycling conditions for cDNA reverse transcription reaction.

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 sec	∞

2.2.12- Quantitative Polymerase Chain Reaction (Q-PCR)

The TaqMan® Probe Detection assay system from Applied Biosystems was used to perform Q-PCR or real time PCR. TaqMan® probes (Applied Byosystems) [Cat No: 4331182] used were Human Oct4 (Hs01895061_u1), Human Nanog (Hs02387400_g1) and Human p53 (Hs99999147_m1). Human GAPDH (Hs99999905_m1) and Human β-Actin (Hs99999903_m1) were used as an endogenous control.

TABLE 2.9- Reagents for Q-PCR reaction

Component	Volume (μl)
TaqMan®Probe	1.0
Taqman Universal PCR Master Mix (Applied Biosystems) [Cat No: PN 4304437]	10.0
Template	9.0
Total per reaction	20.0

1. Briefly, samples were prepared by mixing all components from Table 2.9.
2. Samples were loaded in triplicate in a Micro-Amp Optical 96-well Reaction Plate (Applied Biosystems) [Cat No: N801-0560] and water was used as a control and then plates were sealed.
3. Samples were run in a 7500 Real-Time PCR System (Applied Biosystems) [Cat No: PN 4351104] using the program thermal cycling conditions indicated on Table 2.10.
4. Data was analyzed using 7500 System SDS Software (Applied Biosystems) [Cat No: PN 4366951].

TABLE 2.10- Thermal cycling conditions for Q-PCR

	Step 1	Step 2	Step 3	
Cycles	1	1	40	
Temperature	50°C	95°C	95°C	60°C
Time	2 min	10 min	15 sec	1min

2.2.13- Statistical analysis

Values were considered to be significantly different when the achieved significance level, or *p*-value, was less than 0.05 (p-value < 0.05 was denoted by *) or less than 0.01 (p-value < 0.01 was denoted by **) using the Student's t-test.

CHAPTER 3

Characterization of hESC apoptosis

Data presented in this chapter was partially published in "p53 is required for etoposide-induced apoptosis of human embryonic stem cells." Grandela C, Pera MF and Wolvetang EJ. Stem Cell Research, 2007. *In Press*.

3.1- hESC exhibit classic apoptotic features

As mentioned in Chapter 1, there is little published work on the occurrence of apoptosis or apoptotic signaling pathways in hESC. So, firstly it was investigated whether hESC display any of the hallmarks of apoptosis in response to DNA damage (reviewed by Lawen, 2003). Inhibition of topoisomerase II with etoposide is known to induce DNA double strand breaks (Hande, 1998; Karpinich *et al.*, 2002a) and thus can be used as a model for environmentally induced DNA damage. Therefore, hESC cultures were incubated with etoposide for different lengths of time and assessed for the occurrence of the typical hallmarks of apoptosis: mitochondrial cytochrome c release to the cytosol, caspase 3 activation, DNA laddering and nuclear condensation. Incubation with 340 nM etoposide for 3 hours causes an increase in soluble cytochrome c in the cytosol of hESC, without affecting the total amount of intracellular cytochrome c (Figure 3.1A). Furthermore, immunofluorescent staining with an antibody specific to activated caspase 3 shows that hESC treated with 340 nM etoposide for 3 hours display caspase 3 activation and that this activation is inhibited by the general caspase inhibitor zVAD-fmk (Figure 3.1B). By 3 to 4 hours after addition of etoposide, hESC start to display the classical 210 bp DNA laddering pattern of apoptosis (Figure 3.1C) accompanied by the nuclear condensation and fragmentation typical of apoptosis (Figure 3.1D). There is no cell cycle arrest in G0/G1 (Figure 3.2A) and no increase in the percentage of necrotic cells (Figure 3.2B) over the time period during which we observe the four diagnostic features of apoptosis. These data altogether strongly indicate that hESC can undergo apoptosis through the intrinsic pathway (mitochondrial) in a caspase-3-dependent mechanism in response to DNA double breaks.

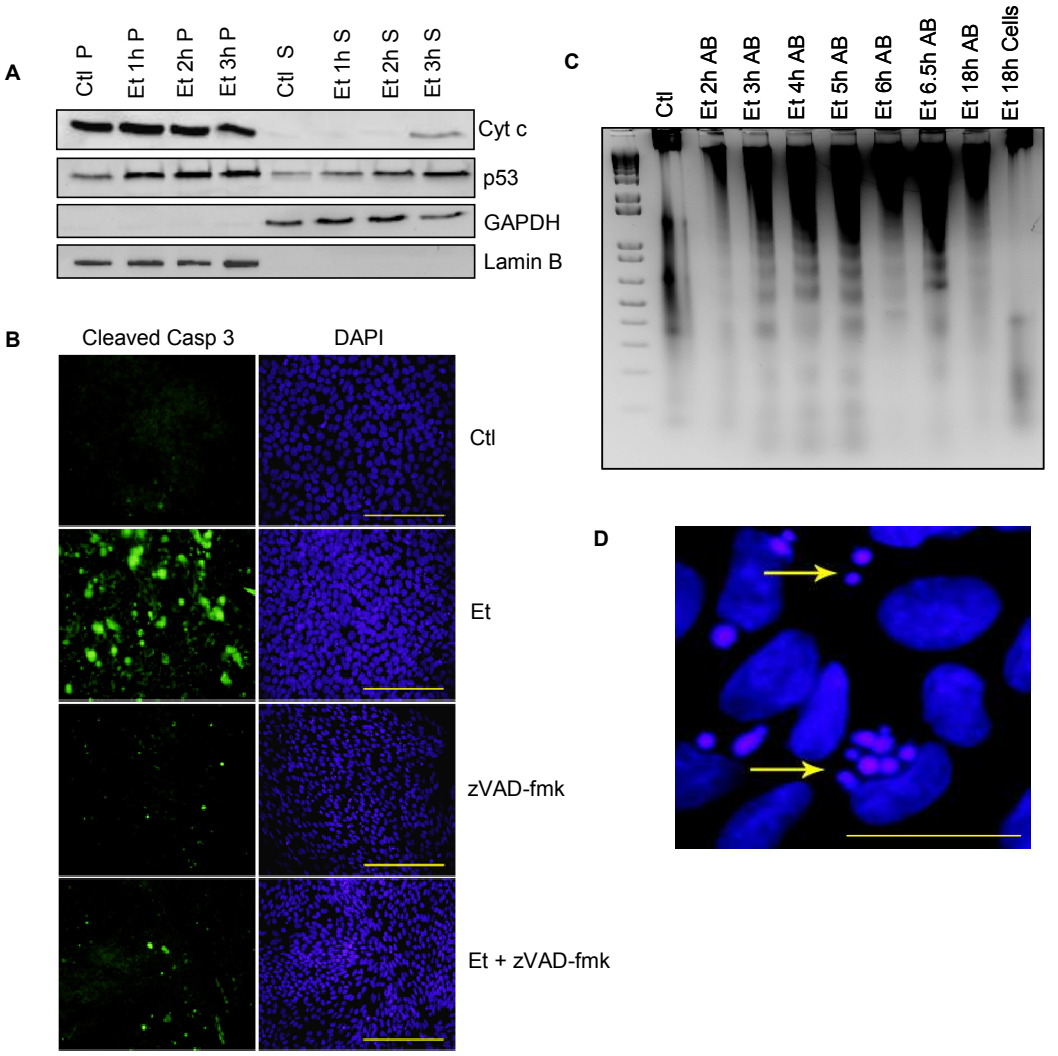

FIGURE 3.1- hESC exhibit classic apoptotic features.
(A) Cytochrome c release. HES-2 cells were treated with 340 nM etoposide for 1, 2 or 3 hours and permeabilized with digitonin. Proteins in the soluble (S- supernant) and non-soluble (P- pellet) fractions were resolved on separate 15 % polyacrylamide gels and Western blotting was performed with p53, cytochrome c, lamin B (nuclear membrane protein) and GAPDH (cytosolic protein) antibodies.
(B) Caspase 3 activation. HES-2 cells were preincubated with 25 μM zVAD-fmk or vehicle for 30 min and then treated with 340 nM etoposide for 3 hours. PFA-fixed adherent colonies were immunostained for cleaved caspase 3 and nuclei visualized with DAPI. Scale bars, 200 μm.
(C) DNA-laddering. HES-2 cells were treated with 340 nM etoposide for 2 to 18 hours and apoptotic bodies and cells were harvested at the indicated time points. DNA was collected and separated on a 1.5% agarose gel. A 1 Kb DNA ladder (Invitrogen) was used as a size standard.
(D) Chromatin condensation HES-2 cells were treated with 340 nM etoposide for 6 hours, fixed with PFA 4% and stained with DAPI. The arrow indicates a hESC with the typical condensed fragmented appearance of an apoptotic nucleus. Scale bars, 50 μm.

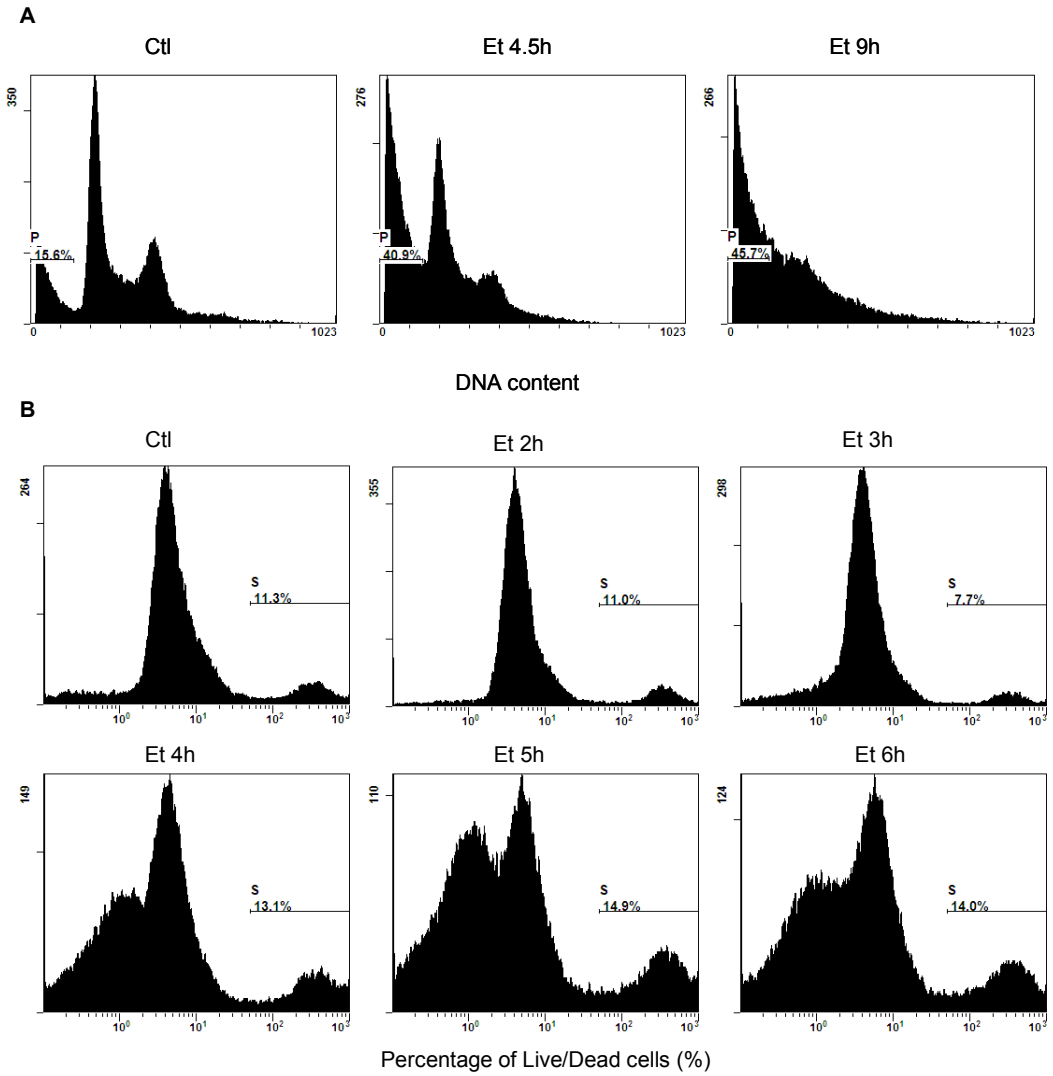

FIGURE 3.2- Etoposide treatment does not induce cell cycle arrest or necrosis.
(A) Etoposide does not induce cell cycle arrest. HES-2 cells were treated with 170 nM etoposide for 4.5 and 9 hours and apoptotic bodies and cells harvested at the indicated time points. Cells were then fixed with 3.7 % PFA, permeabilized with Triton 0.01% and incubated with DAPI (5 μg/ml) diluted in PBS. A representative experiment of three independent experiments is shown.
(B) Etoposide does not induce necrosis. HES-2 cells were treated with 340 nM etoposide for up to 6 hours. Apoptotic bodies and cells were harvested at the indicated time points. Cells were then incubated with green fluorescence reactive dye (LIVE/DEAD® Fixable Dead cell stain) diluted in SR medium for 30 min on ice in the dark, fixed in 4% PFA and analyzed by flow cytometry. A representative experiment of three independent experiments is shown.

3.2- TNFα-induced apoptosis is not operational in hESC

In order to investigate if the extrinsic apoptotic pathway is operational in hESC, the response of hESC to TNFα was investigated. TNFα however failed to induce apoptosis in hESC at concentrations similar to those that induce robust apoptosis in susceptible cell lines (Figure 3.3) (Ghosh *et al.*, 2000; Segui *et al.*, 2001). HepG2 cells, a liver cancer cell line, display resistance to TNFα-induced apoptosis and only die through extrinsic apoptotic stimuli such as TNFα after

preincubation with cycloheximide (CHX) (Santiago-Lomeli *et al.*, 2005). CHX inhibits the NF-κB pathway and consequently blocks the expression of survival factors. When the NF-κB activity subsides, extrinsic proapoptotic signals can induce cell death. In order to determine whether hESC only undergo TNFα-induced apoptosis after CHX treatment, hESC were treated with TNFα in the presence of 10 or 40 μM CHX. However, as shown in Fig 3.3, CHX treatment of hESC induced a substantial amount of apoptosis by itself. Despite the fact that the percentage of apoptotic cells is slightly higher when TNFα is added after preincubation with CHX, this difference is not statistically significant (Figure 3.3). Inhibition of NF-κB activity other than through CHX treatment should be done to confirm that the NF-κB pathway is indeed involved in the inhibition of the activation of the extrinsic apoptotic pathway in hESC.

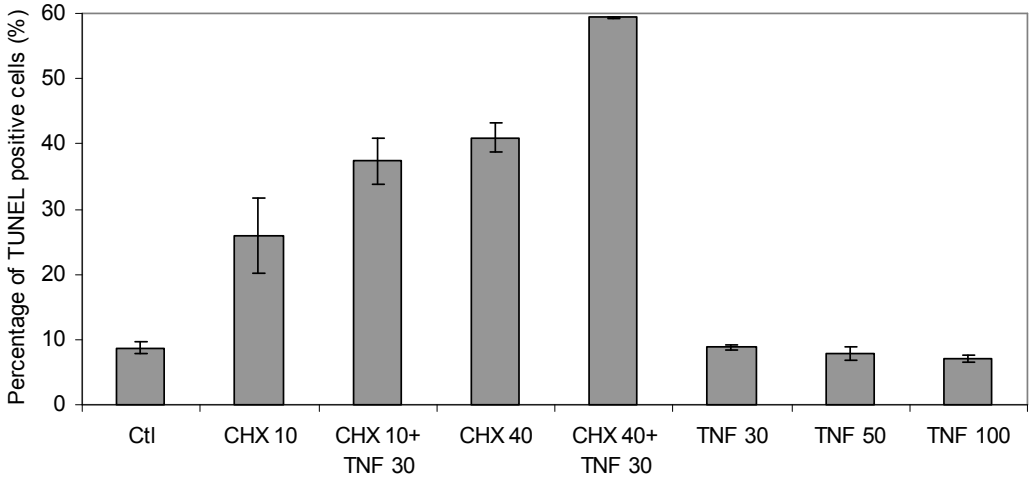

FIGURE 3.3- TNFα fails to induce apoptosis.

HES-4 cells grown in serum-free conditions were preincubated with vehicle alone or with inhibitor (10 μM and 40 μM CHX were added 1 hour before the treatment with TNFα) and subsequently exposed to different concentrations of TNFα (30, 50 and 100 ng/ml) for 24 hours. Floating and adherent cells were harvested, stained with TUNEL and analyzed by flow cytometry. Results are expressed as percentage means ± S.D. (n=2).

3.3- hESC are very sensitive to intrinsic apoptotic stimuli

To further assess the susceptibility of hESC to various different intrinsic apoptosis inducing agents, hESC were subjected to serum removal (Figure 3.4A), addition of hydrogen peroxide (Figure 3.4B), gamma irradiation (Figure 3.4C) and etoposide (Figure 3.4D). Each of these treatments causes a clear increase in apoptosis. Because this study focuses on the role of p53 in hESC and the strict dependence of etoposide-induced apoptosis on p53 in other model systems, the kinetics and dose response of hESC to this drug were carefully determined. The data in Figure 3.4D-F demonstrate the time and concentration dependence of etoposide-induced apoptosis of hESC. hESC are clearly far more sensitive to etoposide-induced apoptosis than other human cell lines (Figure 3.5) or primary cell cultures (Karpinich *et al.*, 2002b; Karpinich *et al.*, 2006; Liu and Joel, 2003). Indeed, exposure of HES2 hESC to 0.17 μM etoposide for 18

hours induces more than 70 % of hESC to undergo apoptosis while the human cell lines HT1080 (fibrosarcoma), 293FT (human embryonic kidney cell line) and CFPac-1 (pancreatic cancer cell line) treated with 0.17 μM etoposide for 24 hours display only 3, 12 and 10 % TUNEL positive apoptotic cells, respectively (Figure 3.5). Because of the exquisite sensitivity of hESC to etoposide and the well established role of p53 in the apoptotic response of human cells to etoposide this drug was selected to further investigate the regulation of apoptosis and mechanism of p53-dependent apoptosis in hESC.

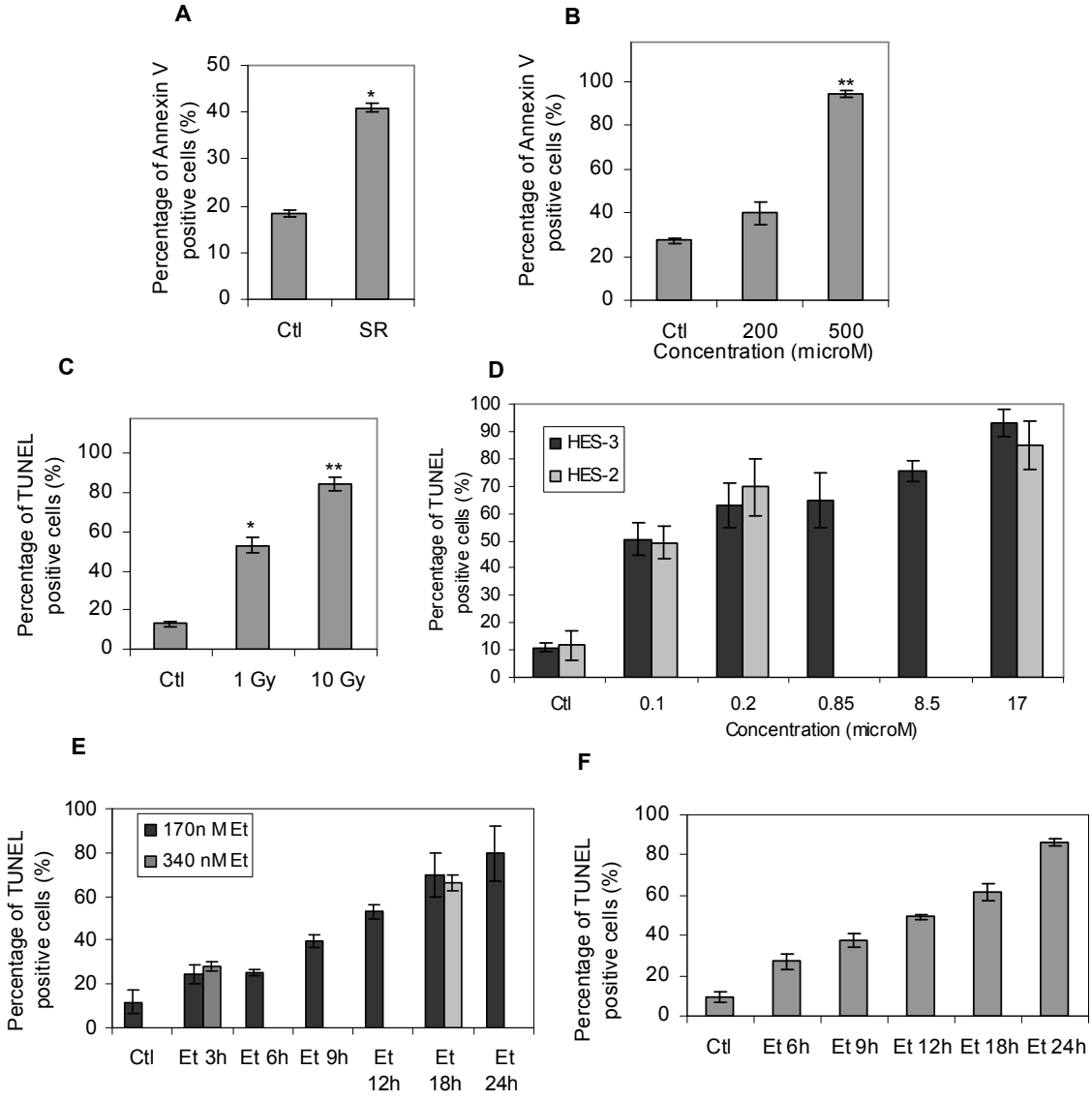

FIGURE 3.4- hESC are very sensitive serum removal, hydrogen peroxide, gamma-irradiation and etoposide.
(A) Serum removal (SR) from HES-2 cells for 22 hours and the level of apoptosis was quantified by Annexin-V-FLUOS Staining Kit (Roche Applied Science).
(B) HES-2 cells were treated with 200 μM or 500 μM hydrogen peroxide for 22 hours and the level of apoptosis quantified by Annexin-V-FLUOS Staining Kit (Roche Applied Science).

(C) HES-2 cells were exposed to 1 Gy and 10 Gy and the level of apoptosis was quantified after 12 hours (*In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science).
(D) HES-2 and HES-3 cells were exposed to increasing etoposide concentrations between 0.17 and 17 μM for 18 hours. Floating and adherent cells were harvested after treatment, TUNEL stained and analyzed by flow cytometry. Results shown are expressed as percentage means ± S.D. (n=3).
(E) HES-2 and (F) HES-4 cells were exposed to 170 nM etoposide for various time points between 3 and 24 hours. Floating and adherent cells were harvested after treatment, TUNEL stained and analyzed by flow cytometry. Results shown are expressed as percentage means ± S.D. (n=3).
(*p*-value < 0.05 by * and *p*-value < 0.01 denoted by ** compared to control).

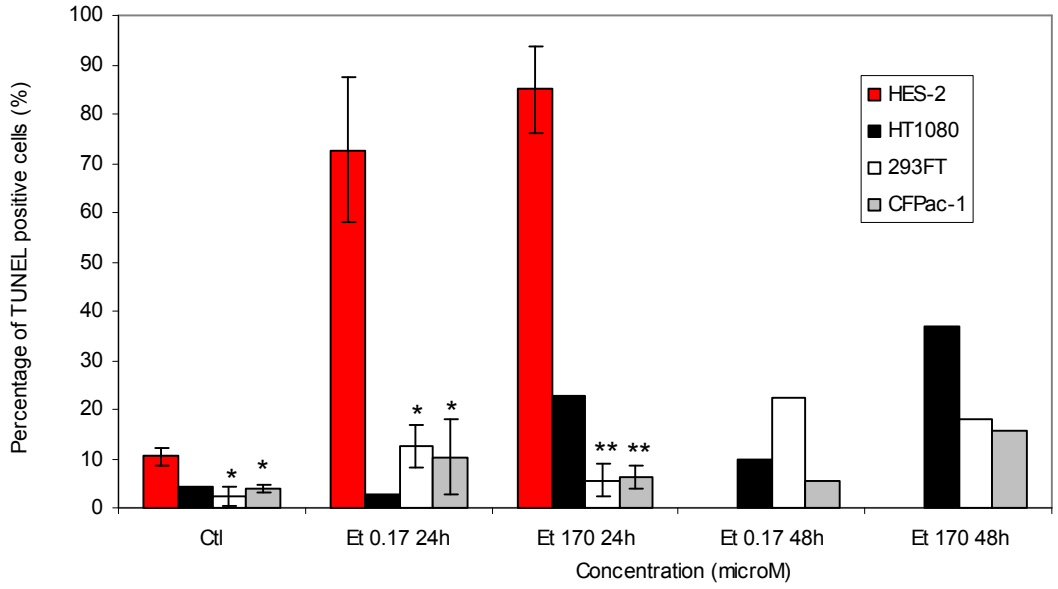

FIGURE 3.5- hESC are more sensitive to etoposide than human cell lines.
HES-2 cells, HT1080, 293FT and CFPac-1 were treated with 0.17 or 170 μM etoposide for 24 or 48 hours. Floating and adherent cells were harvested after treatment, stained with TUNEL and analyzed by flow cytometry. (*p*-value < 0.05 by * and *p*-value < 0.01 denoted by ** compared to HES-2).

3.4- Reduced apoptotic response to etoposide in late passage hESC

As mentioned in Chapter 1, hESC cultured for long periods of time under serum-free conditions are likely to adapt to culture conditions. This adaptation process result most likely from genetic alterations that promote self-renewal and limit differentiation or apoptosis (Baker *et al*., 2007; Enver *et al*., 2005). It has been noted that these adapted hESC tend to gain chromosomal abnormalities resembling the ones observed in hECCs (Baker *et al*., 2007). In order to test if karyotypically abnormal adapted hESC are more resistant to etoposide-induced apoptosis than non-adapted cells, early passage (lower than 10 with normal karyotype) and late passage (higher than 80 with an abnormal karyotype (46XX, t(1;6)(p22;q15)) HES-3 cells (cultured per Amit *et al*, 2000) were exposed to different concentrations of etoposide (Figure 3.6). Interestingly, late passage karyotypically abnormal adapted hESC indeed exhibited reduced apoptotic response to etoposide when compared to early passage cells. It remains to be determined whether the

reduced propensity to undergo apoptosis of late passage adapted hESC is the cause or the consequence of karyotypic abnormalities in hESC. This observation is particularly important when comparing apoptosis studies in hESC from other laboratories, with the present data. All the experiments performed in this study used early passage hESC (P<18) freshly established from standard cultures while most other laboratories routinely use late to very late passage hESC for experimentation.

Nevertheless it is noteworthy that although resistant to low levels of etoposide-induced apoptosis, late passage hESC are still more sensitive than the cancer cell lines examined in this study (see section 3.3).

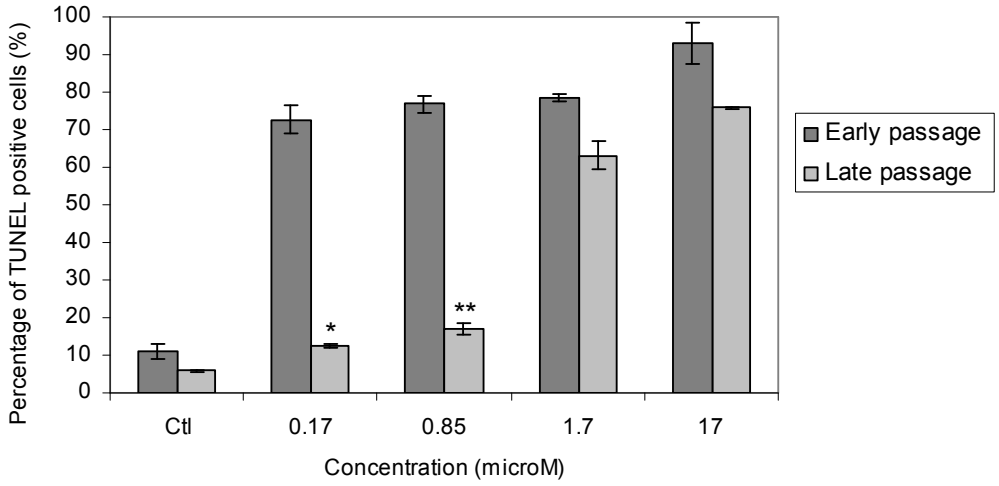

FIGURE 3.6- Early passage hESC with normal karyotype are more sensitive to apoptosis than late passage with abnormal karyotype cells.
HES-3 cells early (P<10) and late passage (P>80) grown in serum-free conditions were treated with 170 or 850 nM etoposide for 18 hours (n=3). Late passage cells were also exposed to 1.7 and 17 μM etoposide (n=2). Floating and adherent cells were harvested after treatment, TUNEL stained and analyzed by flow cytometry.

3.5- The state of differentiation of hESC affects their sensitivity to etoposide-induced apoptosis

hESC cultured on MEF feeder cells in the presence of KSR supplemented with 4 ng/ml FGF-2 (Amit *et al.*, 2004) appear to be a morphologically homogeneous population of undifferentiated cells. However, hESC cultured under these conditions are heterogeneous and display various degrees of differentiation as judged by the expression of the stem cell marker Oct4 when assayed by immunofluorescence or flow cytometry (Figures 3.7, 3.8 and 3.9). Cells at the outer rim of the hESC colonies, usually display reduced expression of Oct4.

Initial observations of hESC treated with etoposide showed that hESC in the center of the colony preferentially died (Figure 3.7A). After treatment, cells at the outer rim of the hESC colonies were more resistant to etoposide apoptosis than hESC in the center of the colonies (Figure 3.7B) and

did no show signs of apoptosis like chromatin condensation (Figure 3.7C). This suggested to us that differentiation may modulate the sensitivity of hESC to etoposide-induced apoptosis.

To quantify this phenomenon, etoposide treated HES-2 and HES-4 cells were double-labeled with both TUNEL and antibody directed against the stem cell marker Oct4. As shown in Figure 3.8, the survival of Oct4-expressing hESC was reduced from around 70% to less than 10% within 18 hours after etoposide treatment. Within this timeframe the proportion of surviving Oct4-negative cells was not significantly reduced, indicating that differentiated hESC are much more resistant to etoposide-induced apoptosis than their less differentiated counterparts. Similar results were obtained for both the HES-2 and HES-4 cell lines, though the latter cell line displaying higher spontaneous differentiation. As shown in Figures 3.7B, 3.8 and 3.10 the Oct4 immunoreactivity of etoposide-treated hESC is lost upon induction of apoptosis. Consequently, the apoptotic hESC do not shift horizontally to the TUNEL-positive quadrant of the flow cytometry plot. To show that the Oct4-positive hESC preferentially undergo apoptosis, the data are expressed as the absolute percentage of surviving TUNEL negative cells (Figure 3.9). The decrease of Oct4-immunoreactivity is due to a loss of the epitope rather than the entire protein because the total amount of Oct4 is not affected by etoposide treatment when analyzed by Western blotting (Figure 3.8). Furthermore, the reduction in Oct4 immunoreactivity in apoptotic hESC is already observed 6-9 hours after etoposide treatment, precluding the possibility that this is somehow due to an in increase in differentiation. Upon exposure to etoposide, immunodetection of cell surface pluripotency markers such as CD9 and GCTM2 is also rapidly lost or takes on an appearance that is not characteristic of those markers (Figure 3.10). These data indicate that the dramatic alterations in nuclear and plasma membrane that accompany apoptosis interfere with the antibody detection of protein epitopes on apoptotic cells. This is possibly due to the activation of transglutaminates during apoptosis that cause cross-linking of proteins, turning them insoluble and presumably less immunogenic (Melino and Piacentini, 1998,Grabarek *et al.*, 2002).

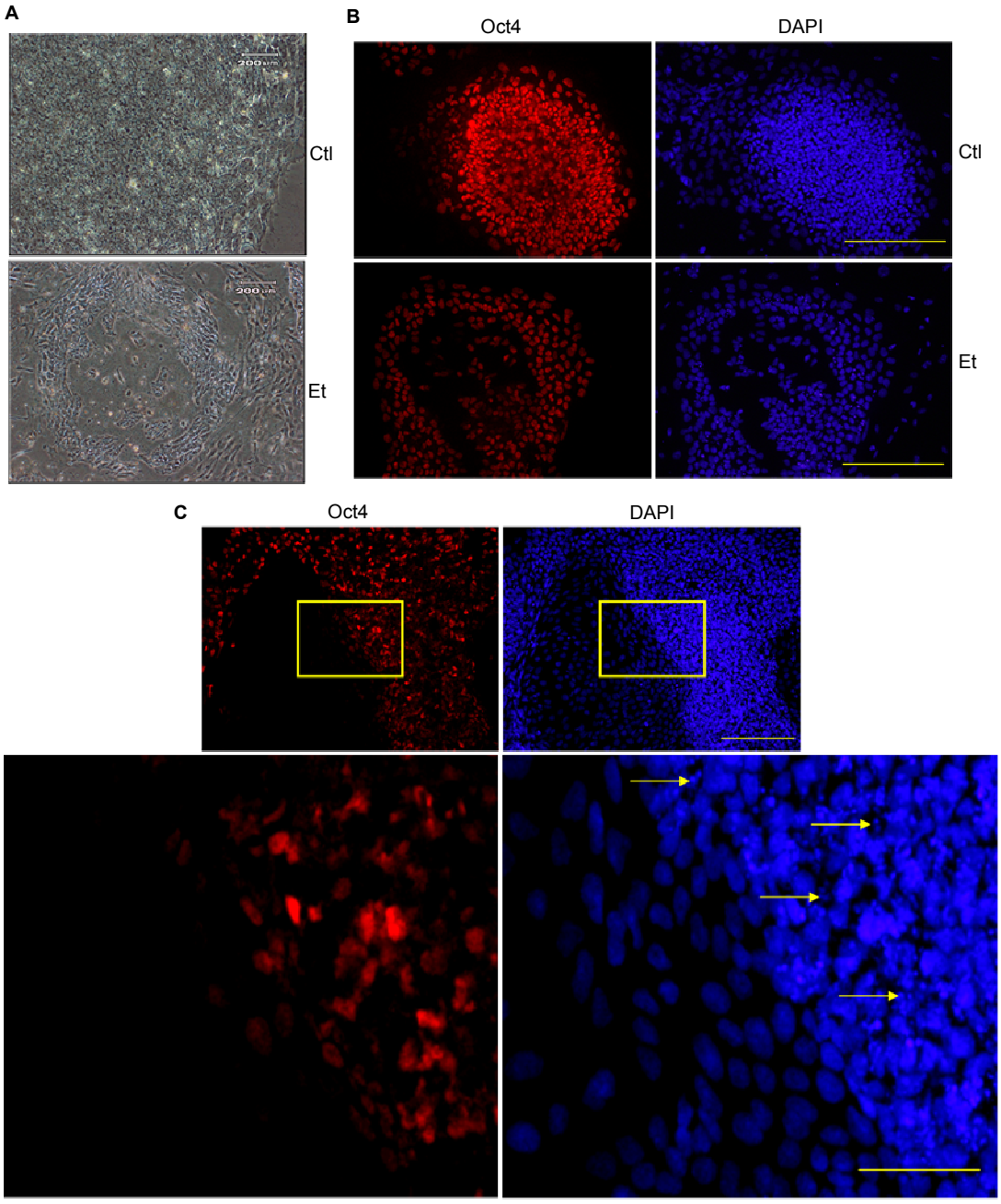

FIGURE 3.7- Oct4 positive cells are more sensitive to etoposide than Oct4 low or negative cells.
(A) HES-4 cells were exposed to vehicle alone or 170 nM etoposide for 18 hours. Scale bars, 200 µm.
(B) HES-2 cells were exposed to vehicle alone or 170 nM etoposide for 6 hours. The hESC cultures were next fixed with 4% PFA and immunostained with the stem cell marker Oct4. Scale bars, 100 µm
(C) HES-2 cells were exposed 170 nM etoposide for 4 hours. Note the reduced non-uniform staining for Oct4 in apoptotic nuclei and that Oct4 positive cells display nuclear condensation and many apoptotic bodies (arrows) while differentiated Oct4 negative cells do not show morphological signs of apoptosis. Scale bar, 200 µm.

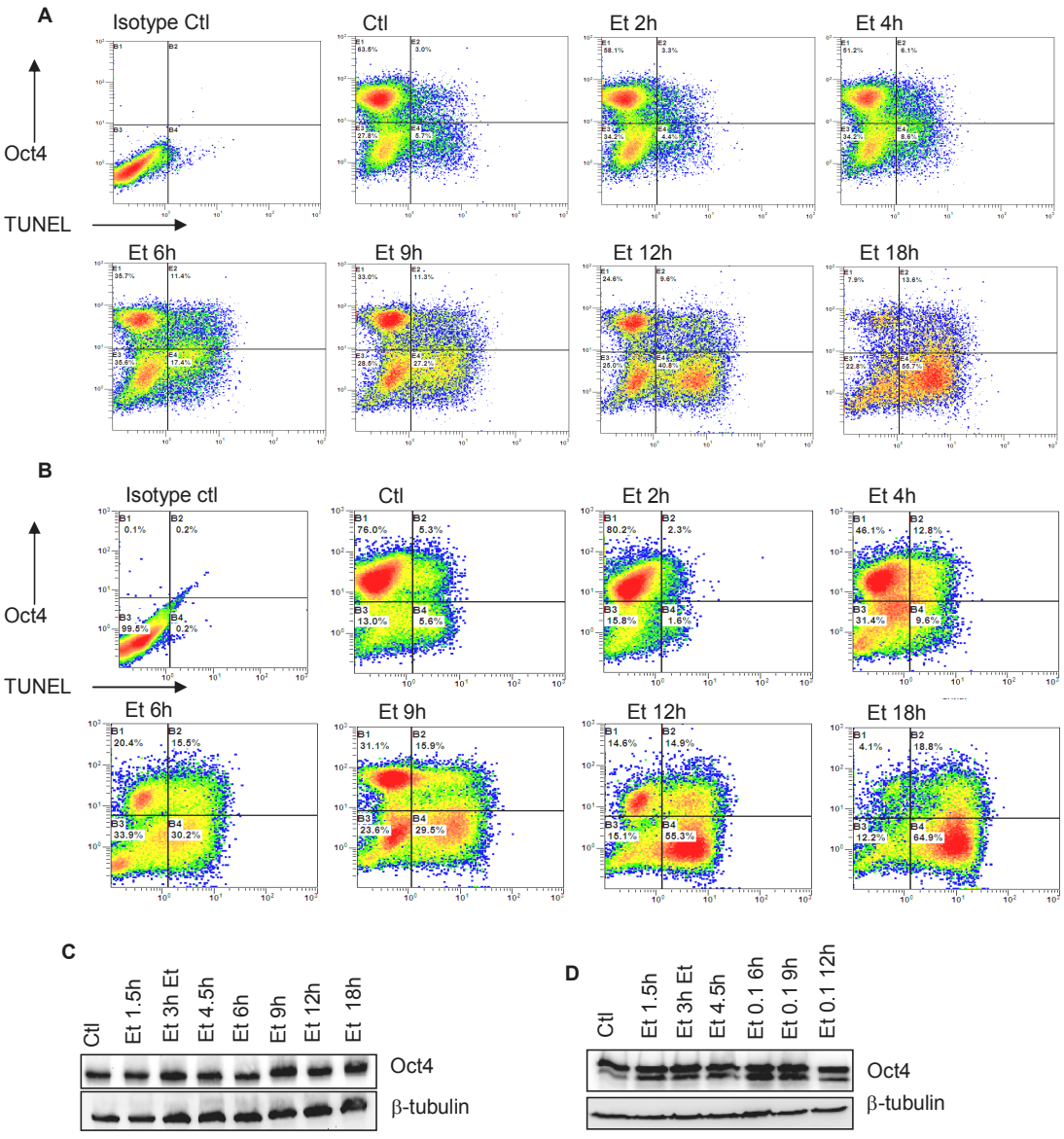

FIGURE 3.8- Flow cytometry analysis of Oct4 expression and TUNEL labelling in hESC treated with etoposide.

(A) HES-4 and (B) HES-2 cells were exposed to 170 nM etoposide and harvested at the indicated time points. Cells were next labeled with TUNEL, immunostained with the stem cell marker Oct4 and analyzed by flow cytometry. Results shown are expressed as percentage means ± S.D. (n=3).

(C) and (D) No reduction of expression or specific degradation of Oct4 occurs during apoptosis in HES-4 (C) and HES-2 (D). hESC were treated with 170 nM etoposide and apoptotic bodies and cells were harvested at the indicated time points. Protein samples were resolved on separate 12 % polyacrylamide gels at 100 V for 1 hour and analyzed by Western blot with Oct4 and β-tubulin antibodies.

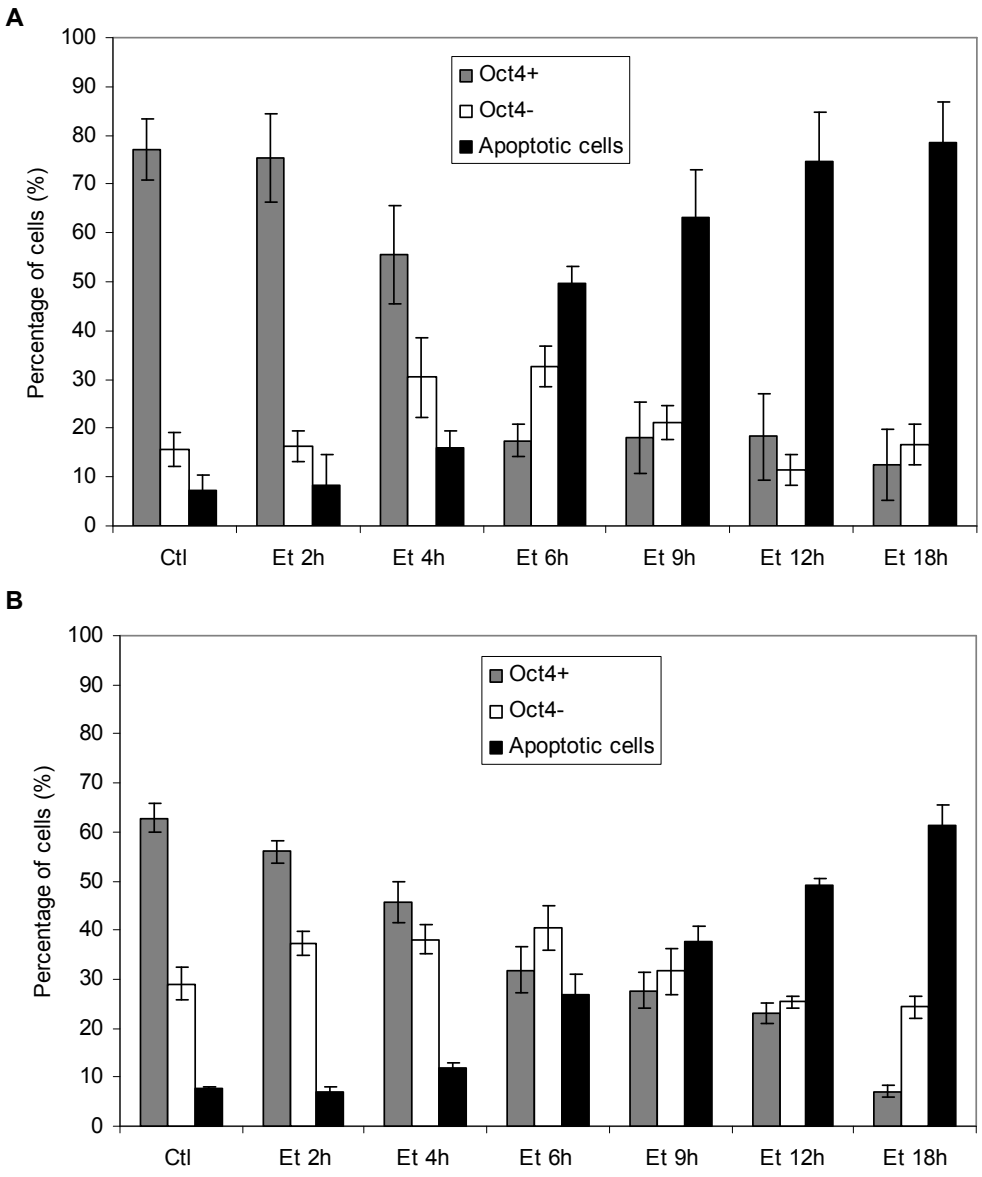

FIGURE 3.9- Differentiation of hESC reduces their sensitivity to etoposide-induced apoptosis.
(A) HES-2 and (B) HES-4 cells grown under serum-free conditions were treated with 170 nM etoposide and harvested after 2, 4, 6, 9, 12 or 18 hours. The etoposide treated hESC were double-labeled with TUNEL and the ESC marker Oct4 and analyzed by flow cytometry. The data are expressed as the percentage of surviving (TUNEL negative) cells in the Oct4 positive (grey bars) and Oct4 negative (white bars) fraction of the hESC culture. Percentage of apoptotic cells is shown by the black bars. The results represent the mean ± S.D. (n=3).

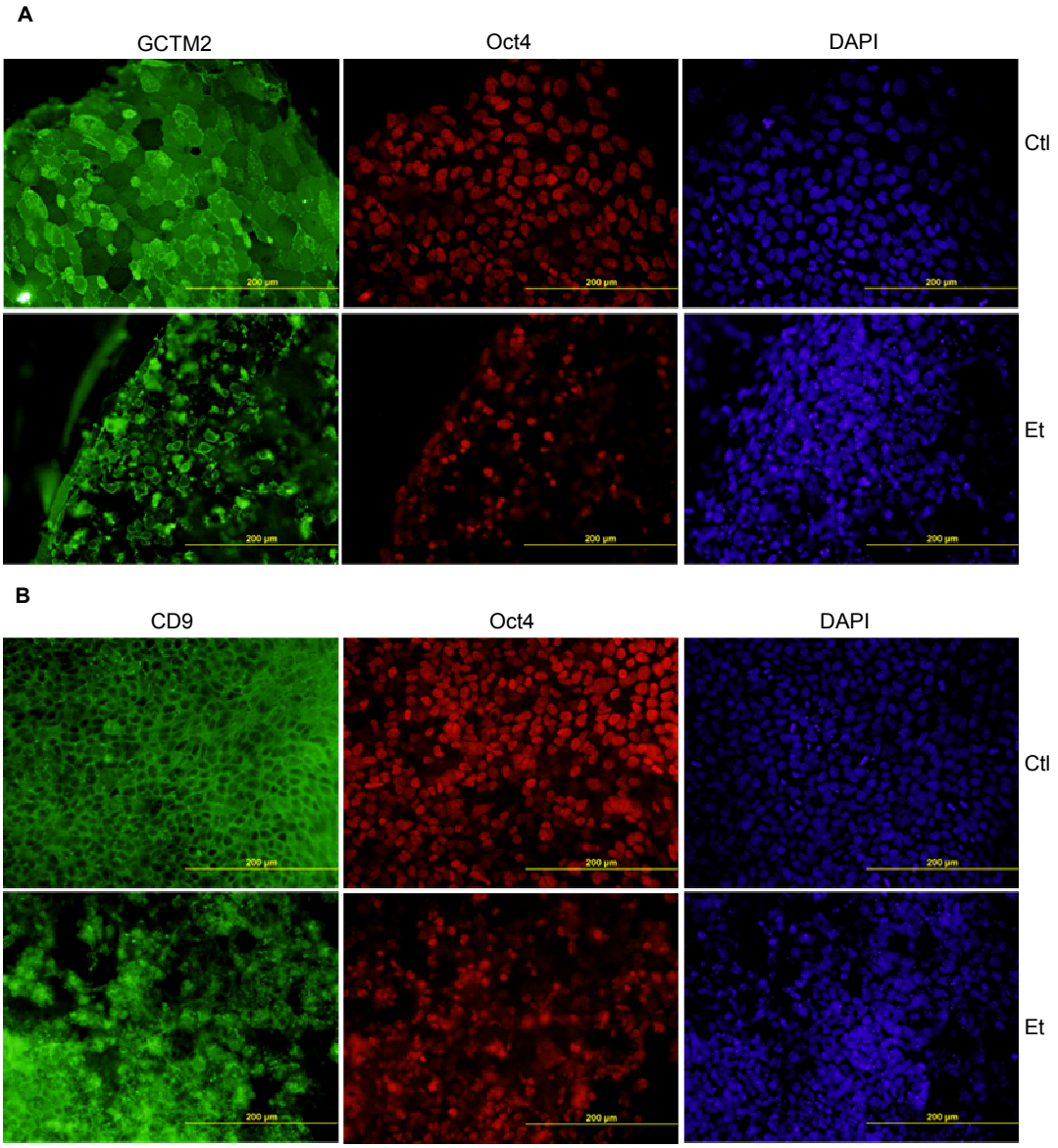

FIGURE 3.10- Reduction in stem cell markers immunoreactivity in apoptotic hESC.
HES-4 cells were exposed 170 nM etoposide for 4 hours and stained for GCTM2, CD9 and Oct4. Scale bars, 200 µm.

3.6- Etoposide-induced apoptosis of hESC requires caspase activation and is partially sensitive to cycloheximide

Cultures of hESC display a high rate of spontaneous apoptosis with 15% of hESC undergoing apoptosis daily under optimal culture conditions (Figure 3.4). Caspases are centrally involved in the regulation and execution of the apoptotic program (Thornberry and Lazebnik, 1998) and caspase 3 is activated in etoposide- treated hESC cultures (Figure 3.1B). To determine whether caspases play a functional role in etoposide-induced and spontaneous apoptosis of hESC, hESC were incubated with the general caspase inhibitor zVAD-fmk prior to etoposide treatment. As

shown in Figure 3.11, zVAD-fmk totally inhibits etoposide-induced, but not spontaneous apoptosis of hESC. Similarly, CHX, an inhibitor of protein translation, is able to prevent 50% of etoposide-induced apoptosis but has no inhibitory effect on spontaneous apoptosis (Figure 3.11). These data therefore indicate that hESC treated with etoposide undergo caspase and protein translation dependent apoptosis whereas spontaneous apoptosis proceeds via caspase- and translation independent pathways. The possibility that spontaneously apoptosing hESC were already committed to die prior to addition of CHX or zVAD-fmk cannot be excluded.

Cyclosporin A, an inhibitor of both mitochondrial permeability transition and calcineurin (Liu *et al.*, 1991), induced apoptosis by itself and did not inhibit etoposide-induced apoptosis (Figure 3.11). Pre-incubation with leptomycin B, a nuclear export inhibitor (Wolff *et al*., 1997), cPFT, a purported inhibitor of p53 mediated transcription (Komarov *et al.*, 1999), also had no or only a very minor inhibitory effect on etoposide-induced hESC apoptosis (Figure 3.11). This is perhaps not surprising since the efficacy of cPFT as an inhibitor of p53 transactivation activity is highly questionable (Walton *et al.,* 2005). PFT-mu, a compound that inhibits association of p53 with mitochondria (Strom *et al*., 2006), was found to reduce etoposide-induced apoptosis of hESC by 50 % (Figure 3.11).

Bongkrekic acid, an inhibitor of the adenine nucleotide translocator and mitochondrial permeability transition pore formation (Pastorino and Hoek, 2000; Scarlett *et al.*, 2000), was not able to inhibit spontaneous or etoposide-induced apoptosis of hESC (Figure 3.12A). In agreement with this observation, there was no detection of loss of mitochondrial membrane potential, measured by JC-1 uptake, during the first 4 hours of induction of apoptosis with etoposide (Figure 3.12B), despite the fact that cytochrome c release in the cytosol was already detected at 3 hours.

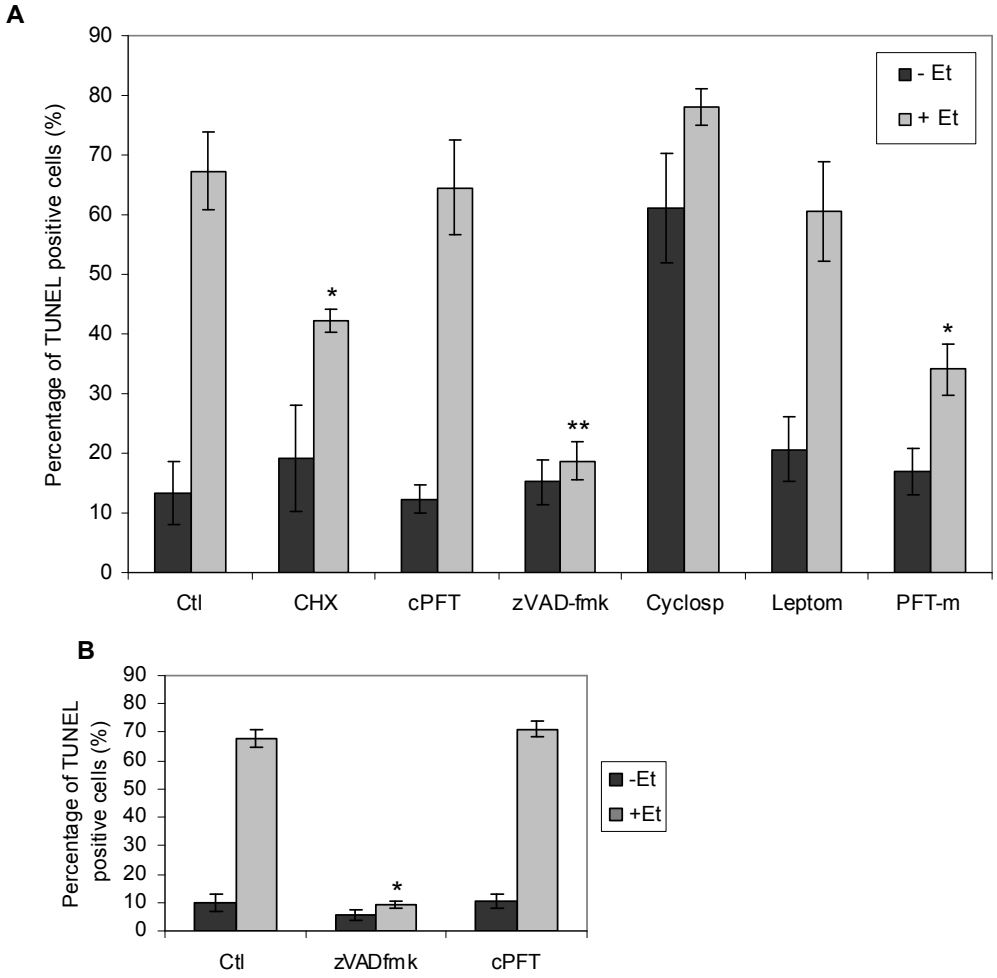

FIGURE 3.11- zVAD-fmk and cycloheximide inhibit etoposide-induced apoptosis of hESC. (A) HES-2 cells and (B) HES-4 grown in serum-free conditions were preincubated with vehicle alone or with inhibitor (25 μM cPFT, 10 μM PFT-mu and 50 μM BA were added 1 h before the treatment with etoposide; 4 μM CHX, 25 μM zVAD-fmk, 5 ng/ml Leptomycin B and 10 μM Cyclosporine A were added 30 min before the treatment with etoposide) and subsequently exposed to 170 nM etoposide for 18 hours. Floating and adherent cells were harvested, stained with TUNEL and analyzed by flow cytometry. Results are expressed as percentage means ± S.D. (n=6 for HES-2 control and etoposide treated cells and n=3 for rest of samples). (p-value < 0.05 by * and p-value < 0.01 denoted by ** compared to treated control cells).

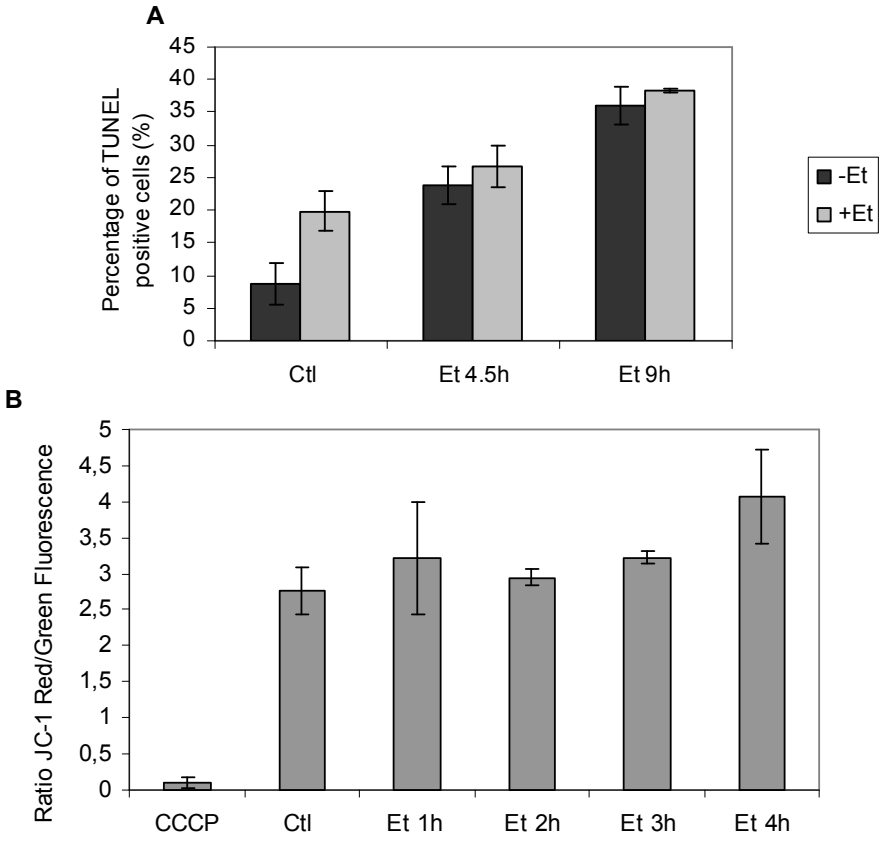

FIGURE 3.12- Bongkrekic acid was not able to inhibit apoptosis of hESC and there was no detection of loss of mitochondrial membrane potential during initial steps of apoptosis.
(A) HES-2 cells were preincubated with vehicle alone or with inhibitor (50 μM BA was added 1 h before the treatment with etoposide) and subsequently exposed to 170 nM etoposide for 4.5 and 9 hours. Floating and adherent cells were harvested, stained with TUNEL and analyzed by flow cytometry. Results are expressed as percentage means ± S.D. (n=3).
(B) HES-2 cells were exposed to 170 nM etoposide for timepoints indicated in graph. Floating and adherent cells were harvested, stained with MitoProbe^{TM}JC-1 and analyzed by flow cytometry. As a positive control, cells were incubated with 100 mM of CCCP for 5 min prior the addition of JC-1. Results are expressed as percentage means ± S.D. (n=3).

3.7- Expression analysis of several pro- and anti-apoptotic proteins in hESC

Gene expression profiling studies of ESC have shown robust expression of p53 and other proteins involved in apoptosis (Bhattacharya *et al.*, 2005; Ginis *et al.*, 2004; Ramalho-Santos *et al.*, 2002). Array analysis of H1 cells indicated (Ginis *et al.*, 2004), that hESC express p53, Mdm2, a negative regulator of p53, Bax a pro-apoptotic downstream target of p53, as well as the Bcl-2 family member Mcl-1. To determine whether hESC express established regulators of p53-dependent apoptosis, immunostaining of *in situ* fixed hESC cultures to detect proteins that are known to possess apoptotic or anti-apoptotic activities was performed. Figure 3.13 shows that p53, Hdm2, Hdm4, Mcl1, Bax and p14ARF are expressed in the HES-4 cell line, whereas Bcl2 was undetectable. hESC grown under serum and serum-free conditions display similar expression patterns for p53, Mcl1 and Bax (Figure 3.13A and 3.13B). In addition to confirming

p53 protein expression, the subcellular localization of p53 in hESC was also examined. In the majority of undifferentiated hESC, p53 has a cytoplasmic localization with only few cells at the edge of the colonies displaying nuclear p53 staining detected by the DO-1 antibody. Similar results were obtained using a different anti-p53 antibody (pAB1801, Figure 3.13C). The high levels of cytoplasmic expression of p53 in untreated hESC was confirmed by Western blotting of cytosolic protein extracts from hESC (Figure 3.1A).

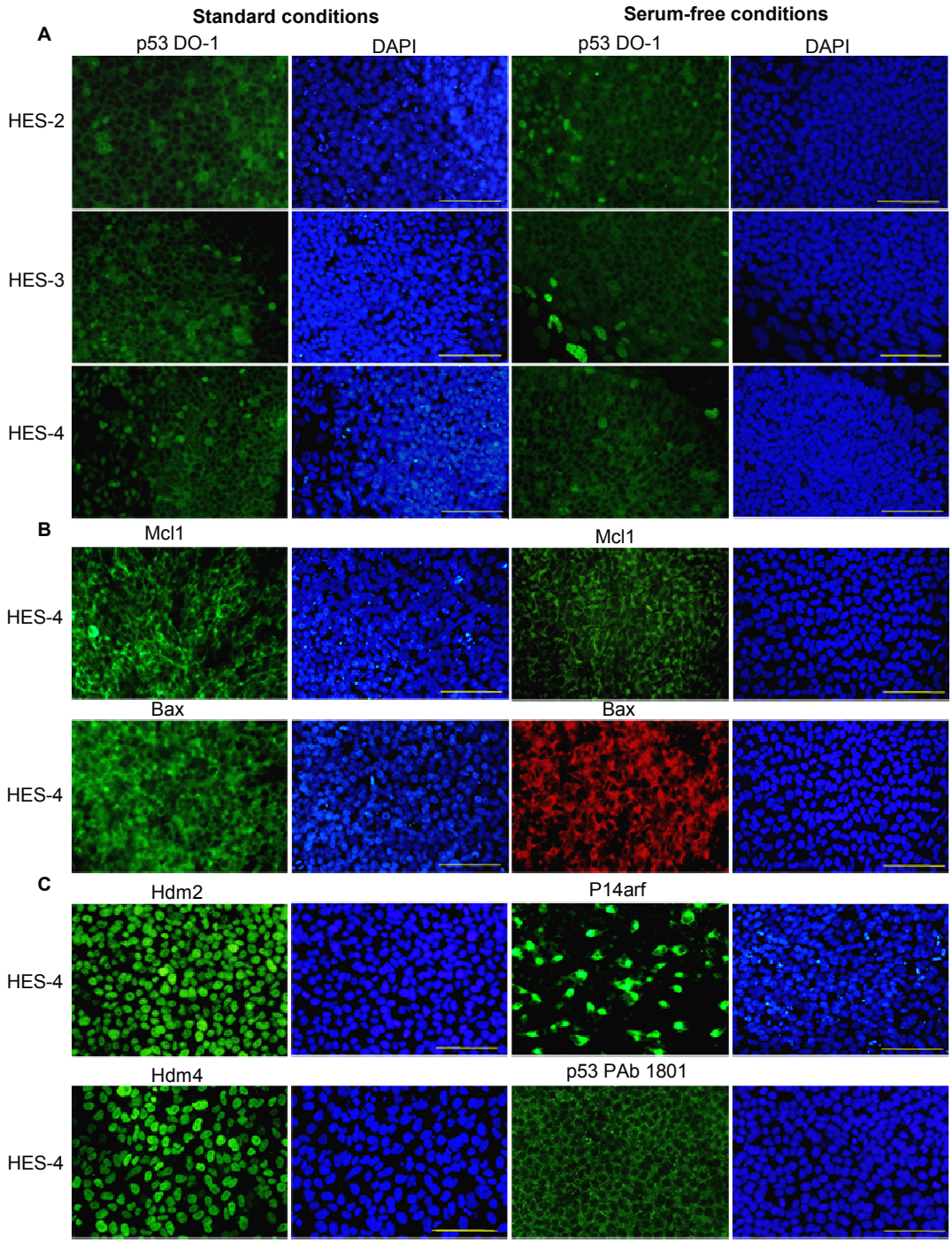

FIGURE 3.13- hESC exhibit a cytoplasmic localization of p53 and express Mcl1, Bax, p14ARF, Hdm4 and Hdm2.
(A) HES-2, HES-3 and HES-4 colonies grown under standard culture conditions and serum-free conditions were fixed and immunostained for p53 (antibody DO-1). Scale bars, 100 µm.
(B) HES-4 colonies grown under standard culture conditions and serum-free conditions were fixed and immunostained for Mcl1 and Bax standard culture conditions and serum-free conditions. Scale bars, 100 µm.
(C) HES-4 colonies grown under standard culture conditions were fixed and immunostained for Hdm2, Hdm4, p14ARF and p53 (antibody pAB1801). Scale bars, 100 µm.

3.8- Etoposide-induced apoptosis in hESC dramatically alters subcellular localization of p53, Bax and Mcl1

In untreated undifferentiated hESC, p53 resides mainly in the cytoplasm in two forms, a soluble fraction and a particulate fraction, as judged by immunofluorescent detection (Figure 3.13) and cell fractionation (Figure 3.1A). Four hours after etoposide treatment co-localization of p53 with the mitochondrial marker antibody 1E6 (Banbury, 1994) was detected, suggesting that p53 is targeted to this organelle (Figure 3.14B). During incubation with etoposide, the subcellular localization of the mitochondria themselves changes from a small punctuate distribution throughout the cytosol to large aggregates of mitochondria in a peri-nuclear region that stain strongly for p53. Simultaneously an increasing proportion of hESC display nuclear staining for p53 (Figure 3.14). These observations suggest that p53 both translocates to the nucleus and associates with the mitochondria in response to etoposide treatment. One of the pre-requisites of mitochondrial permeability transition and cytochrome c release is thought to be the insertion of Bax into the outer mitochondrial membrane. When the subcellular localization was examined of Bax and Mcl1 between 3 and 18 hours after etoposide treatment, Bax increasingly co-localized with Mcl1 in perinuclear structures reminiscent of the mitochondrial aggregates described above (Figure 3.15).

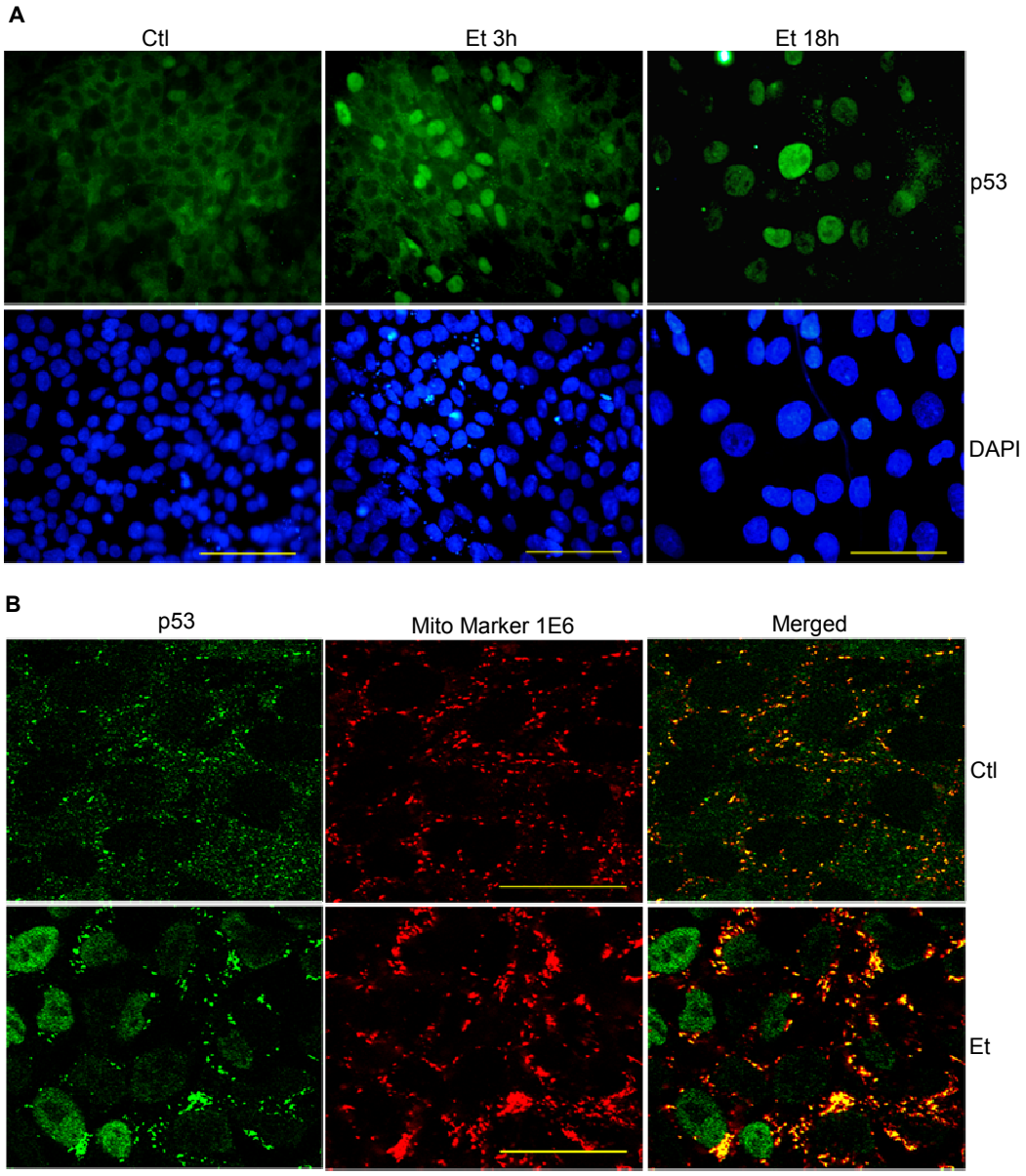

FIGURE 3.14- hESC treated with etoposide show redistribution of cytosolic p53 to mitochondria and nucleus.
(A) HES-2 cells were exposed to 340 nM etoposide for 3 or 18 hours, PFA-fixed and immunostained for p53 and nuclei (DAPI). A representative example of 3 independent experiments is shown. Scale bars, 100 µm. (B) Confocal microscope images of HES-2 cells treated with 340 nM etoposide for 4 hours. p53 relocalizes from the cytosol to the nucleus as well as to the mitochondria (1E6 is a mitochondrial marker). Scale bars, 50 µm.

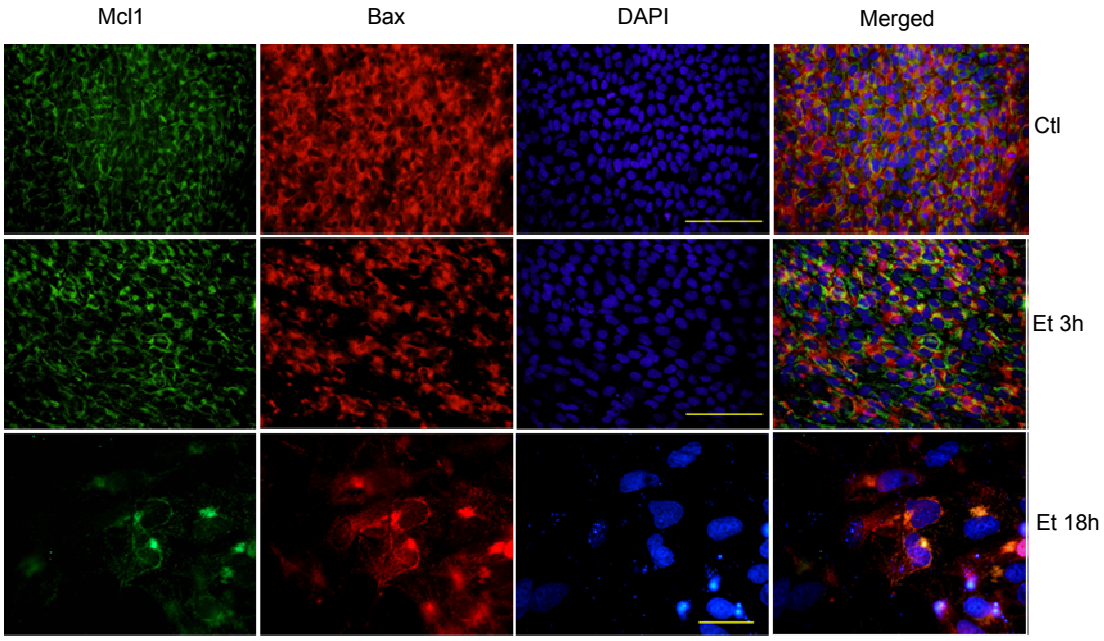

FIGURE 3.15- Upon treatment with etoposide, Bax and Mcl1 co-localize.
HES-4 cells were exposed to 340 nM etoposide for 3 and 18 hours, PFA-fixed and immunostained for Mcl1, Bax and nuclei (DAPI). Upon induction of apoptosis with etoposide Mcl1 and Bax co-localize in a perinuclear region in structures reminiscent of mitochondrial aggregates. Scale bars, 100 µm (control and 3 hours etoposide 340 nM) and 50 µm (18 hours etoposide 340 nM).

3.9- Altered expression of key apoptosis regulators upon etoposide and gamma irradiation-induced apoptosis in hESC

In most model systems, etoposide-induced apoptosis is dependent on p53 (Karpinich *et al*., 2002a, Lowe *et al*., 1993, Yin *et al*., 2000). In agreement with these previous observations, an increase in p53 expression was detected as early as 3 hours after etoposide addition to hESC. Expression of p53 then peaks around 6-9 hours of exposure to etoposide and wanes thereafter (Figure 3.16A). PUMA, one of the downstream targets of p53, is upregulated between 4.5 hours and 6 hours after exposure to etoposide, as is nuclear p53. Furthermore, consistent with the release of cytochrome c at 3 hours after exposure to etoposide, increased activation of caspase 3 is maintained for 6 to 8 hours after etoposide addition. Similar kinetics of upregulation was observed for Hdm2. In contrast, no alteration in Mcl1, Hdm4, Noxa or Oct4 expression were found in etoposide treated hESC (Figure 3.16A). However, Nanog is downregulated in hESC after etoposide-induced upregulation of p53, in agreement with the previously reported (Lin *et al.*, 2005) ability of p53 to supress Nanog expression in mESC. Only a slight upregulation of the p53 target Bax in response to etoposide treatment was detected, as well as an increased processing of Bax to the more potent pro-apoptotic 18 kDa form with time (Gao and Dou, 2000; Wood and Newcomb, 2000). There was no expression of p21 protein, a potential downstream target of p53 involved in cell cycle arrest in many cellular model systems, in either untreated or etoposide treated hESC by Western blotting or immunofluorescence. This was not due to an inability of the

antibody to detect p21 since was readily detected expression of p21 in protein extracts of HEF (data not shown). Since CHX is able to inhibit 50% of etoposide-induced apoptosis, it was interesting to find out how this relates to the expression of p53 regulated apoptosis modulators. As shown in Figure 3.16B preincubation with CHX does not prevent the upregulation of p53, suggesting that the majority of its increased expression is due to increased stabilization of p53 protein and not to *de novo* synthesis. However, the expression of Bax and PUMA (p53 downstream targets) and Mcl1 are clearly reduced by this protein synthesis inhibitor, suggesting that Bax and PUMA may be important determinants of etoposide-induced apoptosis of hESC.

In order to determine whether p53 is able to induce upregulation of its gene targets in response to other DNA damaging agents hESC were exposure to gamma-irradiation. Upregulation of p53 expression as well as PUMA was detected as soon as 3h after gamma radiation (Figure 3.16C).

Because late passage karyotypically abnormal hESC displayed a dramatic reduction in etoposide-induced apoptosis as compared to early passage hESC (section 3.4 of this Chapter) we assessed the kinetics of p53 upregulation in response to etoposide between both cell lines (Figure 3.16D). Under control conditions, levels of p53 are already higher in early passage hESC than in late passage cells. Upon exposure to 170 nM of etoposide p53 is clearly upregulated in early passage hESC after 4.5 h, but in late passage it is only slightly upregulated at 4.5 and 9h. About 75 % of early passage HES-3 cells died at this concentration but late passage show resistance to apoptosis. This is not due to a difference in differentiation of late passage cells. In fact, late passage cells display higher levels of Oct4 then early passage cells (most likely because the population contains a higher proportion of Oct4 positive hESC). Upon exposure of late passage hESC to higher etoposide concentrations (1.7 μM) the cells die via apoptosis and p53 is upregulated at this concentration. These data shows that there is an association or relationship between the response to DNA damage induced apoptosis and p53 levels in hESC and it shows that the regulation of this pathway is altered in late passage cells.

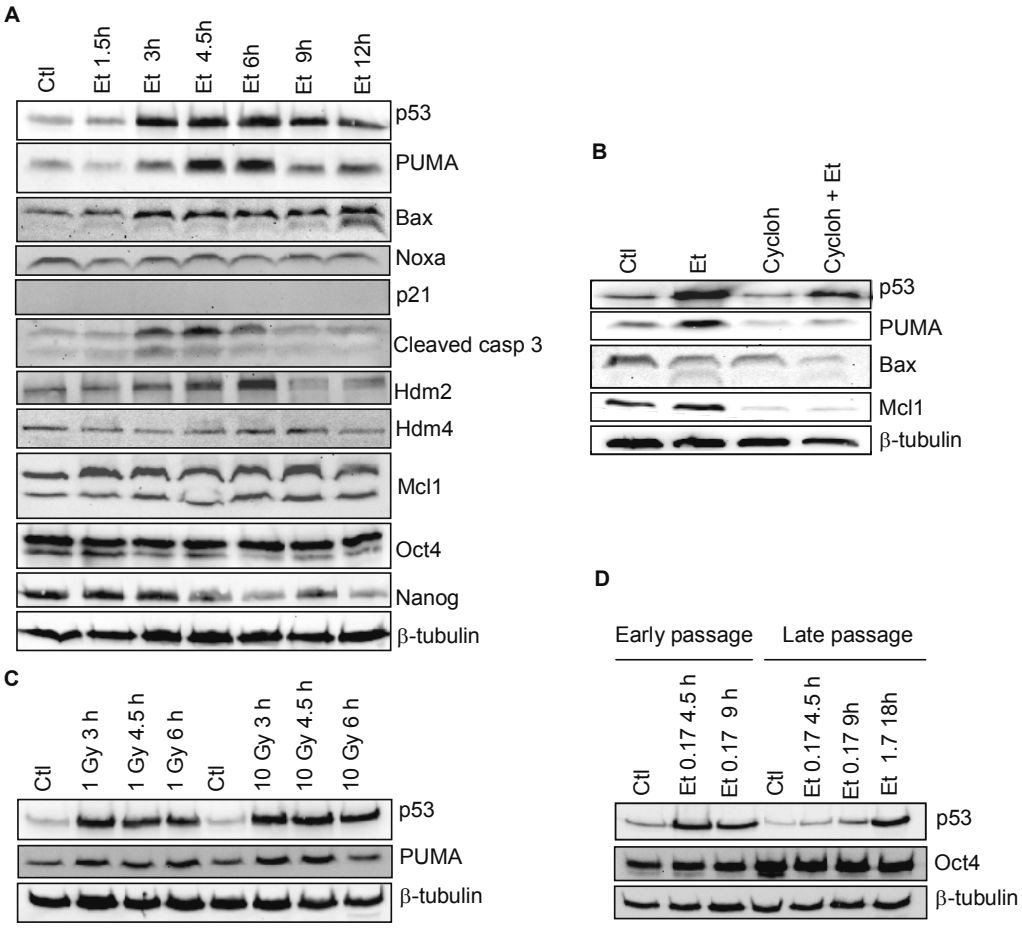

FIGURE 3.16- Increased expression of p53 and altered expression of p53 downstream targets in hESC treated with etoposide and gamma-irradiation.

(A) HES-2 cells were harvested 1.5, 3, 4.5, 6, 9 and 12 hours after treatment with 170 nM etoposide. Protein extracts were resolved on separate polyacrylamide gels and Western blotted with antibodies against: p53, PUMA, Bax, cleaved caspase-3, Hdm2, Hdm4, Mcl1 and Oct4. β-tubulin was used to ensure equal loading. A representative example of 3 independent experiments is shown.

(B) CHX treatment interferes with etoposide-induced upregulation of PUMA and Bax in hESC. HES-2 cells were preincubated with or without 1μg/ml CHX for 30 min and treated with 170 nM etoposide (Et) for 18 hours. Protein extracts were resolved on separate polyacrylamide gels and Western blotted with antibodies against p53, Mcl1, Bax, PUMA and β-tubulin. A representative example of 3 independent experiments is shown.

(C) HES-2 cells were harvested 3, 4.5 and 6h after treatment with gamma-irradiation (1Gy and 10 Gy). Protein extracts were resolved on separate polyacrilamide gels and Western blotted with antibodies against p53, PUMA and β-tubulin

(D) HES-3 cells early and late passage were treated with 170 or 1700 nM etoposide for the indicated timepoints. Protein extracts were resolved on separate polyacrylamide gels and Western blotted with antibodies against: p53, Oct4 and β-tubulin.

3.10- Discussion

Because no reports on apoptosis of hESC had been published at the start of this thesis it was important to first determine whether hESC display any of the hallmarks of apoptosis. Furthermore this is also a prerequisite if one wants to use the established apoptosis assays such as TUNEL (as used throughout this study). hESC indeed the display hallmarks of apoptosis (cytochrome c release, DNA laddering, caspase activation and chromatin condensation) upon exposure to an apoptotic stimulus. In initial experiments, the Annexin V assay was used but because the levels of apoptosis in controls were too high, reflecting most likely non-specific damage to the plasma membrane during processing of cells rather then translocation of phosphatidylserine to the outer layer of the plasma membrane. Therefore, TUNEL was preferentially used to measure apoptosis throughout this study.

Having established that they can die via the intrinsic apoptotic pathway, the effect of different apoptotic stimuli on these cells was studied. The data show that hESC are extremely sensitive to spontaneous apoptosis, as well as gamma irradiation-, hydrogen peroxide- and etoposide-induced apoptosis. Indeed, hESC appear to be far more sensitive to etoposide-induced apoptosis than other cell lines or primary cell cultures. This high sensitivity of hESC to etoposide induced apoptosis is similar to the sensitivity of inner cell mass cells of pre-implantation mouse embryos (Exley *et al*., 1999). Although zygotes were induced to undergo apoptosis after 72 h of incubation in 1 µM staurosporine, most of the zygotes did not undergo fragmentation. In contrast, blastocysts, from which ESC are derived, can be induced to undergo apoptosis after 6 h of incubation in only 10 nM staurosporine. Thus, zygotes appear to be more resistant to the effects of staurosporine than blastocysts.

On the other hand, the extrinsic apoptotic pathway seems not to be operational in hESC since TNFα failed to induce apoptosis. This result is similar to a study performed by Sleeper and colleagues (Sleeper *et al*., 2002) who showed that adult neural stem cells did not die upon exposure to Fas ligand, despite being very sensitive to intrinsic apoptotic stimuli. Arrays performed on H1 cells (Ginis *et al*., 2004) have shown that hESC expressed several genes associated with TNF receptor signaling (TNF-R2, TRAF1, TRAF4 and TRAF6), but Fas and Fas ligand were not expressed at detectable levels in both mESC and hESC. Perhaps the extrinsic apoptotic pathway is not functional in these cells. To confirm this, Fas ligand mediated apoptosis should be also investigated.

When cultured for long periods of time under serum-free conditions hESC tend to adapt to culture conditions. Most likely this adaptation process results from genetic alterations that promote self-renewal and limit differentiation or apoptosis. Late passage HES-3 cell line with an abnormal karyotype showed reduced apoptotic response to etoposide and lower p53 protein levels when compared to early passage hESC. The late passage HES-3 cells express the TNF receptor superfamily member CD30 on their surface (Tung-Liang Chung, personal communication) and CD30 positive cells have been reported to show lower apoptotic response to hydrogen peroxide and reduced dependency on (Herszfeld *et al*., 2006). Furthermore Survivin, an inhibitor of apoptosis, is negatively regulated by p53 and is a gene upregulated in CD30 expressing hESC

(Ernst Wolvetang, personal communication). This is one of the reasons why the assays performed in this study hESC lines were only used up to passage 18 when cultured as per Amit *et al* (2000). It would be very interesting to investigate the apoptotic response of late passage hESC with normal karyotypes. Such experiments and regular karyotype analysis (or CGH) of hESC overexpressing anti-apoptotic genes such as Mcl1 or Bcl2 during prolonged passage of hESCcould provide indications whether changes in the apoptotic threshold of hESC are a cause or consequence of the chromosomal abnormalities.

The sensitivity of hESC (HES-2 and HES-4) to etoposide-induced apoptosis is also dependent on their differentiation status. Undifferentiated hESC that express Oct4 are the least resistant to etoposide-induced apoptosis as compared to their more differentiated progeny. Given the profound effect of the differentiation status of hESC on their rate of apoptosis meaningful data on apoptosis in hESC can only be obtained when TUNEL labelled cells are double labelled with at least one pluripotency marker, as performed in this study.

Like mESC, hESC express high levels of p53 protein in the cytoplasm under standard culture and serum-free conditions. How ESC can tolerate such high levels of p53 is still unknown. Probably post-translational modifications and protein interactions are involved in regulation of p53 localization in these cells. Parc, a Parkin-like ubiquitin ligase, has been implicated in sequestering p53 in the cytoplasm by direct interaction by p53. Together they form a 1 MDa complex in the cytoplasm in unstressed cells that serves as a cytoplasmic anchor for p53. Additionally, inactivation of this protein even in unstressed cells leads to nuclear localization of endogenous p53 and activates p53-dependent apoptosis. On the other hand, Parc overexpression promotes cytoplasmic sequestration of ectopic p53 (Nikolaev *et al*., 2003). Perhaps Parc serves as a bridge between p53 and a cytoplasmic structure. In fact, cytoskeletal proteins such as vimentin have been shown to contribute to the cytoplasmic sequestration of p53 (Klotzsche *et al*., 1998). Recently, Parc has been shown to form complexes with its related homolog Cullin 7 (Skaar *et al*., 2007). Cullin 7 binds to p53's tetramerization domain. Although its overexpression does not sequester p53 in the cytoplasm, it functions to promote cell growth through, in part, antagonizing the function of p53 through non-proteolytic ubiquitination (Andrews *et al*., 2006). Mortalin or Mot2, a member of the heat-shock protein Hsp70, is another cytoplasmic protein that has been implicated in p53 cytoplasmic sequestration by binding directly to it (Wadhwa *et al*., 2002). It would be very interesting to investigate if these proteins are anchoring p53 to the cytoplasm in hESC. Since hESC represent a non-transformed model, it will be also a good opportunity to unravel new protein interactions and to obtain more insight into the mechanisms that regulate the subcellular localization of p53 in a non-transformed background.

Members of Bcl2 family are essential for the survival of stem cells. In HSC, growth factors including stem cell factor increase transcription of the Mcl1 gene and Mcl1 protein is required to increase the survival rate of purified bone marrow progenitors (Opferman *et al*., 2005). In fact, Mcl-1 is the most abundantly and consistently expressed Bcl2 family member in the preimplantation embryo (Jurisicova and Acton, 2004) and is the only pro-survival Bcl-2 family member whose disruption results in preimplantation embryonic lethality (Rinkenberger *et al*.,

2000). The data in this study show that Mcl1 is the main Bcl-2 family member expressed in hESC and that Bcl2 protein is not expressed in hESC.

To unravel the mechanism of apoptosis, the different known regulators mentioned above and features of apoptosis were investigated. Initiation of etoposide-induced apoptosis in hESC commences around 3 hours after etoposide addition with translocation of the highly-expressed cytoplasmic p53 protein to the mitochondria and release of cytochrome c. There is no detectable loss of mitochondrial membrane potential, as judged by the maintenance of JC-1 red/green fluorescence. Cytochrome c release from mitochondria without loss of mitochondrial membrane potential has previously been reported (Finucane *et al*., 1999; Grubb *et al*., 2001; Krohn *et al*., 1999). Because caspase 3 is activated at three hours after etoposide treatment, apoptosome formation and initiation of the apoptotic cascade has most likely already commenced at this stage. The dramatic increase in nuclear p53, which occurs at the same time, suggests that there are two fractions of cytoplasmic p53 with different functions in apoptosis induction in response to etoposide, one that associates with the mitochondria and one that translocates to the nucleus. In support of this idea, inhibition of p53 association with mitochondria with PFT-mu prevents only 50 % of etoposide-induced apoptosis of hESC. Due to failure of cPFT to inhibit p53 transcription activity, it is impossible to discriminate one from the other. But since CHX only partially inhibits apoptosis this can also indicate that etoposide-induced apoptosis of hESC involves both direct mitochondrial transcription independent, and nuclear, transcription dependent functions of p53. When p53 associates with the mitochondria upon apoptotic stimuli, it has been reported to interact with anti-apoptotic proteins of the Bcl2 family members sequestering anti-apoptotic proteins (like Bcl2 and Bcl-xL) and to promote the activation of proapotpotic proteins (such as inducing Bax and Bak oligomerization). It is still not completely clear what signals are responsible for translocation of p53 to the mitochondria. A study by Dumont and colleagues found that two different polymorphisms of the p53 gene gave rise proteins with different subcellular localizations (Dumont *et al*., 2003). At position 72, wild-type p53 can encode either a proline or an arginine and these authors found that Arg72 was more effective at inducing apoptosis than Pro72 under stress and that this was due to enhanced mitochondrial localization of Arg72 after DNA damage or other types of cellular stress. It would be of interest to examine possible differences in post translational modifications between nuclear and cytosolic p53 fractions in hESC that may explain the simultaneous targeting of p53 to two different subcellular locations.

The regulation and kinetics of apoptosis in hESC are very similar to those observed for *in vivo* irradiated thymocytes, with a rapid p53 transcription independent first wave of apoptosis followed by a second p53 transcription dependent wave *(Erster et al*., 2004) and a very early upregulation of PUMA and Bax coincident with caspase 3 activation. The data suggest that in hESC these two waves occur either simultaneously or very soon after one another. In both model systems, however, the p53-dependent upregulation of PUMA and Bax appear to be critical determinants.

In mESC, cytoplasmic p53 protein translocates inefficiently to the nucleus upon nucleotide depletion and the cells undergo p53-independent apoptosis in response to DNA damage. This may be due to differences between the DNA damage response mechanisms in etoposide-treated

cells and cells exposed to nucleotide depletion or to intrinsic differences between mouse and human ESC. Nuclear accumulation of p53 in hESC is accompanied by an early upregulation of the p53 target gene PUMA, but does not cause an increase in p21. This finding may explain why hESC that have suffered etoposide induced DNA damage fail to arrest the cell cycle in G_0/G1 and preferentially die via apoptosis (as indicated by flow cytometric analysis of DAPI stained hESC cultures). In this respect hESC resemble mESC (Xu *et al*., 2002), which also do not exhibit cell cycle arrest or slowdown upon UV-induced DNA damage. In fact, DNA damage response and cell-cycle regulation differ markedly between mESC and somatic cells (Hong and Stambrook, 2004). Spontaneous mutation frequency in mouse somatic cells is approximately 100-fold higher than in mESC. mESC also lack a G1 checkpoint and are hypersensitive to ionizing radiation and other DNA-damaging agents (Hong and Stambrook, 2004) and these characteristics facilitate apoptosis and the removal of cells with a mutational burden from the population.

In other model systems PUMA, a BH3-only derepressor molecule, counteracts the anti-apoptotic function of Mcl1 and induces a pro-apoptotic conformational change in Bax (Kuwana *et al*., 2005). BH3-only Bcl2 family members such as PUMA trigger mitochondrial cytochrome c release without loss of mitochondrial membrane potential (Shimizu and Tsujimoto, 2000), as is the case in etoposide treated hESC. Alternatively PUMA may be involved in the release of mitochondrially associated p53 to further enhance nuclear p53 mediated apoptotic events, as recently demonstrated by Chipuk and colleagues (Chipuk *et al*., 2005). The fact that in hESC p53 upregulation results in an upregulation of only a subset of p53 target genes, such as PUMA, suggests that p53 exhibits target specificity. This may be due to differences in p53 postranslational modifications, histone modifications or interactions with other transcription factors. These data are in contrast to the study by Qin and colleagues (Qin *et al*., 2006), that was published during the course of this study, which indicated that UV- and gamma-radiation induced apoptosis of hESC, although p53 dependent, does not lead to accumulation of Bax or Hdm2 and does not cause activation of any p53 downstream target genes in luciferase reporter assays. The authors postulated that post translational modifications of p53 render the p53 protein transcriptionally inactive without interfering with p53's ability to repress Oct4 and Nanog expression. It was consequently concluded that p53 induces apoptosis solely through a mitochondrial pathway. However, in that study 30-40 % of the Annexin V positive cells were stained with propidium iodide (PI) indicating the occurrence of necrosis, which does not involve gene regulation. Indeed, the use of PI staining to detect apoptosis in hESC, as used by Qin and colleagues *(*Qin *et al., 2006)*, should be discouraged since this assay does not discriminate between primary and secondary necrosis and apoptosis. In order to determine whether the differing results in terms of p53 downstream target activation between our study (using etoposide) and the Qin *et al* study (using UV and gamma radiation) could be due to the different methods to induced DNA damage, PUMA modulation was assessed in response to gamma radiation. Similar to what we observed for etoposide-induced apoptosis gamma-radiation-induced apoptosis of hESC causes increased p53 expression that is accompanied by upregulation of the p53 downstream target gene PUMA and Hdm2. This leaves the possibility that the differences

between the two studies may be due to intrinsic cell line differences (H1 versus HES-2, HES-3 and HES-4 in the present study), the passage number of the hESC (P42-68 H1 versus less than P15 in our study) or the method of propagation (dispase versus collagenase in this study). There are probably mechanistic differences between the responses of hESC to the different apoptotic stimuli, namely UV irradiation and etoposide treatment (Attardi *et al*., 2004). For example, UVB treatment generally causes activation of p38 and/or wip1 (Takekawa *et al*., 2000). p38-mediated phosphorylation and stabilization of p53 can subsequently cause cytoplasmic sequestration of wild-type p53, leading to an absence of p53 target gene modulation (Chouinard *et al*., 2002) while wip1 expression has been shown to suppress both p53-mediated transcription and apoptosis in response to UV radiation (Takekawa *et al*., 2000)

A working model for etoposide induced apoptosis in hESC is proposed on Figure 3.17. In this model, DNA damage leads to stabilization and increased expression of p53 followed by translocation of p53 to both the mitochondria, where it possibly interacts with Mcl1, and to the nucleus, where it leads to transcriptional upregulation of Bax and PUMA. Bax and PUMA next cooperate in triggering cytochrome c release from the mitochondria without loss of membrane potential and this subsequently leads to activation of a caspase 3 dependent apoptotic program. The presence of two simultaneous pathways to induce p53-mediated apoptosis may be a failsafe mechanism reflecting their origin from pluripotent embryonic cells in vivo, where it would be important to ensure that genetically abnormal cells do not contribute to the developing tissues and the germ line. In order to confirm that p53 is required for apoptosis and to gain more insight into the role of p53 in hESC, p53 shRNA transduced cell lines were generated.

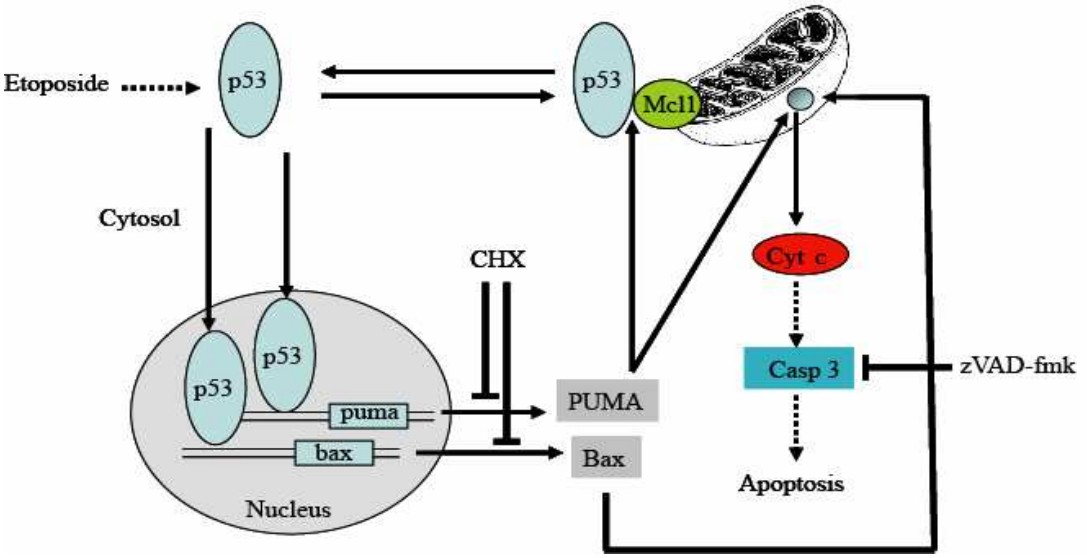

FIGURE 3.17- A model depicting the role of p53 and p53 downstream targets in the regulation of etoposide-induced apoptosis of hESC.
In response to DNA damage p53 translocates to both the mitochondria, where it possibly interacts with Mcl1, as well as to the nucleus where it triggers upregulation of PUMA and maintenance of Bax expression. PUMA and Bax subsequently cooperate in facilitating cytochrome c release without loss of membrane potential and initiation of the apoptotic cascade. See text for details.

CHAPTER 4

Lentiviral-mediated RNAi to explore the role of p53 in hESC

4.1- p53 is required for etoposide-induced apoptosis

p53 is considered a critical regulator of genomic integrity due to its ability to trigger apoptosis and/or cell cycle arrest in response to genotoxic stress. Because hESC display remarkable genomic stability and are highly sensitive to ionising radiation and other DNA-damaging agents, the modulation of p53 and other proteins involved in the regulation of apoptosis was examined in detail in the last Chapter. In order to determine the requirement of p53 for etoposide-induced apoptosis of hESC HES-2 hESC were stably transduced with a lentivirus carrying shRNA designed to knockdown p53 mRNA. To control for differences due to the lentiviral transduction itself or activation of the RNAi pathway, a cell line stably transduced with GFP (an shRNA directed against a gene not expressed in hESC) was used as control. These GFP shRNA transduced hESC were found to display a very similar response to etoposide as compared to untransduced hESC.

Using bioinformatic analysis (Invitrogen RNAi Design Services) of the p53 mRNA, two shRNA sequences (p53A and p53B) were designed and cloned into the lentiviral construct and stably transduced hESC. Western blotting analysis showed that p53A shRNA was most efficient at knocking down p53 expression. This construct was next selected for subsequent experiments described in this section.

As shown in Figure 4.1B and 4.1C, p53 shRNA transduced hESC constitutively expressed only 20% of p53 protein as compared to untransduced HES-2 cells or GFP shRNA transduced HES-2 cells, and displayed a reduced upregulation of p53 in response to etoposide addition. Importantly, these p53 shRNA transduced hESC displayed an 80 % reduction in etoposide-induced apoptosis that is consistent with the 80 % reduction in p53 protein expression (Figure 4.1A). These data therefore unequivocally demonstrate that etoposide-induced apoptosis of hESC is dependent on p53. The reduction of p53 dependent apoptosis in the p53 shRNA transduced hESC is accompanied by a constitutive downregulation of Bax expression and an attenuated upregulation of PUMA in response to etoposide addition. Therefore, these results strongly suggest that the reduction of etoposide-induced apoptosis in p53 knockdown hESC is due to a reduced upregulation of PUMA and a constitutively reduced expression of Bax. As shown previously, levels of Nanog decreased during the timecourse of exposure to etoposide in control cells, but not in p53 shRNA transduced cells (Figure 4.1B). The observation that this no longer occurs in the p53 shRNA transduced cells confirms that p53 is indeed required for repression of Nanog expression in response to etoposide. (Figure 4.1B). The reduced sensitivity of the p53 shRNA transduced hESC to etoposide-induced apoptosis is not due to an increased differentiation since levels of Oct4 are similar to levels in control cells (Figure 4.1B).

4.2- p53 is required for spontaneous apoptosis

Between 10-15 % of hESC (HES-2, HES-3 and HES-4) grown under serum free culture conditions (Amit *et al*., 2004) undergo spontaneous apoptosis over a 18h hour period. As shown in Figure 4.1B, HES-2 stably transduced with p53 shRNA and displaying only 20% residual p53 expression as compared to untransduced or GFP shRNA transduced hESC show a reduction in

spontaneous apoptosis from 11.4 ± 5.5% to only 2.6 ± 1.1 % (Figure 4.1A). GFP shRNA transduced HES-2 cells display a similar level of spontaneous apoptosis to untransduced HES-2, indicating that the reduction in spontaneous apoptosis is not due to the lentiviral transduction *per se*. Although the reason for the relatively high rate of spontaneous apoptosis in hESC serum free culture is unknown, these data indicate that spontaneous apoptosis is dependent on p53.

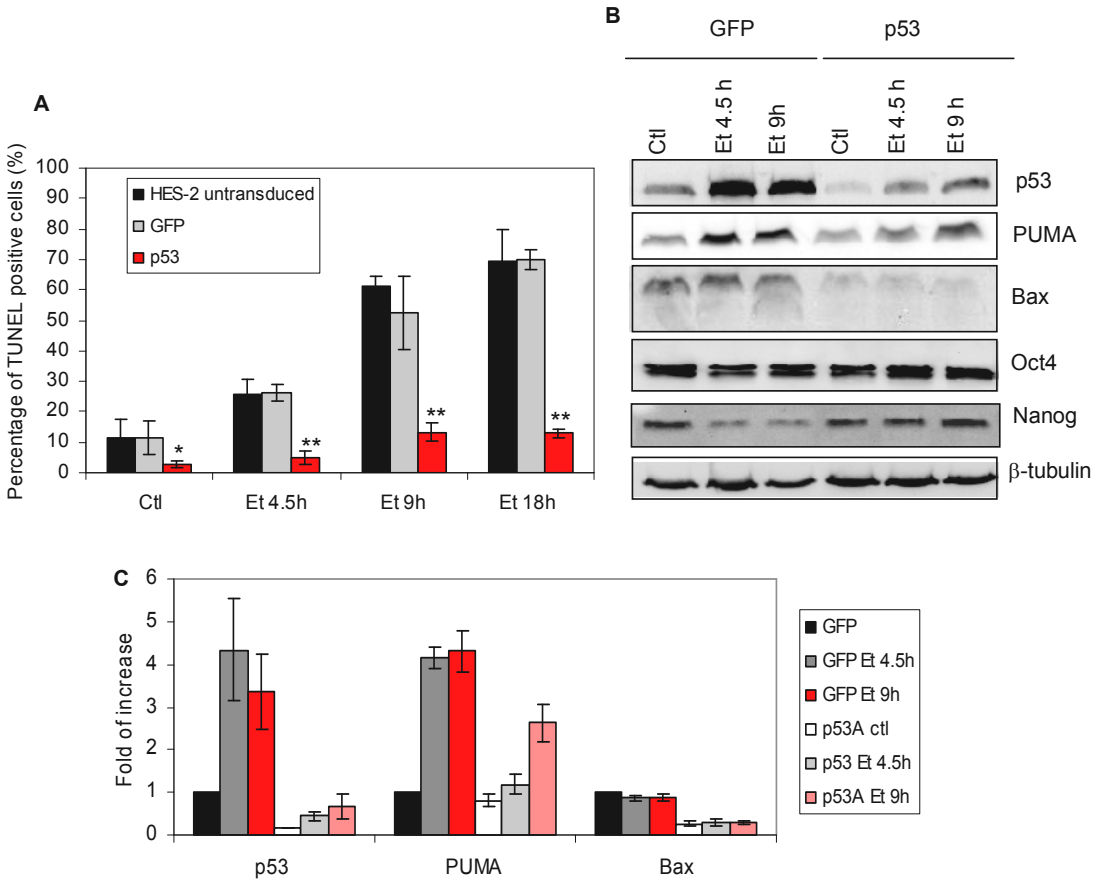

FIGURE 4.1- p53 is required for etoposide-induced apoptosis of hESC.
(A) HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP and grown in serum-free conditions. Untransduced, GFP and p53 shRNA transduced hESC were harvested 4.5, 9 and 18 hours after treatment with 170 nM etoposide. Cells were subsequently PFA-fixed, labeled with TUNEL, immunostained with the stem cell marker Oct4 and analyzed by flow cytometry. Results shown are expressed as percentage means ± S.D. (n=4). (*p*-value < 0.05 denoted by *and *p*-value < 0.01 denoted by ** compared to GFP shRNA transduced cell line).
(B) Downregulation of p53 and p53 downstream targets Bax and PUMA in p53 shRNA transduced HES-2 cells. HES-2 cells stably expressing p53 shRNA or GFP shRNA were harvested 4.5 and 9 hours after treatment with 170 nM etoposide. Protein extracts were boiled in Laemmli buffer, resolved on separate polyacrylamide gels and analyzed by Western blotting with p53, PUMA, Bax, Nanog and Oct4 antibodies. β-tubulin was used to ensure equal protein loading.
(C) Quantification of protein expression using software based densitometric analysis on cross-reactive bands and the β-tubulin loading controls.

4.3- Cell proliferation and cell cycle of p53 shRNA transduced cells

In many model systems p53 can exert control over cell cycle progression through regulation of the expression of cell cycle checkpoint regulators (reviewed by Pietenpol and Stewart, 2002; Stewart and Pietenpol, 2001). In order to determine the role of p53 in cell cycle control of hESC we measured cell cycle entry via BrdU incorporation in both p53 shRNA and GFP shRNA transduced hESC. The CD9 pluripotency marker was use to gate on undifferentiated hESC. This is important since cell cycle control of hESC is dramatically altered upon differentiation (Filipczyk *et al*., 2007). No significant differences were found between cell cycle entry of GFP and p53 shRNA transduced cell lines or distribution cell cycle phases (Figure 4.2). In both cases, a higher proportion of cells resides in the S phase of cell cycle as compare to other phases. These data therefore indicate that p53 does not play a role in cell cycle entry of hESC or that 80 % reduction of p53 is not sufficient to unmask such an effect.

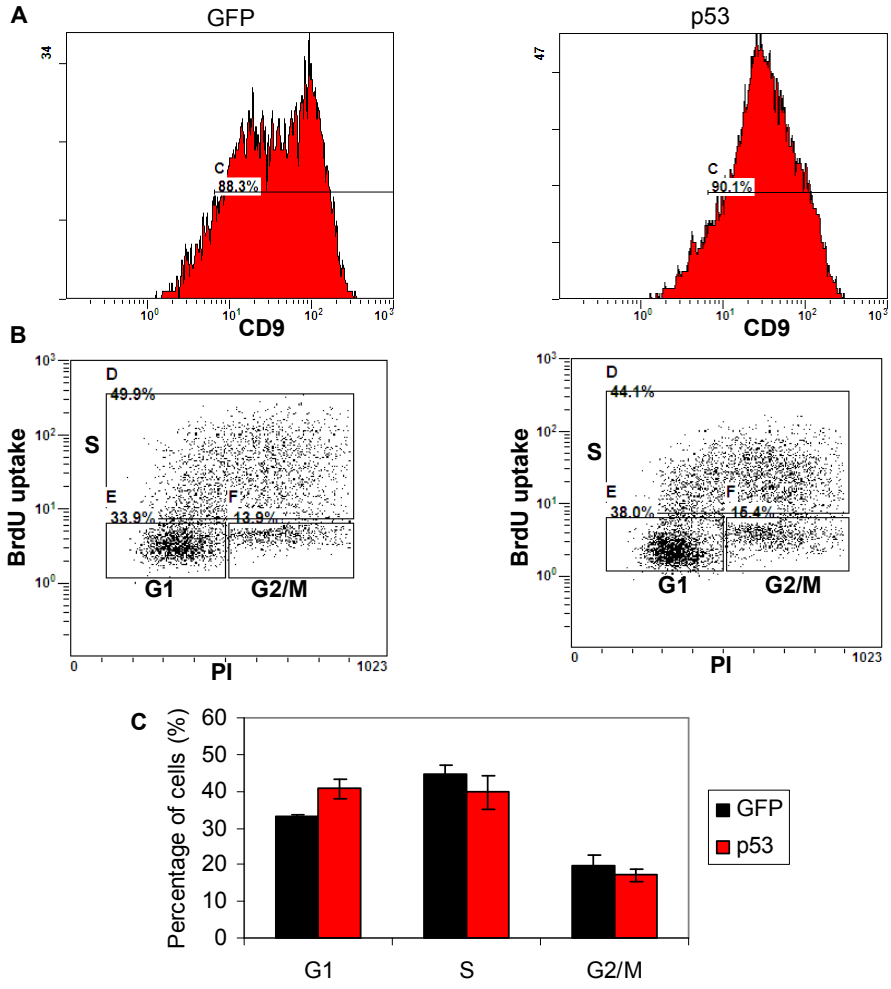

FIGURE 4.2- No differences in cell proliferation and distribution of cell cycle phases in p53 shRNA transduced hESC.
HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP and grown in serum-free conditions. Both p53 shRNA transduced and GFP shRNA transduced (control) hESC immunostained with the stem cell marker CD9 (A), labelled with BrdU with *In Situ* Cell Proliferation Kit, FLUOS (Roche Applied Science) (B) and subsequently analyzed by flow cytometry (n=2). (C) Quantification of different phases of cell cycle.

4.4- Karyotype of p53 shRNA cells

The role of p53 in regulation of apoptosis, centrosome duplication and control over G2/M and the G1 cell cycle checkpoints can all contribute to the maintenance of genetic stability in other model systems. Therefore, the potential role of p53 in the maintenance of genetic stability of hESC was investigated. GFP and p53 shRNA transduced cell lines cultured under standard conditions in the presence of serum and passaged with mechanical dissociation did not develop karyotypic abnormalities up to passage 20 (Figure 4.3) when analysed by G-banding karyotype analysis. This analysis was not carried out on hESC cultured in serum-free culture conditions because karyotypic abnormalities frequently occur under these conditions and this would make it difficult to determine the specific role of p53 in genetic stability of hESC. A more refined analysis, like CHG arrays, should be carried out in order to assess the role of p53 in maintenance genetic stability in more detail because G-banding karyotype analysis is a low resolution assay that does not identify small deletions or mutations.

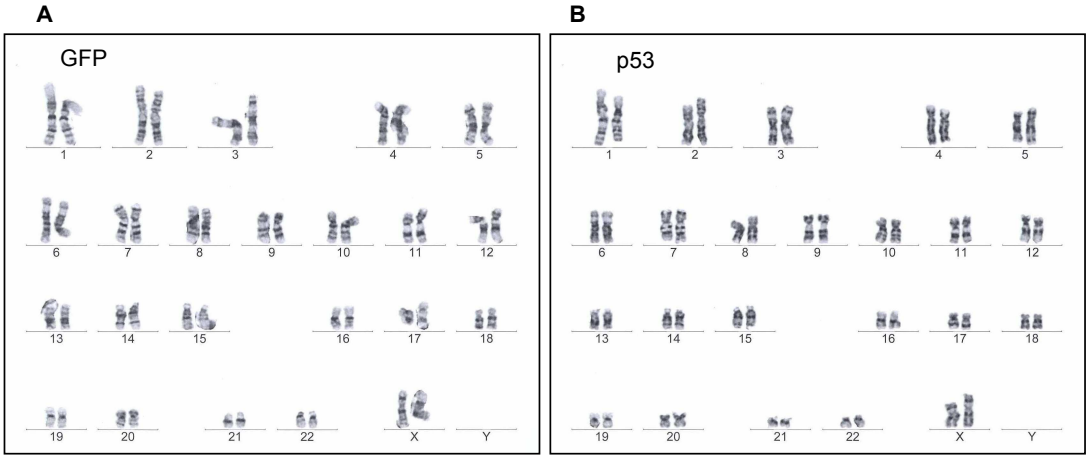

FIGURE 4.3- Karyotype of p53 shRNA transduced hESC is normal.
HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP and grown under standard conditions. Cells were processed for G-band karyotyping analysis by Cytogenetics Labotory, Monash Medical Centre, Clayton, Victoria, Australia.

4.5- p53 controls gene expression under normal hESC culture conditions: Transcriptome analysis of GFP and p53 shRNA transduced hESC

Three biological replicates of hESC (HES-2 and HES-4) transduced with either GFP (control) or p53 shRNA were FACS sorted using the cell surface markers CD9 and GCTM2 to obtain a population of undifferentiated hESC. RNA was isolated from these fractions and subjected to microarray analysis on the 46 k Human Illumina platform. Firstly, the gene expression profile results confirmed that p53 mRNA levels in p53 shRNA transduced hESC are reduced (Figure 4.4). The data shown in Figure 4.5 demonstrate that biological replicates of transduced cell lines display highly similar transcriptome profiles and that a similar set of genes was consistently altered between GFP and p53 shRNA transduced cells, independently of the hESC line used.

Since the data show that p53 shRNA transduced hESC display a reduction in spontaneous apoptosis as compared to GFP shRNA transduced hESC this set of differentially expressed

genes is presumably responsible for this phenotype. There were 148 differentially expressed genes in both HES-2 and HES-4 p53 shRNA tranduced cell lines in comparison to their controls (94 genes upregulated and 54 downregulated). Consistently differentially expressed genes between GFP and p53 shRNA transduced hESC and their functions are shown in Tables A 2.1-A 2.4 (Appendix II). About 70 % of these genes were found to contain p53 responsive elements in their promoters using p53FamTaG database. This database contains p53 family direct target genes selected in the human genome and searches for the presence of the p53 responsive elements (RE) and the expression profile of these target genes obtained by microarray experiments (Sbisa *et al*., 2007). Some of those genes (like DYRK3, VIM, TRIM22, GALNT13 and ISG20L1) were previously shown to bind p53 in their promoters (Wei *et al*., 2006). There are genes with known function that have been previously implicated in apoptosis and tumorigenesis, among other functions and other genes whose function is unknown. The main ontology groups of genes differentially expressed on the network that includes p53 revealed by Ingenuity pathway analysis are: 1. Cancer, 2. Cell Cycle, 3. DNA Replication, Recombination and Repair (Figure 4.6).

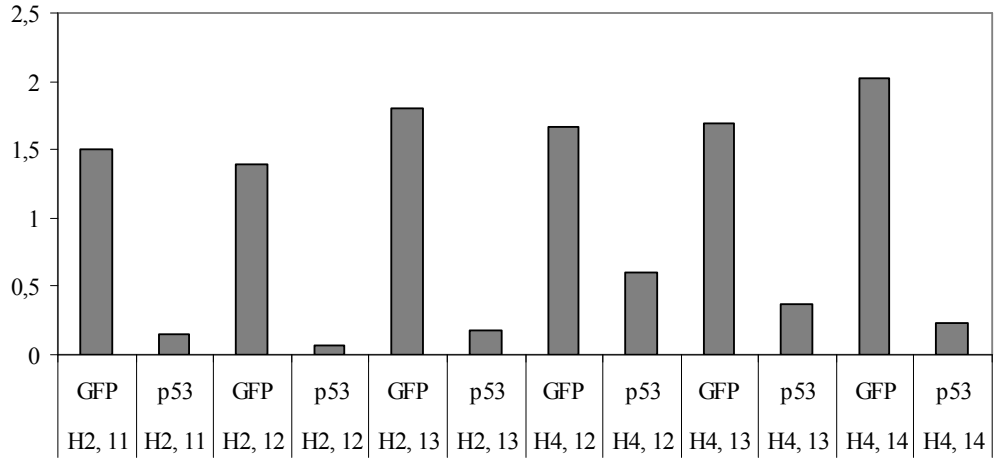

FIGURE 4.4- Levels of p53 in GFP and p53 shRNA transduced hESC grown under serum-free conditions measured by gene profile analysis. (H2 = HES-2, H4 = HES-4).

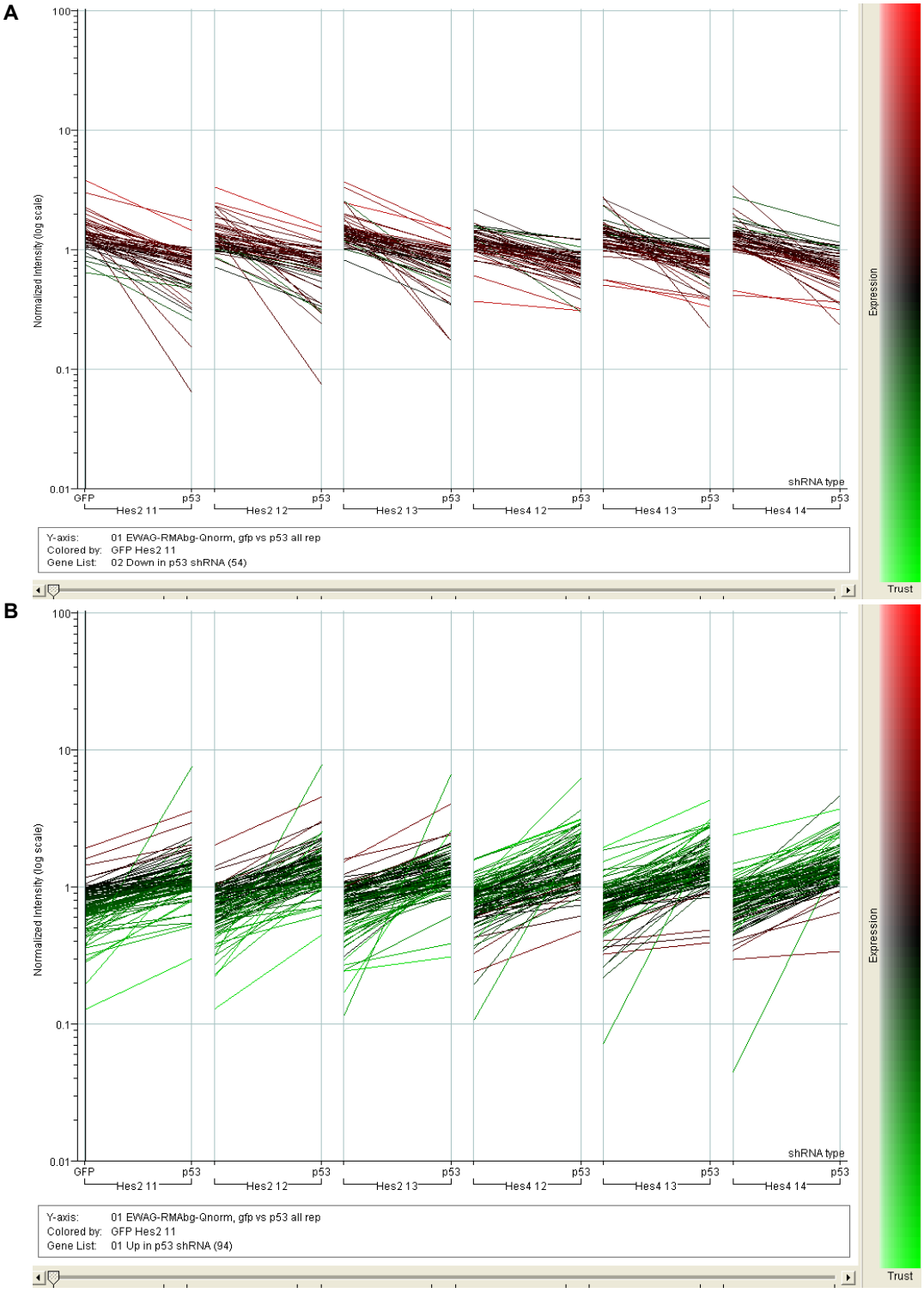

FIGURE 4.5– Gene Spring analysis: Genes that are (A) downregulated (54 genes) and (B) upregulated (94 genes) in p53 shRNA transduced HES-2 and HES-4 cells in comparison to control cell line.

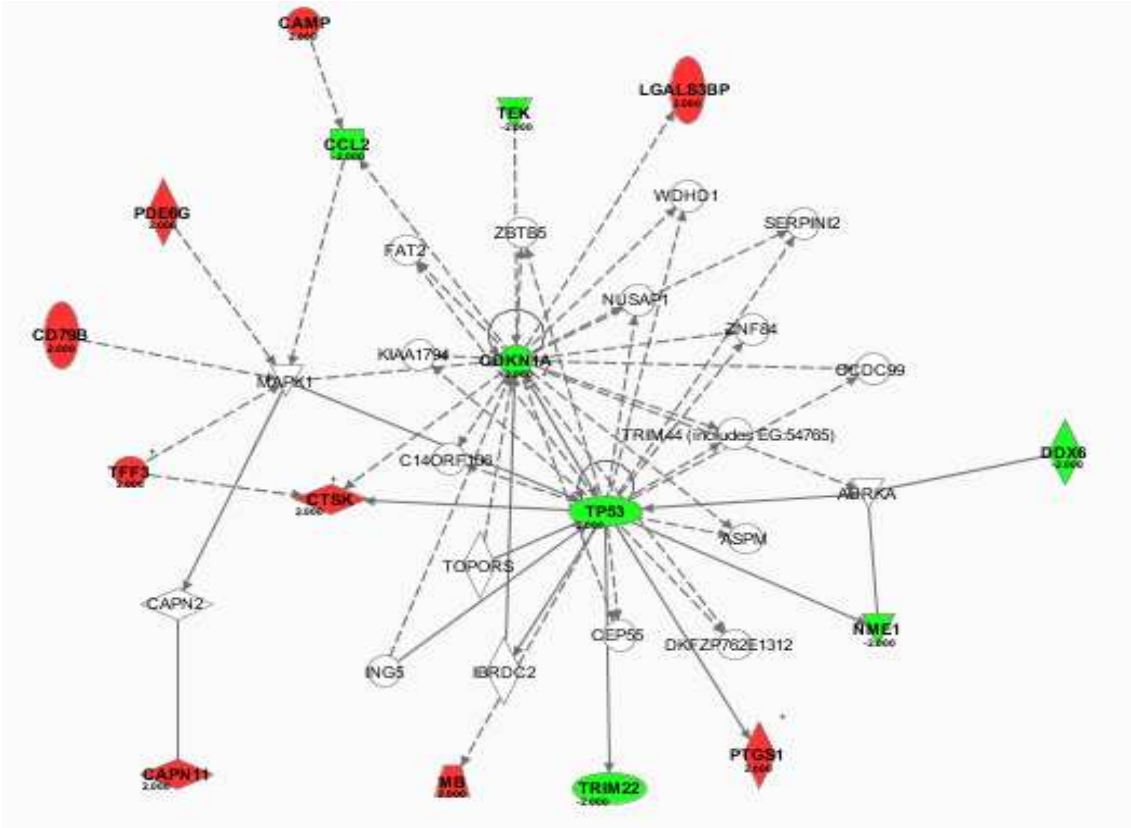

FIGURE 4.6- Best scoring network which includes p53 (Ingenuity pathways analysis on genes with Bstat > 0 in pairwise analysis). Top Functions: 1. Cancer, 2. Cell Cycle, 3. DNA Replication, Recombination, and Repair.

4.6- p53 has a small but reproducible effect on spontaneous differentiation

Even under the best culture conditions a proportion of hESC are known to spontaneously differentiate. In order to determine whether p53 plays a role in spontaneous differentiation of hESC, Oct4 expression was assessed in spontaneously differentiating p53 shRNA transduced hESC and GFP shRNA transduced controls.

The data show that 80 % reduction in p53 expression has a small but reproducible inhibitory effect on spontaneous differentiation of cultured hESC as determined by the slight increase in Oct4 expression by flow cytometric analysis in p53 shRNA transduced hESC lines (92.6 % ± 1.52 % (n=5) as compared to the GFP shRNA transduced controls (82.4 % ± 4.65 % n=5, $p<0.01$), similar to what was observed by Qin *et al* (Qin *et al*., 2006) (Figure 4.7).

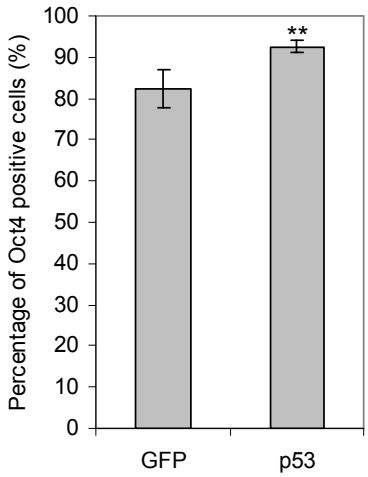

FIGURE 4.7- p53 has a small but reproducible inhibitory effect on spontaneous differentiation. HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP and grown in serum-free conditions. Both p53 shRNA transduced and GFP shRNA transduced were harvested and subsequently PFA-fixed, immunostained with the stem cell marker Oct4 and analyzed by flow cytometry. Results shown are expressed as percentage means ± S.D. (n=5). (*p*-value < 0.01 denoted by ** compared to GFP shRNA transduced cell line).

4.7- Role of p53 in induced differentiation of hESC with Activin A and BMP4

To investigate the role of p53 in direct differentiation of hESC, HES-2 cells stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP were differentiated for 7 days with Activin A (20 and 40 ng/ml) or BMP4 (25 and 50 ng/ml) on Matrigel coated culture flasks (Figures 4.8 and 4.9). The morphology of the colonies was assessed and the levels of p53, Nanog and Oct4 were quantified by real time PCR (Q-PCR).

In terms of morphology, both treatments induced the appearance of differentiated cells, which are characterized by increased cell size, higher ratio of cytoplasm nucleus and darker nuclei, forming less compacted areas of cells (Figure 4.8). In case of BMP4 treatment, p53 shRNA transduced hESC seemed to have a slightly higher proportion of undifferentiated cells, but with Activin A treatment the opposite was observed. Real-time PCR analysis gave an unexpected result, namely lower levels of ESC marker in p53 shRNA transduced cell lines compared to control even in untreated samples (Figure 4.9). This is surprising because p53 was been shown to suppress Nanog expression. The concentrations of BMP4 used were more effective in inducing differentiation than Activin A. Although both cell lines showed a significant reduction of Nanog and Oct4, p53 shRNA transduced cell line expressed higher levels of these ESC markers after BMP4 treatment (Figure 4.9A), confirming the morphological observations. Levels of p53 decreased with BMP4 treatment in GFP shRNA transduced cells.

Activin A at 20 ng/ml promoted the maintenance of hESC in undifferentiated state. At 40 ng/ml Activin A induced downregulation of Nanog and Oct4 in both cell lines, but p53 shRNA transduced cells expressed lower levels of these hESC markers (Figure 4.9B).

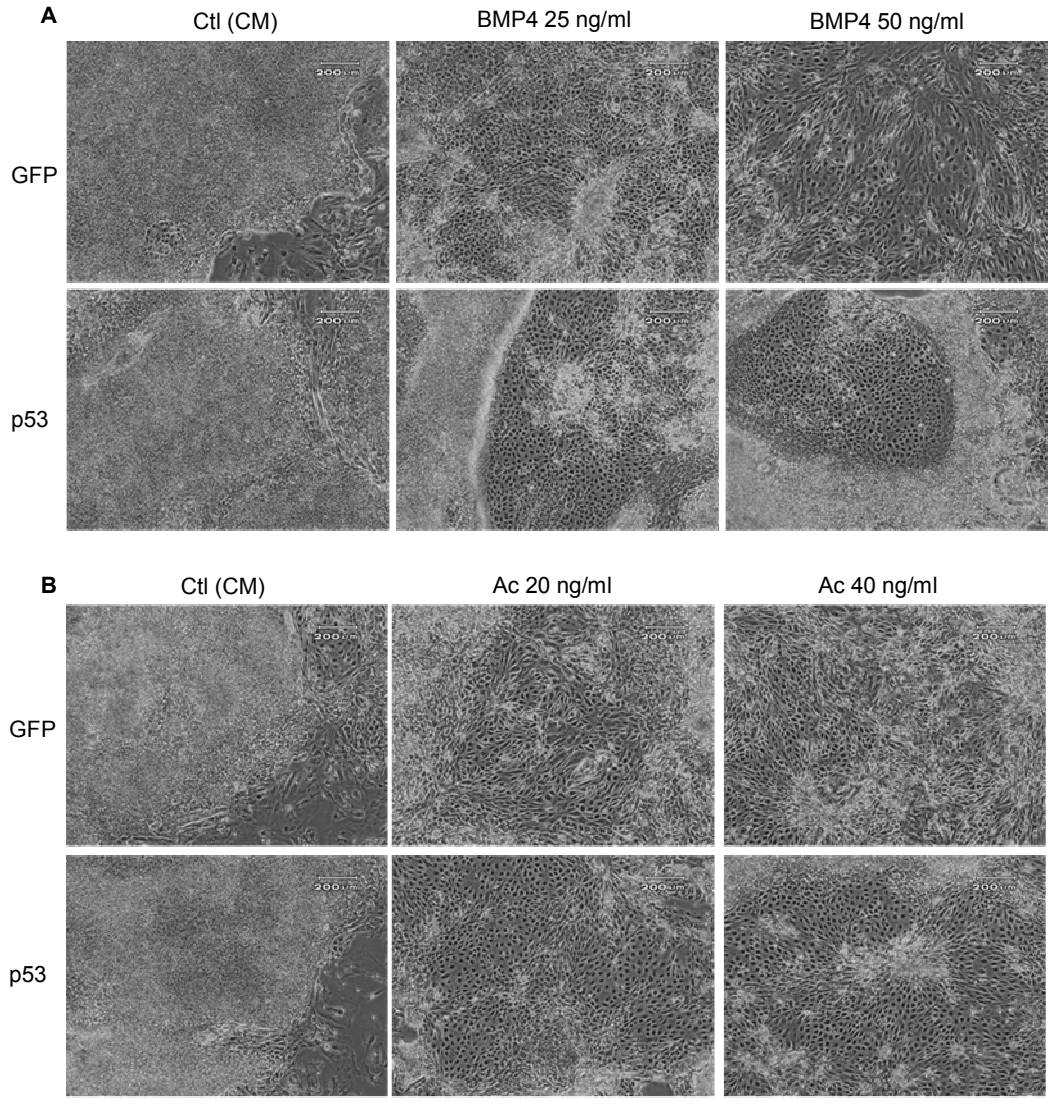

FIGURE 4.8- Colony morphology of p53 and GFP shRNA transduced hESC upon differentiation with Activin A and BMP4.
HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP. Both p53 shRNA transduced and GFP shRNA transduced hESC were differentiated for 7 days with (A) BMP4 (25 and 50 ng/ml) and (B) Activin A (Ac, 20 and 40 ng/ml) in Matrigel. Control cells were fed with conditioned media (CM). Scale bars = 200 μm.

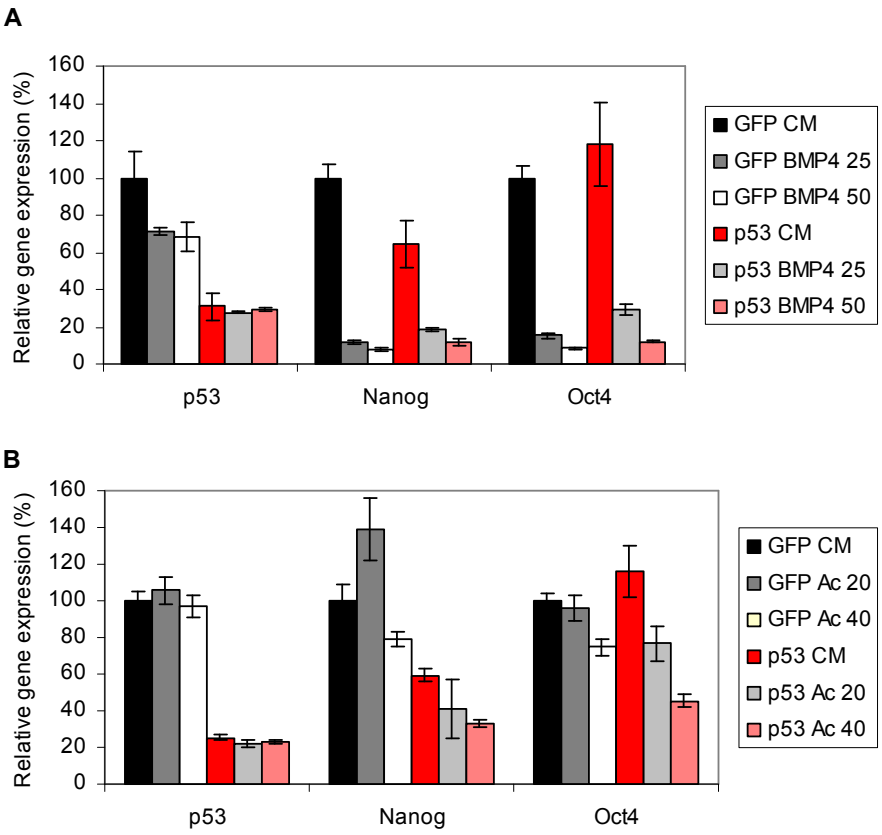

FIGURE 4.9- Differentiation of p53 and GFP shRNA transduced hESC with BMP4 and Activin A analysed by Q-PCR analysis.
HES-2 cells were stably transduced with lentivirus carrying shRNA designed to knockdown p53 or GFP. Both p53 shRNA transduced and GFP shRNA transduced hESC were differentiated for 7 days with (A) BMP4 (25 and 50 ng/ml) and (B) Activin A (Ac, 20 and 40 ng/ml) on Matrigel. Control cells were fed with conditioned media (CM). Total RNA was isolated with RNeasy Mini Kit (Qiagen) and reversed transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan® probes (Applied Byosystems) Human p53, Human Oct4 and Human Nanog were used to perform Q-PCR. Human GAPDH was used as an endogenous control. Samples were run in a 7500 Real-Time PCR System (Applied Biosystems) and data was analyzed using 7500 System SDS Software (Applied Biosystems).

4.8- Role of p53 hESC in teratoma formation

As mentioned in Chapter 2, the ability of hESC to form teratomas is the best test of pluripotency presently available. This assay was used to evaluate if p53 shRNA transduced hESC showed resistance to differentiation or if they have a bias towards a particular cell type. Both GFP and p53 shRNA transduced cell lines formed teratomas when implanted beneath the testis capsule of SCID mice. GFP shRNA transduced cell line teratomas contained mature adipose tissue, immature neural tissue, mature epithelium of uncertain histotype. The remainder of tissue was difficult to classify, but an immature stroma (mesenchyme) with a small epithelial component was observed. On the other hand, the p53 shRNA transduced cell line teratomas showed immature neural tissue, immature loose connective tissue/mesenchyme including some cystic spaces,

mature adipose tissue and mature epithelial structures, a very small amount of immature cartilage and small amount of squamous epithelium. The main differences between the teratomas developed by this two cell lines was that p53 shRNA transduced cell line teratomas were bigger, exhibited a higher proportion of immature neural tissue and had cystic spaces. Perhaps these cells are more prone undergo differentiation to the neural lineage, or mature more slowly.

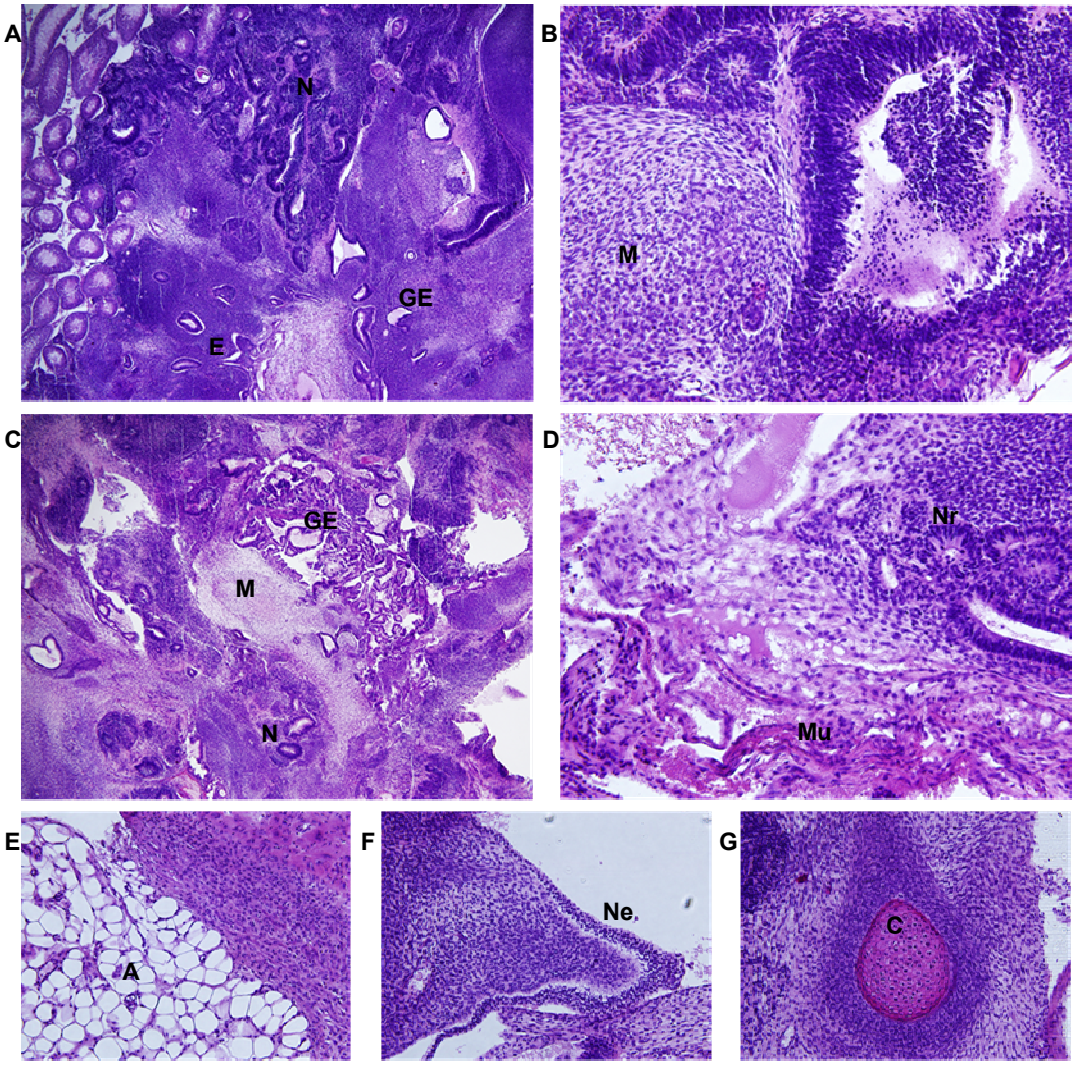

FIGURE 4.10- Histological analysis of differentiated tissues found in teratomas formed in the testis capsule of SCID mice after transplantation of GFP and p53 shRNA transduced hESC. Tumours were grown for a period of 6 weeks. Figures show bright field micrographs of tissues prepared in sections and stained with H&E.
(A) and (B) Teratomas formed from GFP shRNA transduced hESC.
(C) to (G) Teratomas formed from p53 shRNA transduced hESC.
N- neural tissue, GE- gut like epithelium, E- epithelium, M- mesenchyme tissue; Nr- neural rosettes; Mu- muscle; A- adipocytes; Ne- neural epithelium; C-cartilage.

4.9- Discussion

Homologous recombination to generate targeted gene deletions has so far proven to be inefficient in hESC. An alternative and more feasible way of investigating the role of a specific gene, such as p53, in hESC is to downregulate its expression through RNAi. Using lentiviral-metiated delivery of shRNA, it was possible to achieve p53 shRNA transduced hESC constitutively expressing around 20 % of p53 protein and around 28% of p53 mRNA as compared to untransduced or control GFP shRNA transduced hESC. Because suppression of transgenes delivered by lentiviral vectors in hESC has been reported, for each assay in which p53 shRNA transduced cell lines were used, p53 levels were measured by western blotting or Q-PCR to confirm that they were indeed reduced to the levels expected. No evidence of shRNA transgene inactivation was ever observed.

Spontaneous and etoposide-induced apoptosis of hESC is mediated by p53 since reduction of p53 expression in HES-2 causes a commensurate reduction in both. These data are in agreement with a recent study by Qin *et al* (Qin *et al*., 2006), who also showed that spontaneous and UV-induced apoptosis of hESC is controlled by p53. Reduction of the pool of p53 by lentivirally delivered shRNA leads to a reduced expression of PUMA and Bax, suggesting that these proteins may be critical p53 downstream targets for induction of etoposide-induced apoptosis in hESC.

As mentioned in Chapter 1, p53 can be involved in maintaining genetic stability. If p53-mediated spontaneous apoptosis is involved in eliminating genetically damaged cells from the ESC population, reduction of p53 function should result in an increased frequency of chromosomal abnormalities and genetic mutations. However, no significant differences were observed between cell cycle of GFP and p53 shRNA transduced cell lines or distribution of cells in cell cycle phases, suggesting that any cell cycle checkpoint operation triggered by spontaneous apoptosis is unaltered following p53 knockdown. The karyotypes of both cell lines were found to be normal at passage 20. Several studies suggest that apoptosis is among the most important p53 functions selected against during tumour progression. The defective cell cycle-checkpoints and aneuploidy that often coincide with p53 inactivation in human cancers are just byproducts of p53 loss (Jeffers *et al*., 2003) (Duensing and Duensing, 2005) (Schmitt *et al*., 2002). Furthermore, some cell lines can retain a diploid karyotype in the presence of mutated p53 (Lengauer *et al*., 1998). However, p53 inactivation synergizes with oncogenic events or other cellular insults to promote aneuploidy and may facilitate chromosomal imbalances through indirect mechanisms. As mentioned before, p53 also has been implicated in regulation of centrosome duplication and abnormal amplification of centrosomes is the major cause of mitotic defects and chromosome instability in cancer cells (Shinmura *et al*., 2007). A study performed by D'Assoro *et al* (D'Assoro *et al*., 2004) showed that cells with partial impairment of p53 function can retain normal centrosome numbers similar to wild-type cells. However, when exposed to DNA damage, these cells readily developed centrosome amplification whereas cells carrying wild-type p53 maintained normal centrosome numbers. Perhaps low levels of p53 under physiological conditions are still sufficient to ensure that karyotypic abnormalities do not occur in hESC (indeed a low level of p53 may also be

sufficient to maintain a normal cell cycle). To test this hypothesis, hESC transduced with shRNA to knockdown p53 could be subjected to DNA damage and followed over time to detect if those cells develop abnormalities. Other possible explanation for an absence of abnormal karyotypes in p53 shRNA transduced hESC are 1) G-banding karyotype analysis is a low resolution assay that does not identify small deletions or mutations 2) it takes longer than 20 passages for those abnormalities to appear, 3) hESC possess efficient and alternative mechanisms besides p53 pathway to delete/suppress abnormal cells or display enhanced repair DNA, and 4) p53 has no role in maintaining genetic stability in hESC.

Microarray analysis of untreated GFP and p53 transduced hESC shows that there is a highly similar set of up and down regulated mRNAs in both HES-2 and HES-4 p53 shRNA transduced hESC in three independent biological replicates. Bioinformatic analysis shows that more than 70 % of these genes contain p53 responsive elements. Some of p53 regulated genes were previously shown to exhibit binding of p53 to consensus p53 binding sites in their promoters by CHIP analysis (Wei *et al*., 2006). These data strongly suggest that the observed gene expression changes are not due to viral insertion effects and that even without etoposide treatment, basal expression of p53 functions in the control of downstream target genes. These genes should be validated by further analysis such as Q-PCR and western blotting. The ontology groups of genes differentially expressed on the network that includes p53 revealed by Ingenuity Pathway Analysis include carcinogenesis, cell cycle regulation and DNA replication, recombination and repair. This may indicate a role of p53 in maintenance of genetic stability in hESC.

The biological processes of the genes upregulated in p53 shRNA transduced cell lines are cell adhesion, transport (amino acid and ion), regulation of pH, G-protein coupled receptor protein signalling pathway, lipid metabolic process, proteolysis, cell cycle and development. It is noteworthy that some of these upregulated genes may be involved in apoptosis regulation and contribute to tumorigenesis. TFF3 (trefoil factor 3) protein decreases apoptosis of colonocytes and etoposide-induced apoptosis of AGS human gastric cancer cell line in a p53 dependent fashion (Taupin *et al*., 2000). On the other hand, some of these genes were reported to be overexpressed in cancer cell lines. For example, MVP (major vault protein) is overexpressed in multidrug-resistant cancer cells (Mossink *et al*., 2003) and its transcription can be suppressed by p53 (Wang and Beck, 1998). Furthermore, MVP has also been claimed to prevent cells from undergoing stress response (Yi *et al*., 2005). Cathepsin K has potent proteolytic activities against several extracellular matrix components and its mRNA and protein were found in samples of primary breast carcinoma (Castiglioni *et al*., 1994) and prostate cancer (Brubaker *et al*., 2003). It has been suggested that this protease, like metalloproteases, may contribute to the invasive potential of certain cancer cells through the digestion of the extracellular matrix (Funicello *et al*., 2007). PTGS1, also known as Cox-1, is highly expressed in a mouse model of epithelial ovarian cancer, which lacks p53 but overexpresses c-myc and K-ras or c-myc and Akt (Daikoku *et al*., 2006). HSPB8 (heat shock 22kDa protein 8) depending on the cell type and the extent of expression can exert both pro- and anti-apoptotic activity (Gober *et al*., 2003; Gober *et al*., 2005)(reviewed by Shemetov *et al*., 2007).

However, there are a few upregulated genes that are known to have a pro-apoptotic role. NRGN (neurogranin), also known as RC3, a calcium/calmodulin binding protein can induce induction of apoptosis due to cytokine IL-2 deprivation in lymphoid cells (Devireddy and Green, 2003). In this model, levels of intracellular Ca^{2+} levels play a role in T-cell apoptosis. Bin1 (bridging integrator 1) is a tumour suppressor that mediates apoptosis by interacting with c-myc (Sakamuro *et al*., 1996), is involved in the negative regulation of progression through cell cycle and can inhibit transformation by mutant p53 (Elliott *et al*., 1999). It has also been reported that Bin1 participates in a caspase-independent cell death program (Elliott *et al*., 2000). These genes can be part of simultaneous or alternative mechanisms to suppress the appearance of genetic abnormalities in the hESC population.

The biological processes of the genes downregulated in p53 shRNA transduced cell lines are genes include transport, regulation of transcription, nucleic acid metabolic process, cell cycle and protein amino acid phosphorylation. Some of the downregulated genes can also contribute to tumorigenesis. NME1 (non-metastatic cells 1) which is involved in negative regulation of progression through cell cycle, was identified because of its reduced mRNA transcript levels in highly metastatic cells (Driouch *et al*., 1998). CST6 (cystatin E/M) is a secreted inhibitor of lysosomal cysteine proteases and its loss of expression is likely associated with the progression of a primary tumour to a metastatic phenotype (Ai *et al*., 2006). TRIM22 (tripartite motif-containing 22) is a p53 target gene that confers reduced clonogenic growth and differentiation of leukemic U-937 cells (Obad *et al*., 2004). This expression of this gene has also been shown to increase in response to DNA-damaging treatments in B-lymphoblastoid cell line TK6 (Amundson *et al*., 2005) and in response to cisplatin in TGCTs (Kerley-Hamilton *et al*., 2005). TMEM43 (transmembrane protein 43) is another gene regulated by p53 identified by a genome-wide analysis under hypoxic conditions (Hammond *et al*., 2006). Although the cell cycle inhibitor p21 (CDKN1A) protein was not detected (section 3.9 of Chapter 2), levels of this p53 downstream target inhibitor of cell cycle were found to be reduced in p53 shRNA transduced cell lines. On the other hand, Bax which was found to be downregulated by western blotting analysis did not display differential expression on the microarray analysis. In order to validate these results Q-PCR should be performed.

p53 had a small but reproducible effect in inhibiting spontaneous differentiation. Preliminary studies to address the role of p53 in differentiation of hESC revealed a slight resistance of p53 shRNA transduced cell lines to BMP4-induced differentiation but the opposite was observed in relation to Activin A treatment, based on expression of Nanog and Oct4. Because these morphogens operate through different pathways, p53 may participate differently in the differentiation process induced by them. In GFP shRNA transduced cells p53 levels decreased upon differentiation with BMP4. Perhaps more hESC differentiated progeny show regulation of p53 similar to somatic cells, where this tumor suppressor gene is kept at low levels. A very interesting study performed by Dazard and colleagues (Dazard *et al*., 2000) explored the p53/MDM2 regulatory loop during different transitions of the human epidermal differentiation program. They showed that maximum expression of p53 was found in proliferating keratinocytes and that a transient induction of MDM2 and a downregulation of p53 characterized the transition

from proliferation to differentiation in primary human keratinocytes. These changes correlated with an increase in cell size, at the time of irreversible commitment to differentiation. It is noteworthy to mention that the differentiation assays performed on p53 and GFP shRNA transduced cell line with Activin A and BMP4 are very simple and were just a first approach to check if p53 has a role on initial steps of differentiation. Additionally, only technical replicates were analyzed. In order to confirm some of the findings, biological replicates should be performed. Furthermore, this study only quantified the expression of ESC genes. Because some ESC markers also appeared in their progeny or have not been silenced yet during the process of differentiation, future studies should use probes to check the expression of known differentiation genes. Longer protocols of differentiation into different cell types should also be performed.

p53 shRNA transduced cells formed teratomas with representative tissues of all three germ layers when implanted beneath the testis capsule of SCID mice, therefore they are still pluripotent. Teratomas formed with these cells exhibited a higher proportion of immature neural tissue compared to control cells, suggesting that these cells are more prone undergo differentiation to the neural lineage, or mature more slowly.

CHAPTER 5

Conclusions and Future directions

hESC are derived from the blastocyst of the preimplantation embryo and give rise into cells of all lineages of the body. Since they were derived in 1998, significant progress has been made in the characterization of the hESC lines. Their differentiation into various cell types has opened the possibility to use these cells in cell replacement therapies and as a model for early human development. However, the biological characterization of undifferentiated hESC is still poorly incomplete. Genetic stability is an aspect that needs also to be addressed in detail in order to ensure safe use of these promising cells.

hESC display unlimited self-renewal, however they do not show obvious signs of transformation if cultured under standard conditions. It is not clear how ESC are able to proliferate indefinitely without acquiring genetic changes that would lead to oncogenic transformation. This could be either due to a very efficient DNA repair or the selective induction of apoptosis in ESC. There is little published work on apoptotic signalling pathways in hESC.

hESC represent a unique cellular model to study regulation of apoptosis, given that they are non-transformed immortal cells with a normal karyotype, and cannot be directly compared to long-established cell lines, in which apoptosis pathways are intrinsically altered as part of the transformation process, or even to primary cell cultures which display senescence, DNA damage-induced cell cycle arrest and contact inhibition. Furthermore, it is anticipated that a detailed understanding of apoptosis pathways in hESC will be useful for future cell replacement therapies using hESC, since it may a allow for the selective elimination of unwanted cell lineages prior or subsequent to lineage specific differentiation. Apoptosis can also be used as a quality control parameter. For example, if levels of apoptosis are abnormally low, this might be indicative of the presence of chromosomal abnormalities, or if levels are too high, this could reflect suboptimal culture conditions, like non-suportive MEFs.

In an effort to better understand apoptosis pathways in hESC, different known regulators of apoptosis were investigated. The current study shows that hESC display the classic features of apoptosis and, like mESC, are extremely sensitive to both spontaneous and DNA-induced apoptosis. Indeed, hESC appear to be far more sensitive to etoposide-induced apoptosis than other cell lines or primary cell cultures. Because pluripotent cells can contribute precursors to all adult cell lineages, damage to their genomes during embryonic development may cause serious developmental malfunctions. Teleologically, it might be better to eliminate pluripotent cells that represent a potential oncogenic danger than to attempt to repair them. The high proliferative capacity of hESC, or their counterparts in the embryo, can compensate for the loss of some cells through apoptosis that would otherwise propagate genetic damage throughout all their descendants. At present it remains to be established whether this spontaneous apoptosis, although p53-dependendent, is due to suboptimal culture conditions, or whether it is an intrinsic feature of hESC that is involved in eliminating cells with acquired DNA damage. However, the extrinsic apoptotic pathway seems not to be operational in hESC since TNFα failed to induce apoptosis. Other extrinsic pathways, such as Fas ligand mediated apoptosis, should be also investigated.

When cultured for long periods of time under serum-free conditions, hESC can adapt to culture conditions and often gain chromosomal abnormalities resembling the ones observed in hECC. This adaptation process result most likely from alterations that lead to better survival. Late passage hESC with an abnormal karyotype exhibited reduced apoptotic response to low doses of etoposide when compared to early passage diploid cells. This is important to take in account especially when studying apoptosis in late passage hESC. Biomarkers of the adapted state, and sensitive bioassays for transformation, will be required to monitor cultures for development of genetic abnormalities that have phenotypic consequences.

hESC cultured on MEF feeder cells in the presence of KSR supplemented with 4 ng/ml FGF-2 (Amit *et al*., 2004) appear to be a morphologically homogeneous population of undifferentiated cells. However, hESC cultured under these conditions are heterogeneous and display various degrees of differentiation. This also occurs in hESC under standard conditions (Laslett *et al*., 2007) and it is not clear if this heterogeneity is due to intrinsic biological properties of hESC or to culture conditions. The sensitivity of hESC to etoposide-induced apoptosis is dependent on their differentiation status. Undifferentiated hESC that express Oct4 are the least resistant to etoposide-induced apoptosis as compared to their more differentiated progeny. When measuring apoptosis or any other biological parameter is important to assess differentiation status of populations, because different subpopulations can display different responses. This is particularly important when hESC are to be used for cytotoxicity assays (as proposed in many publications). In the case of mESC, the relationship between the level of differentiation and the sensitivity of mESC to apoptotic stimuli remains unexplored to present knowledge.

In this study a mixed effect of p53 on differentiation of hESC was observed. Its downregulation slightly inhibits spontaneous differentiation and BMP4-induced differentiation, but seems to promote differentiation when cells are treated with Activin A. Longer differentiation protocols with these morphogens and measurement of differentiation markers of different cell lineages would be required to further confirm these preliminary findings. The teratomas formed from p53 shRNA transduced cells exhibited a higher proportion of immature neural tissue, suggesting that these cells are more prone to undergo differentiation to the neural lineage, or mature more slowly.

p53 is considered a critical regulator of genomic integrity because of its ability to trigger apoptosis and/or cell cycle arrest in response to genotoxic stress. Induction of of apoptosis by p53 can occur via both transcription-independent and transcription dependent pathways. The current study shows that p53 is required for etoposide-induced apoptosis of hESC and that p53 response in these cells involves both mitochondrial and nuclear apoptotic pathways. The presence of two simultaneous pathways to induce p53-mediated apoptosis may be a failsafe mechanism of pluripotent cells to ensure that abnormal cells do not contribute to the developing tissues and the germ line.

It would be interesting to perform timed gene expression profile analysis to identify known and novel genes involved in the apoptotic response of hESC. This is a technical challenge since apoptosis progresses very quickly in hESC and RNA in apoptotic cells that have to be sorted by flow cytometry (to obtain a population of undifferentiated hESC) can degrade quickly during this

process. Such experiments would further represent a good opportunity to study p53 post-translational modifications involved in the response to different apoptotic stimuli and elucidate the differences between mitochondrially and nuclear targeted p53.

If p53-mediated spontaneous apoptosis is involved in eliminating genetically damaged cells from the hESC population, reduction of p53 function should result in an increased frequency of chromosomal abnormalities and genetic mutations. This hypothesis is under further investigation. G-banding did not reveal an abnormal karyotype of cell lines transduced with p53 shRNA mediated knockdown after 20 passages under standard culture conditions. As mentioned above, more refined techniques like CGH arrays may identified include other more subtle genetic changes in these cells. Also studying the epigenetic status of these cells could elucidate if p53 plays a role in genetic stability.

It is puzzling how hESC can tolerate high levels of functional p53 without undergoing cell cycle arrest or apoptosis. Gene expression profile analysis of hESC transduced with p53 shRNA and GFP shRNA shows that there is a highly similar set of up and down regulated mRNAs in both HES-2 and HES-4. Some genes have previously been reported to play a role in apoptosis and tumorigenesis, among other functions. Novel p53 regulated genes whose function is unknown were also identified. Perhaps future studies should address the validation of some of the genes that are differentially expressed, due to reduction of p53 levels and investigate their role in hESC biology. The main ontology of genes differentially expressed in the network that includes p53 relate to carcinogenesis, cell cycle regulation and DNA replication, recombination and repair. This may point to a role for p53 in maintenance of genetic stability in hESC. However, the fact that so far no chromosomal abnormalities were observed in p53 shRNA transduced cell lines may indicate that alternative mechanism may also be operational in hESC. This could involve genes like Bin1, responsible for inhibition of transformation. It would be very interesting to downregulate both these tumour suppressors to evaluate if they play complementary or alternative roles in maintaining genomic stability in hESC by eliminating from the population cells that can become transformed.

As hESC são derivadas a partir do blastocisto do embrião preimplantatório e dão origem às células de todas as lineagem do corpo. Desde a sua descoberta em 1998, progresso tem sido feito na caracterização das linhas celulares derivadas. A diferenciação em vários tipos celulares abriu a possibilidade de utilizar estas células em terapias de substituição de células e como modelo para o devolvimento humano. Contudo, a caracterizção biológica das hESC indiferenciadas continua incompleta. A estabilidade genética é também um aspecto que é preciso ser estudado em detalhe de modo a assegurar o uso seguro destas células tão promissoras.

hESC possuem um capacidade de auto-renovação ilimitada, contudo elas não exibem sinais de transformação óbvios quando cultivadas nas condições padrão. Não é ainda claro como elas são capazes de proliferar infinitamente sem adquirirem alterações genéticas que possam levar à transformação oncogénica. Isto poder ser devido a uma eficaz reparação no ADN ou a uma indução selectiva da apoptose. Existem poucos estudos publicados em vias de sinalização de apoptose em hESC.

hESC representam uma modelo celular único para o estudo de regulação da apoptose, dado que são células não transformadas imortais com cariótipo normal e não podem ser comparadas directamente com as já há muito estabelecidas linhas celulares, nas quais as vias de sinalização de apoptose estão intrinsicamente alteradas como parte do processo de transformação, ou até das linhas primárias que exibem senescência e paragem no ciclo celular devido a danos no ADN. Por um outro lado, é antecipado que uma melhor compreensão das vias de apoptose das hESC irá ser útil em futuras terapias de substituição celular, mas vez que poderá permitir a eliminação selectiva de linhagem celulares não desejáveis antes ou depois da diferenciação específica. A apoptose pode também ser usada como um parâmetro de controlo de qualidade. Por exemplo, se a apoptose for muito baixa poderá ser indicativo da presença de alguma anomalia cromossómica ou se for muito elevada poderá ser indicativo de condições de cultura sub-óptimas, como MEF não suportivos.

No sentido de melhor compreender as vias de sinalização de apoptose em hESC, diferentes reguladores da apoptose já conhecidos foram investigados. O presente estudo demonstra que as hESC exibem características clássicas da apoptose e, como as mESC, são extremamente sensíveis à apoptose espontânea e induzida por danos no ADN. De facto, as hESC parecem ser mais sensíveis à apoptose induzida por etopósido do que outras linhas celulares ou culturas primárias. Devido ao facto das ESC contribuírem para os precurssores de todas a linhagem celulares adultas, danos no genoma das ESC pode causar sérias disfuncções no desenvolvimento. Teleologicamente, o embrião em desenvolvimento poderá escolher em eliminar as células que representam um potencial perigo oncogénico do que tentar repará-las. A alta capacidade proliferativa das hESC poderá compensar a perda de algumas que podem pôr em risco a população. Presentemente, ainda não se sabe se a apoptose espontânea, embora dependente do p53, é devida a condições de cultura sub-óptimas ou a característica intrínsica das hESC envolvida na eliminação de danos adquiridos no ADN. Contudo, a via extrínsica de

apoptose parece não estar operacional em hESC uma vez que TNFα falhou na indução da apoptose. A apoptose mediada por Fas ligante deverá ser também investigada.

Quando cultivadas por longos períodos de tempo com meios sem soro, as hESC tendem a adaptar às condições de cultura e desenvolvem com frequência abnormalidades cromossómicas semelhantes às observadas em células embrionárias do carcinoma humanas (hECC). Este processo de adaptação resulta muito provavelmente de alterações que levam a melhor uma sobrevivência. hESC com muitas passagem e um cariótipo anormal exibem resposta apoptótica reduzida a baixas concentrações de etopósido quando comparadas com células passadas poucas vezes. Isto é importante ter em conta especialmente qando se estuda a apoptose em hESC com elevado número de passagens. Biomarcadores do estado adaptado e bioensaios sensíveis à tranformação serão necessárias para monitorizar as culturas relativamente ao desenvolvimento de anormalidades genéticas que têm consequências fenotípicas.

As hESC cultivadas sob camadas de MEF e na presença de KSR suplementado com 4 ng/ml de FGF-2 (Amit *et al*., 2004) aparentam ser uma população morfologicamente homogénea de células indiferenciadas. Contudo, hESC cultivadas nestas condições são heterogéneas e exibem vários graus de diferenciação. Isto também ocorre hESC cultivadas em condições padrão (Laslett *et al*., 2007) e ainda não é claro se estas diferenças fenotípicas são devidas a propriedades biológicas das hESC ou a condições da cultura. A sensibilidade de hESC à apoptose induzida pelo etopósido é dependente do estado da sua diferenciação. hESC indiferenciadas que expressam Oct4 são menos resistentes à apoptose induzida pelo etopósido quando comparadas com a sua progenia mais diferenciada. Quando se mede a apoptose ou qualquer outro parâmetro biológico é importante também verificar o estado de diferenciação das populações dada porque diferentes subpopulações poderão exibir diferentes respostas. Isto é particularmente importante quando as hESC são usado em ensaios citotóxicos (como tem sido proposto em muitas publicações). No caso das mESC, a relação entre o estado de diferenciação e a sensibilidade a estímulos apoptóticos continua por ser investigada.

Neste estudo é observado um efeito misto do p53 na differenciação das hESC. A diminuição dos seus níveis inibe a diferenciação espontânea e a induzida por BMP4, mas promove a diferenciação quando as células são exposta à Activina A. Protocolos de diferenciação mais longos com estas morfógenos e medição dos níveis de marcadores de diferenciação de diferentes linhagens celulares poderá confirmar estes resultados preliminares. Os teratomas formados por estas células exibem uma maior proporção de tecido neural imaturo, o que sugere que elas preferencialmente se diferenciam numa linhagem neural ou o seu processo de maturação é mais lento.

O p53 é considerado o regulador crítico da integridade genómica devido à sua capacidade de desplotar apoptose e/ou paragem no ciclo celular em resposta a stresse no genoma. A indução da apoptose pelo p53 pode ocorre através de vias dependentes ou indepedentes da transcripção. O presente estudo mostra que o p53 é requerido na apoptose de hESC induzida por etopósido e a resposta do p53 nestas células envolve ambas as vias apoptóticas mitocondrial e nuclear. A presença destas duas vias em simultâneo poderá constituir um mecanismo para assegurar que

as hESC com mutações não contribuam para os tecidos em desenvolvimento e para a linha germinal.

Seria interessante realizar um estudo do perfil genético para identificar genes com funções conhecidas e até no genes involvidos na resposta apoptótica das hESC. A nível técnico isto é um desafio porque a apoptose progride muito rapidamene e o RNA pode degradar-se durante o processo de isolar as células indiferenciadas. Este modelo representa também uma boa oportunidade para estudar as modificações pós-tradução envolvidas nas resposta a diferentes estímulos apoptóticas e as enzimas envolvidas.

Caso a apoptose espontânea mediada pelo p53 esteja envolvida na eliminação de células com danos genéticos da população de hESC, a redução da função do p53 deverá resultar um aumento da frequência de anormalidades cromossómicas e mutações. Esta hipótese está correntemente a ser testada com a passagem destas células e cariotipagem. Este método ainda não revelou um cariótipo anormal nas células após 20 passagens sob condições padrão. Como anteriormente foi mencionado, técnicas mais refinadas poderão identificar alterações genéticas mais subtis. Também o estudo do estado epigenético poderá elucidar se o p53 tem um papel na estabilidade genética.

É intrigante como as ESC podem tolerar elevados níveis de expressão de p53 functional sem que ocorra paragem no ciclo celular ou apoptose. Análise dos perfis de expressão genética células infectadas com shRNA para o p53 e GFP revela que um conjunto muito similar de mRNAs sofre aumento ou diminuição em ambas HES-2 and HES-4. Alguns desses genes já form implicados na apoptose e na tumorigénesis, entre outras funções. Outros têm função desconhecida. Estudos futuros deverão validar estes resultados de genes expressos diferentemente devido a uma redução nos níveis de p53 e investigar o papel na biologia das hESC. As funções principais dos genes diferentemente expressos que contêm o p53 estão envolvidos em carcinogénesis, regulação do ciclo celular e replicação, recombinação e reparação do ADN. Isto poderá evidenciar o papel do p53 na manutenção da estabilidade das hESC. O facto de não terem sido detectadas anormalidades cromossómicas nas hESC que expressam shRNA para diminuir os níveis de p53 poderá sugerir que mecanismos alternativos são operacionais em hESC, envolvendo genes como o Bin1, responsáveis pela inibição da transformação. Seria muito interessante também diminiuir os níveis de expressão desses supressores de tumor para avaliar que eles desempenham mecanismos complementares ou alternativos na manutenção da estabilidade genómica das hESC através da eliminação de populações de células que poderam tornar-se transformadas.

CHAPTER 6

BIBLIOGRAPHY

Abeyta, M. J., Clark, A. T., Rodriguez, R. T., Bodnar, M. S., Pera, R. A., and Firpo, M. T. (2004). Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum Mol Genet* **13,** 601-8.

Academic Pathology. Queen's University of Belfast. Last Modified February 26, 2003. Available at http://www.qub.ac.uk

Adrain, C., Brumatti, G., and Martin, S. J. (2006). Apoptosomes: protease activation platforms to die from. *Trends Biochem Sci* **31,** 243-7.

Adrain, C., and Martin, S. J. (2001). The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* **26,** 390-7.

Ai, L., Kim, W. J., Kim, T. Y., Fields, C. R., Massoll, N. A., Robertson, K. D., and Brown, K. D. (2006). Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression. *Cancer Res* **66,** 7899-909.

Akita, K., Ohtsuki, T., Nukada, Y., Tanimoto, T., Namba, M., Okura, T., Takakura-Yamamoto, R., Torigoe, K., Gu, Y., Su, M. S., Fujii, M., Satoh-Itoh, M., Yamamoto, K., Kohno, K., Ikeda, M., and Kurimoto, M. (1997). Involvement of caspase-1 and caspase-3 in the production and processing of mature human interleukin 18 in monocytic THP.1 cells. *J Biol Chem* **272,** 26595-603.

Aladjem, M. I., Spike, B. T., Rodewald, L. W., Hope, T. J., Klemm, M., Jaenisch, R., and Wahl, G. M. (1998). ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. *Curr Biol* **8,** 145-55.

Allegrucci, C., Wu, Y. Z., Thurston, A., Denning, C. N., Priddle, H., Mummery, C. L., Ward-van Oostwaard, D., Andrews, P. W., Stojkovic, M., Smith, N., Parkin, T., Jones, M. E., Warren, G., Yu, L., Brena, R. M., Plass, C., and Young, L. E. (2007). Restriction landmark genome scanning identifies culture-induced DNA methylation instability in the human embryonic stem cell epigenome. *Hum Mol Genet* **16,** 1253-68.

Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., Itskovitz-Eldor, J., and Thomson, J. A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* **227,** 271-8.

Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* **70,** 837-45.

Amundson, S. A., Do, K. T., Vinikoor, L., Koch-Paiz, C. A., Bittner, M. L., Trent, J. M., Meltzer, P., and Fornace, A. J., Jr. (2005). Stress-specific signatures: expression profiling of p53 wild-type and -null human cells. *Oncogene* **24,** 4572-9.

Andrews, P., He, Y. J., and Xiong, Y. (2006). Cytoplasmic localized ubiquitin ligase cullin 7 binds to p53 and promotes cell growth by antagonizing p53 function. *Oncogene* **25,** 4534-48.

Andrews, P. W., Benvenisty, N., McKay, R., Pera, M. F., Rossant, J., Semb, H., Stacey, G. N., and Steering Committee of the International Stem Cell, I. (2005). The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. *Nature Biotechnology* **23,** 795-7.

Armstrong, J. F., Kaufman, M. H., Harrison, D. J., and Clarke, A. R. (1995). High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* **5,** 931-6.

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. L., and Tzukerman, M. (2001). Insulin production by human embryonic stem cells. *Diabetes* **50,** 1691-7.

Attardi, L. D., de Vries, A., and Jacks, T. (2004). Activation of the p53-dependent G1 checkpoint response in mouse embryo fibroblasts depends on the specific DNA damage inducer. *Oncogene* **23,** 973-80.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* **17,** 126-40.

Baker, D. E., Harrison, N. J., Maltby, E., Smith, K., Moore, H. D., Shaw, P. J., Heath, P. R., Holden, H., and Andrews, P. W. (2007). Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* **25,** 207-15.

Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* **41,** 899-906.

Banbury, D. N. (1994). New monoclonal antibodies to visualize vesicular compartments. *Thesis (D.Phil.) University of Oxford*.

Banerjee, D., Lenz, H. J., Schnieders, B., Manno, D. J., Ju, J. F., Spears, C. P., Hochhauser, D., Danenberg, K., Danenberg, P., and Bertino, J. R. (1995). Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. *Cell Growth Differ* **6,** 1405-13.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281-97.

Beachy, P. A., Karhadkar, S. S., and Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature* **432,** 324-31.

Beattie, G. M., Lopez, A. D., Bucay, N., Hinton, A., Firpo, M. T., King, C. C., and Hayek, A. (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* **23,** 489-95.

Becker, K. A., Ghule, P. N., Therrien, J. A., Lian, J. B., Stein, J. L., van Wijnen, A. J., and Stein, G. S. (2006). Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. *J Cell Physiol* **209,** 883-93.

Benard, J., Douc-Rasy, S., and Ahomadegbe, J. C. (2003). TP53 family members and human cancers. *Hum Mutat* **21,** 182-91.

Bendall, S. C., Stewart, M. H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., Ramos-Mejia, V., Rouleau, A., Yang, J., Bosse, M., Lajoie, G., and Bhatia, M. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature*.

Ben-Hur, T., Idelson, M., Khaner, H., Pera, M., Reinhartz, E., Itzik, A., and Reubinoff, B. E. (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. *Stem Cells* **22,** 1246-55.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409,** 363-6.

Bhattacharya, B., Cai, J., Luo, Y., Miura, T., Mejido, J., Brimble, S. N., Zeng, X., Schulz, T. C., Rao, M. S., and Puri, R. K. (2005). Comparison of the gene expression profile of undifferentiated human embryonic stem cell lines and differentiating embryoid bodies. *BMC Dev Biol* **5,** 22.

Bhattacharya, B., Miura, T., Brandenberger, R., Mejido, J., Luo, Y., Yang, A. X., Joshi, B. H., Ginis, I., Thies, R. S., Amit, M., Lyons, I., Condie, B. G., Itskovitz-Eldor, J., Rao, M. S., and Puri, R. K. (2004). Gene expression in human embryonic stem cell lines: unique molecular signature. *Blood* **103,** 2956-64.

Bieberich, E., Silva, J., Wang, G., Krishnamurthy, K., and Condie, B. G. (2004). Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. *J Cell Biol* **167,** 723-34.

Bikfalvi, A. (1995). Significance of angiogenesis in tumour progression and metastasis. *Eur J Cancer* **31A,** 1101-4.

Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci U S A* **98,** 14428-33.

Bode, A. M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* **4,** 793-805.

Boeuf, H., Hauss, C., Graeve, F. D., Baran, N., and Kedinger, C. (1997). Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. *J Cell Biol* **138,** 1207-17.

Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19,** 185-93.

Bourdon, J. C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D. P., Saville, M. K., and Lane, D. P. (2005). p53 isoforms can regulate p53 transcriptional activity. *Genes Dev* **19,** 2122-37.

Bourdon, J. C., Laurenzi, V. D., Melino, G., and Lane, D. (2003). p53: 25 years of research and more questions to answer. *Cell Death Differ* **10,** 397-9.

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., and Young, R. A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122,** 947-56.

Boyer, L. A., Mathur, D., and Jaenisch, R. (2006). Molecular control of pluripotency. *Curr Opin Genet Dev* **16,** 455–462.

Brenner, L., Munoz-Antonia, T., Vellucci, V. F., Zhou, Z. L., and Reiss, M. (1993). Wild-type p53 tumor suppressor gene restores differentiation of human squamous carcinoma cells but not the response to transforming growth factor beta. *Cell Growth Differ* **4,** 993-1004.

Brimble, S. N., Sherrer, E. S., Uhl, E. W., Wang, E., Kelly, S., Merrill, A. H., Jr., Robins, A. J., and Schulz, T. C. (2007). The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. *Stem Cells* **25,** 54-62.

Brimble, S. N., Zeng, X., Weiler, D. A., Luo, Y., Liu, Y., Lyons, I. G., Freed, W. J., Robins, A. J., Rao, M. S., and Schulz, T. C. (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. *Stem Cells Dev* **13,** 585-97.

Brubaker, K. D., Vessella, R. L., True, L. D., Thomas, R., and Corey, E. (2003). Cathepsin K mRNA and protein expression in prostate cancer progression. *J Bone Miner Res* **18,** 222-30.

Brynczka, C., Labhart, P., and Merrick, B. A. (2007). NGF-mediated transcriptional targets of p53 in PC12 neuronal differentiation. *BMC Genomics* **8,** 139.

Burden, D. A., and Osheroff, N. (1998). Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim Biophys Acta* **1400,** 139-54.

Burdon, T., Smith, A., and Savatier, P. (2002). Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol* **12,** 432-8.

Buzzard, J. J., Gough, N. M., Crook, J. M., and Colman, A. (2004). Karyotype of human ES cells during extended culture. *Nat Biotechnol* **22,** 381-2; author reply 382.

Carpenter, M. K., Inokuma, M. S., Denham, J., Mujtaba, T., Chiu, C. P., and Rao, M. S. (2001). Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* **172,** 383-97.

Castiglioni, T., Merino, M. J., Elsner, B., Lah, T. T., Sloane, B. F., and Emmert-Buck, M. R. (1994). Immunohistochemical analysis of cathepsins D, B, and L in human breast cancer. *Hum Pathol* **25,** 857-62.

Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., and et al. (1992). Molecular cloning of the interleukin-1 beta converting enzyme. *Science* **256,** 97-100.

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., and Bhatia, M. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* **102,** 906-15.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113,** 643-55.

Chao, C., Saito, S., Kang, J., Anderson, C. W., Appella, E., and Xu, Y. (2000). p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. *EMBO Journal* **19,** 4967-75.

Chen, G., and Goeddel, D. V. (2002). TNF-R1 signaling: a beautiful pathway. *Science* **296,** 1634-5.

Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D., and Green, D. R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science* **309,** 1732-5.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303,** 1010-4.

Chouinard, N., Valerie, K., Rouabhia, M., and Huot, J. (2002). UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochem J* **365,** 133-45.

Clark, A. T., Bodnar, M. S., Fox, M., Rodriquez, R. T., Abeyta, M. J., Firpo, M. T., and Pera, R. A. (2004). Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet* **13,** 727-39.

Cleary, M. L., and Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci U S A* **82,** 7439-43.

Cory, S., and Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2,** 647-56.

Couzin, J. (2002). Breakthrough of the year. Small RNAs make big splash. *Science* **298,** 2296-7.

Csipo, I., Montel, A. H., Hobbs, J. A., Morse, P. A., and Brahmi, Z. (1998). Effect of Fas+ and Fas- target cells on the ability of NK cells to repeatedly fragment DNA and trigger lysis via the Fas lytic pathway. *Apoptosis* **3,** 105-14.

Daikoku, T., Tranguch, S., Trofimova, I. N., Dinulescu, D. M., Jacks, T., Nikitin, A. Y., Connolly, D. C., and Dey, S. K. (2006). Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. *Cancer Res* **66,** 2527-31.

Daheron, L., Opitz, S. L., Zaehres, H., Lensch, W. M., Andrews, P. W., Itskovitz-Eldor, J., and Daley, G. Q. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22,** 770-8.

D'Assoro, A. B., Busby, R., Suino, K., Delva, E., Almodovar-Mercado, G. J., Johnson, H., Folk, C., Farrugia, D. J., Vasile, V., Stivala, F., and Salisbury, J. L. (2004). Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint. *Oncogene* **23,** 4068-75.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1993). LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* **260,** 1805-8.

Dazard, J. E., Piette, J., Basset-Seguin, N., Blanchard, J. M., and Gandarillas, A. (2000). Switch from p53 to MDM2 as differentiating human keratinocytes lose their proliferative potential and increase in cellular size. *Oncogene* **19,** 3693-705.

Devireddy, L. R., and Green, M. R. (2003). Transcriptional program of apoptosis induction following interleukin 2 deprivation: identification of RC3, a calcium/calmodulin binding protein, as a novel proapoptotic factor. *Mol Cell Biol* **23,** 4532-41.

Dohn, M., Zhang, S., and Chen, X. (2001). p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene* **20,** 3193-205.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356,** 215-21.

Draper, J. S., Smith, K., Gokhale, P., Moore, H. D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T. P., Thomson, J. A., and Andrews, P. W. (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* **22,** 53-4.

Dravid, G., Ye, Z., Hammond, H., Chen, G., Pyle, A., Donovan, P., Yu, X., and Cheng, L. (2005). Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. *Stem Cells* **23,** 1489-501.

Driouch, K., Briffod, M., Bieche, I., Champeme, M. H., and Lidereau, R. (1998). Location of several putative genes possibly involved in human breast cancer progression. *Cancer Res* **58,** 2081-6.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102,** 33-42.

Duensing, A., and Duensing, S. (2005). Guilt by association? p53 and the development of aneuploidy in cancer. *Biochem Biophys Res Commun* **331,** 694-700.

Dumont, P., Leu, J. I., Della Pietra, A. C., 3rd, George, D. L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat Genet* **33,** 357-65.

Dvash, T., Mayshar, Y., Darr, H., McElhaney, M., Barker, D., Yanuka, O., Kotkow, K. J., Rubin, L. L., Benvenisty, N., and Eiges, R. (2004). Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. *Hum Reprod* **19,** 2875-83.

Ehinger, M., Nilsson, E., Persson, A. M., Olsson, I., and Gullberg, U. (1995). Involvement of the tumor suppressor gene p53 in tumor necrosis factor-induced differentiation of the leukemic cell line K562. *Cell Growth Differ* **6,** 9-17.

Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15,** 188-200.

Elliott, K., Ge, K., Du, W., and Prendergast, G. C. (2000). The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program. *Oncogene* **19,** 4669-84.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Staller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G. C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms. *Oncogene* **18,** 3564-73.

Enver, T., Soneji, S., Joshi, C., Brown, J., Iborra, F., Orntoft, T., Thykjaer, T., Maltby, E., Smith, K., Dawud, R. A., Jones, M., Matin, M., Gokhale, P., Draper, J., and Andrews, P. W. (2005). Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Hum Mol Genet* **14,** 3129-40.

Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol* **24,** 6728-41.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154-6.

Evsikov, S., and Verlinsky, Y. (1998). Mosaicism in the inner cell mass of human blastocysts. *Hum Reprod* **13,** 3151-5.

Exley, G. E., Tang, C., McElhinny, A. S., and Warner, C. M. (1999). Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. *Biology of Reproduction* **61,** 231-9.

Fabian, D., Koppel, J., and Maddox-Hyttel, P. (2005). Apoptotic processes during mammalian preimplantation development. *Theriogenology* **64,** 221-31.

Feinstein, E., Gale, R. P., Reed, J., and Canaani, E. (1992). Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene* **7,** 1853-7.

Filipczyk, A., Laslett, A., Mummery, C., and Pera, M. F. (2007). Differentiation is Coupled to Changes in the Cell Cycle Regulatory Apparatus of Human Embryonic Stem Cells. *Stem Cell Research. In press.*

Fink, S. L., and Cookson, B. T. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun* **73,** 1907-16.

Finucane, D. M., Waterhouse, N. J., Amarante-Mendes, G. P., Cotter, T. G., and Green, D. R. (1999). Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. *Exp Cell Res* **251,** 166-74.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391,** 806-11.

Fisher, L. J. (1997). Neural precursor cells: applications for the study and repair of the central nervous system. *Neurobiol Dis* **4,** 1-22.

Funicello, M., Novelli, M., Ragni, M., Vottari, T., Cocuzza, C., Soriano-Lopez, J., Chiellini, C., Boschi, F., Marzola, P., Masiello, P., Saftig, P., Santini, F., St-Jacques, R., Desmarais, S., Morin, N., Mancini, J., Percival, M. D., Pinchera, A., and Maffei, M. (2007). Cathepsin K null mice show reduced adiposity during the rapid accumulation of fat stores. *PLoS ONE* **2,** e683.

Gao, G., and Dou, Q. (2000). N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem* **80,** 53-72.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., and et al. (1992). The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* **255,** 1434-7.

Gerami-Naini, B., Dovzhenko, O. V., Durning, M., Wegner, F. H., Thomson, J. A., and Golos, T. G. (2004). Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. *Endocrinology* **145,** 1517-24.

Gerecht-Nir, S., Cohen, S., Ziskind, A., and Itskovitz-Eldor, J. (2004a). Three-dimensional porous alginate scaffolds provide a conducive environment for generation of well-vascularized embryoid bodies from human embryonic stem cells. *Biotechnol Bioeng* **88,** 313-20.

Gerecht-Nir, S., Dazard, J. E., Golan-Mashiach, M., Osenberg, S., Botvinnik, A., Amariglio, N., Domany, E., Rechavi, G., Givol, D., and Itskovitz-Eldor, J. (2005). Vascular gene expression and phenotypic correlation during differentiation of human embryonic stem cells. *Dev Dyn* **232,** 487-97.

Gerecht-Nir, S., Osenberg, S., Nevo, O., Ziskind, A., Coleman, R., and Itskovitz-Eldor, J. (2004b). Vascular development in early human embryos and in teratomas derived from human embryonic stem cells. *Biol Reprod* **71,** 2029-36.

Ghosh, A. K., Majumder, M., Steele, R., Meyer, K., Ray, R., and Ray, R. B. (2000). Hepatitis C virus NS5A protein protects against TNF-alpha mediated apoptotic cell death. *Virus Res* **67,** 173-8.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Carpenter, M. K., Itskovitz-Eldor, J., and Rao, M. S. (2004). Differences between human and mouse embryonic stem cells. *Dev Biol* **269,** 360-80.

Gober, M. D., Smith, C. C., Ueda, K., Toretsky, J. A., and Aurelian, L. (2003). Forced expression of the H11 heat shock protein can be regulated by DNA methylation and trigger apoptosis in human cells. *J Biol Chem* **278,** 37600-9.

Gober, M. D., Wales, S. Q., and Aurelian, L. (2005). Herpes simplex virus type 2 encodes a heat shock protein homologue with apoptosis regulatory functions. *Front Biosci* **10,** 2788-803.

Goldfarb, M. (2001). Signaling by fibroblast growth factors: the inside story. *Sci STKE* **2001,** PE37.

Grabarek, J., Ardelt, B., Kunicki, J., and Darzynkiewicz, Z. (2002). Detection of in situ activation of transglutaminase during apoptosis: correlation with the cell cycle phase by multiparameter flow and laser scanning cytometry. *Cytometry* **49,** 83-9.

Grandela, C., and Wolvetang, E. (2007). hESC Adaptation, Selection and Stability. *Stem Cell Rev* **3,** 183-91.

Greber, B., Lehrach, H., and Adjaye, J. (2007). Fibroblast growth factor 2 modulates transforming growth factor beta signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. *Stem Cells* **25,** 455-64.

Grubb, D. R., Ly, J. D., Vaillant, F., Johnson, K. L., and Lawen, A. (2001). Mitochondrial cytochrome c release is caspase-dependent and does not involve mitochondrial permeability transition in didemnin B-induced apoptosis. *Oncogene* **20,** 4085-94.

Hammond, E. M., Mandell, D. J., Salim, A., Krieg, A. J., Johnson, T. M., Shirazi, H. A., Attardi, L. D., and Giaccia, A. J. (2006). Genome-wide analysis of p53 under hypoxic conditions. *Mol Cell Biol* **26,** 3492-504.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404,** 293-6.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293,** 1146-50.

Hande, K. R. (1998). Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur J Cancer* **34,** 1514-21.

Hannon, G. J. (2002). RNA interference. *Nature* **418,** 244-51.

Hardy, K., Handyside, A. H., and Winston, R. M. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. *Development* **107,** 597-604.

Hart, A. H., Hartley, L., Ibrahim, M., and Robb, L. (2004). Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev Dyn* **230,** 187-98.

Hayflick, L., and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res* **25,** 585-621.

He, J. Q., Ma, Y., Lee, Y., Thomson, J. A., and Kamp, T. J. (2003). Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res* **93,** 32-9.

Heidersbach, A., Gaspar-Maia, A., McManus, M. T., and Ramalho-Santos, M. (2006). RNA interference in embryonic stem cells and the prospects for future therapies. *Gene Ther* **13,** 478-86.

Heng, B. C., Clement, M. V., and Cao, T. (2007). Caspase Inhibitor Z-VAD-FMK Enhances the Freeze-Thaw Survival Rate of Human Embryonic Stem Cells. *Biosci Rep* **27,** 257-64.

Heng, B. C., Ye, C. P., Liu, H., Toh, W. S., Rufaihah, A. J., Yang, Z., Bay, B. H., Ge, Z., Ouyang, H. W., Lee, E. H., and Cao, T. (2006). Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. *J Biomed Sci* **13,** 433-45.

Herszfeld, D., Wolvetang, E., Langton-Bunker, E., Chung, T. L., Filipczyk, A. A., Houssami, S., Jamshidi, P., Koh, K., Laslett, A. L., Michalska, A., Nguyen, L., Reubinoff, B. E., Tellis, I., Auerbach, J. M., Ording, C. J., Looijenga, L. H., and Pera, M. F. (2006). CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. *Nat Biotechnol* **24,** 351-7.

Hill, M. M., Adrain, C., and Martin, S. J. (2003). Portrait of a killer: the mitochondrial apoptosome emerges from the shadows. *Mol Interv* **3,** 19-26.

Hoffman, L. M., and Carpenter, M. K. (2005). Characterization and culture of human embryonic stem cells. *Nat Biotechnol* **23,** 699-708.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers. *Science* **253,** 49-53.

Hong, Y., and Stambrook, P. J. (2004). Restoration of an absent G1 arrest and protection from apoptosis in embryonic stem cells after ionizing radiation. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 14443-8.

Humphrey, R. K., Beattie, G. M., Lopez, A. D., Bucay, N., King, C. C., Firpo, M. T., Rose-John, S., and Hayek, A. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* **22,** 522-30.

Hussain, S. P., and Harris, C. C. (1998). Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res* **58,** 4023-37.

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003). Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* **31,** e15.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* **6,** 88-95.

Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., Ihle, J. N., McKinnon, P. J., Cleveland, J. L., and Zambetti, G. P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4,** 321-8.

Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378,** 206-8.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410,** 549-54.

Jurisicova, A., and Acton, B. M. (2004). Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. *Reproduction* **128,** 281-91.

Karpinich, N. O., Tafani, M., Rothman, R. J., Russo, M. A., and Farber, J. L. (2002a). The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. *J Biol Chem* **277,** 16547-52.

Karpinich, N. O., Tafani, M., Rothman, R. J., Russo, M. A., and J.L., F. (2002b). The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. *J Biol Chem* **277,** 16547-52.

Karpinich, N. O., Tafani, M., Schneider, T., Russo, M. A., and Farber, J. L. (2006). The course of etoposide-induced apoptosis in Jurkat cells lacking p53 and Bax. *J Cell Physiol* **208,** 55-63.

Kastan, M. B., Radin, A. I., Kuerbitz, S. J., Onyekwere, O., Wolkow, C. A., Civin, C. I., Stone, K. D., Woo, T., Ravindranath, Y., and Craig, R. W. (1991). Levels of p53 protein increase with maturation in human hematopoietic cells. *Cancer Res* **51,** 4279-86.

Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R., and Thomson, J. A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **98,** 10716-21.

Kehat, I., Amit, M., Gepstein, A., Huber, I., Itskovitz-Eldor, J., and Gepstein, L. (2003). Development of cardiomyocytes from human ES cells. *Methods Enzymol* **365,** 461-73.

Kehat, I., Gepstein, A., Spira, A., Itskovitz-Eldor, J., and Gepstein, L. (2002). High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel in vitro model for the study of conduction. *Circ Res* **91,** 659-61.

Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* **108,** 407-14.

Keim, A. L., Chi, M. M., and Moley, K. H. (2001). Hyperglycemia-induced apoptotic cell death in the mouse blastocyst is dependent on expression of p53. *Mol Reprod Dev* **60,** 214-24.

Kerley-Hamilton, J. S., Pike, A. M., Li, N., DiRenzo, J., and Spinella, M. J. (2005). A p53-dominant transcriptional response to cisplatin in testicular germ cell tumor-derived human embryonal carcinoma. *Oncogene* **24,** 6090-100.

Khurana, R., and Simons, M. (2003). Insights from angiogenesis trials using fibroblast growth factor for advanced arteriosclerotic disease. *Trends Cardiovasc Med* **13,** 116-22.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14,** 5579-88.

Klein, N. P., and Schneider, R. J. (1997). Activation of Src family kinases by hepatitis B virus HBx protein and coupled signaling to Ras. *Mol Cell Biol* **17,** 6427-36.

Klotzsche, O., Etzrodt, D., Hohenberg, H., Bohn, W., and Deppert, W. (1998). Cytoplasmic retention of mutant tsp53 is dependent on an intermediate filament protein (vimentin) scaffold. *Oncogene* **16,** 3423-34.

Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285,** 1733-7.

Krohn, A. J., Wahlbrink, T., and Prehn, J. H. (1999). Mitochondrial depolarization is not required for neuronal apoptosis. *J Neurosci* **1,** 7394-404.

Kumar, M., and Carmichael, G. G. (1998). Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol Mol Biol Rev* **62,** 1415-34.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. (2005a). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Molecular Cell* **17,** 525-35.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. (2005b). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* **17,** 525-35.

Lamkanfi, M., Festjens, N., Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2007). Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* **14,** 44-55.

Laslett, A. L., Filipczyk, A. A., and Pera, M. F. (2003). Characterization and culture of human embryonic stem cells. *Trends in Cardiovascular Medicine* **13,** 295-301.

Laslett, A. L., Grimmond, S., Gardiner, B., Stamp, L., Lin, A., Hawes, S. M., Wormald, S., Nikolic-Paterson, D., Haylock, D., and Pera, M. F. (2007). Transcriptional analysis of early lineage commitment in human embryonic stem cells. *BMC Dev Biol* **7,** 12.

Lavon, N., Yanuka, O., and Benvenisty, N. (2004). Differentiation and isolation of hepatic-like cells from human embryonic stem cells. *Differentiation* **72,** 230-8.

Lawen, A. (2003). Apoptosis-an introduction. *Bioessays* **25,** 888-96.

Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature* **396,** 643-9.

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol* **6,** 443-50.

Levenberg, S., Golub, J. S., Amit, M., Itskovitz-Eldor, J., and Langer, R. (2002). Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **99,** 4391-6.

Levenstein, M. E., Ludwig, T. E., Xu, R. H., Llanas, R. A., VanDenHeuvel-Kramer, K., Manning, D., and Thomson, J. A. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* **24,** 568-74.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323-31.

Li, X. J., Du, Z. W., Zarnowska, E. D., Pankratz, M., Hansen, L. O., Pearce, R. A., and Zhang, S. C. (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* **23,** 215-21.

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., and Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nature Cell Biology* **7,** 165-71.

Liu, G., Nozell, S., Xiao, H., and Chen, X. (2004). DeltaNp73beta is active in transactivation and growth suppression. *Mol Cell Biol* **24,** 487-501.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66,** 807-15.

Liu, W. M., and Joel, S. P. (2003). The schedule-dependent effects of etoposide in leukaemic cell lines: a function of concentration and duration. *Cancer Chemother Pharmacol* **51,** 291-6.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86,** 147-57.

Lu, S. J., Feng, Q., Caballero, S., Chen, Y., Moore, M. A., Grant, M. B., and Lanza, R. (2007). Generation of functional hemangioblasts from human embryonic stem cells. *Nat Methods* **4,** 501-9.

Lu, X. (2005). p53: a heavily dictated dictator of life and death. *Curr Opin Genet Dev* **15,** 27-33.

Ludwig, T. E., Bergendahl, V., Levenstein, M. E., Yu, J., Probasco, M. D., and Thomson, J. A. (2006a). Feeder-independent culture of human embryonic stem cells. *Nat Methods* **3,** 637-46.

Ludwig, T. E., Levenstein, M. E., Jones, J. M., Berggren, W. T., Mitchen, E. R., Frane, J. L., Crandall, L. J., Daigh, C. A., Conard, K. R., Piekarczyk, M. S., Llanas, R. A., and Thomson, J. A. (2006b). Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* **24,** 185-7.

Maherali, M., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., and Hochedlinger, K. (2007). Directly Reprogrammed Fibroblasts Show Global Epigenetic Remodeling and Widespread Tissue Contribution. *Cell Stem Cell* **1**.

Maitra, A., Arking, D. E., Shivapurkar, N., Ikeda, M., Stastny, V., Kassauei, K., Sui, G., Cutler, D. J., Liu, Y., Brimble, S. N., Noaksson, K., Hyllner, J., Schulz, T. C., Zeng, X., Freed, W. J., Crook, J., Abraham, S., Colman, A., Sartipy, P., Matsui, S., Carpenter, M., Gazdar, A. F., Rao, M., and Chakravarti, A. (2005). Genomic alterations in cultured human embryonic stem cells. *Nat Genet* **37,** 1099-103.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78,** 7634-8.

Martin, G. R., and Evans, M. J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc Natl Acad Sci U S A* **72,** 1441-5.

Martinon, F., and Tschopp, J. (2004). Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117,** 561-74.

Massague, J. (1990). Transforming growth factor-alpha. A model for membrane-anchored growth factors. *J Biol Chem* **265,** 21393-6.

Massague, J. (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* **1,** 169-78.

Matas, D., Milyavsky, M., Shats, I., Nissim, L., Goldfinger, N., and Rotter, V. (2004). p53 is a regulator of macrophage differentiation. *Cell Death Differ* **11,** 458-67.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *Embo J* **18,** 4261-9.

McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002). RNA interference in adult mice. *Nature* **418,** 38-9.

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., and Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57,** 79-88.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. *Nature* **407,** 796-801.

Melino, G., and Piacentini, M. (1998). 'Tissue' transglutaminase in cell death: a downstream or a multifunctional upstream effector? *FEBS Lett* **430,** 59-63.

Metcalfe, A. D., Hunter, H. R., Bloor, D. J., Lieberman, B. A., Picton, H. M., Leese, H. J., Kimber, S. J., and Brison, D. R. (2004). Expression of 11 members of the BCL-2 family of apoptosis regulatory molecules during human preimplantation embryo development and fragmentation. *Molecular Reproduction & Development* **68,** 35-50.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria. *Molecular Cell* **11,** 577-90.

Mills, A. A. (2005). p53: link to the past, bridge to the future. *Genes Dev* **19,** 2091-9.

Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398,** 708-13.

Mitalipova, M. M., Rao, R. R., Hoyer, D. M., Johnson, J. A., Meisner, L. F., Jones, K. L., Dalton, S., and Stice, S. L. (2005). Preserving the genetic integrity of human embryonic stem cells. *Nat Biotechnol* **23,** 19-20.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113,** 631-42.

Moley, K. H. (2001). Hyperglycemia and apoptosis: mechanisms for congenital malformations and pregnancy loss in diabetic women. *Trends Endocrinol Metab* **12,** 78-82.

Moley, K. H., Chi, M. M., Knudson, C. M., Korsmeyer, S. J., and Mueckler, M. M. (1998). Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat Med* **4,** 1421-4.

Moll, U. M., and Slade, N. (2004). p63 and p73: roles in development and tumor formation. *Mol Cancer Res* **2,** 371-86.

Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378,** 203-6.

Mossink, M. H., van Zon, A., Scheper, R. J., Sonneveld, P., and Wiemer, E. A. (2003). Vaults: a ribonucleoprotein particle involved in drug resistance? *Oncogene* **22,** 7458-67.

Mummery, C., Ward, D., van den Brink, C. E., Bird, S. D., Doevendans, P. A., Opthof, T., Brutel de la Riviere, A., Tertoolen, L., van der Heyden, M., and Pera, M. (2002). Cardiomyocyte differentiation of mouse and human embryonic stem cells. *J Anat* **200,** 233-42.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A. B., Passier, R., and Tertoolen, L. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* **107,** 2733-40.

Nakamura, T., Arai, T., Takagi, M., Sawada, T., Matsuda, T., Yokota, T., and Heike, T. (1998). A selective switch-on system for self-renewal of embryonic stem cells using chimeric cytokine receptors. *Biochem Biophys Res Commun* **248,** 22-7.

Ng, E. S., Davis, R. P., Azzola, L., Stanley, E. G., and Elefanty, A. G. (2005). Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood* **106,** 1601-3.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95,** 379-91.

Nikolaev, A. Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003). Parc: a cytoplasmic anchor for p53. *Cell* **112,** 29-40.

Nistor, G. I., Totoiu, M. O., Haque, N., Carpenter, M. K., and Keirstead, H. S. (2005). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia* **49,** 385-96.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev* **12,** 2048-60.

Niwa, H., Miyazaki, J., and Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24,** 372-6.

Niwa, H. (2007). How is pluripotency determined and maintained? *Development* **134,** 635-646.

Nykanen, A., Haley, B., and Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107,** 309-21.

Obad, S., Brunnstrom, H., Vallon-Christersson, J., Borg, A., Drott, K., and Gullberg, U. (2004). Staf50 is a novel p53 target gene conferring reduced clonogenic growth of leukemic U-937 cells. *Oncogene* **23,** 4050-9.

Oka, M., Tagoku, K., Russell, T. L., Nakano, Y., Hamazaki, T., Meyer, E. M., Yokota, T., and Terada, N. (2002). CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells. *Mol Biol Cell* **13,** 1274-81.

Okada-Ban, M., Thiery, J. P., and Jouanneau, J. (2000). Fibroblast growth factor-2. *Int J Biochem Cell Biol* **32,** 263-7.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature*.

Opferman, J. T., Iwasaki, H., Ong, C. C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S. J. (2005). Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* **307,** 1101-4.

Ornitz, D. M., and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol* **2,** REVIEWS3005.

Park, S., Lee, K. S., Lee, Y. J., Shin, H. A., Cho, H. Y., Wang, K. C., Kim, Y. S., Lee, H. T., Chung, K. S., Kim, E. Y., and Lim, J. (2004). Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors. *Neurosci Lett* **359,** 99-103.

Pastorino, J. G., and Hoek, J. B. (2000). Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. *Hepatology* **31,** 1141-52.

Pebay, A., Wong, R. C., Pitson, S. M., Wolvetang, E. J., Peh, G. S., Filipczyk, A., Koh, K. L., Tellis, I., Nguyen, L. T., and Pera, M. F. (2005). Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. *Stem Cells* **23,** 1541-8.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). p63 identifies keratinocyte stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 3156-61.

Pera, M. F., Andrade, J., Houssami, S., Reubinoff, B., Trounson, A., Stanley, E. G., Ward-van Oostwaard, D., and Mummery, C. (2004). Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *Journal of Cell Science* **117,** 1269-80.

Pera, M. F., Reubinoff, B., and Trounson, A. (2000). Human embryonic stem cells. *J Cell Sci* **113 (Pt 1),** 5-10.

Pera, M. F., and Trounson, A. O. (2004). Human embryonic stem cells: prospects for development. *Development* **131,** 5515-25.

Perrier, A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., Harrison, N. L., and Studer, L. (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* **101,** 12543-8.

Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W., and Sontheimer, E. J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell* **117,** 83-94.

Piek, E., Heldin, C. H., and Ten Dijke, P. (1999). Specificity, diversity, and regulation in TGF-beta superfamily signaling. *Faseb J* **13,** 2105-24.

Pietenpol, J. A., and Stewart, Z. A. (2002). Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. *Toxicology* **181-182,** 475-81.

Poon, E., Clermont, F., Firpo, M. T., and Akhurst, R. J. (2006). TGFbeta inhibition of yolk-sac-like differentiation of human embryonic stem-cell-derived embryoid bodies illustrates differences between early mouse and human development. *J Cell Sci* **119,** 759-68.

Powers, C. J., McLeskey, S. W., and Wellstein, A. (2000). Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* **7,** 165-97.

Pyle, A. D., Lock, L. F., and Donovan, P. J. (2006). Neurotrophins mediate human embryonic stem cell survival. *Nat Biotechnol* **24,** 344-50.

Qin, H., Yu, T., Qing, T., Liu, Y., Zhao, Y., Cai, J., Li, J., Song, Z., Qu, X., Zhou, P., Wu, J., Ding, M., and Deng, H. (2006). Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J Biol Chem*.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C., and Melton, D. A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298,** 597-600.

Rambhatla, L., Chiu, C. P., Kundu, P., Peng, Y., and Carpenter, M. K. (2003). Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant* **12,** 1-11.

Rao, R. R., Calhoun, J. D., Qin, X., Rekaya, R., Clark, J. K., and Stice, S. L. (2004). Comparative transcriptional profiling of two human embryonic stem cell lines. *Biotechnol Bioeng* **88,** 273-86.

Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T. (2001). Neural progenitors from human embryonic stem cells.[see comment]. *Nature Biotechnology* **19,** 1134-40.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nature Biotechnology* **18,** 399-404.

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414,** 105-11.

Rich, T., Allen, R. L., and Wyllie, A. H. (2000). Defying death after DNA damage. *Nature* **407,** 777-83.

Richards, M., Fong, C. Y., Chan, W. K., Wong, P. C., and Bongso, A. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* **20,** 933-6.

Richards, M., Tan, S. P., Tan, J. H., Chan, W. K., and Bongso, A. (2004). The transcriptome profile of human embryonic stem cells as defined by SAGE. *Stem Cells* **22,** 51-64.

Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S. J. (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev* **14,** 23-7.

Roelofs, H., Mostert, M. C., Pompe, K., Zafarana, G., van Oorschot, M., van Gurp, R. J., Gillis, A. J., Stoop, H., Beverloo, B., Oosterhuis, J. W., Bokemeyer, C., and Looijenga, L. H. (2000). Restricted 12p amplification and RAS mutation in human germ cell tumors of the adult testis. *Am J Pathol* **157,** 1155-66.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33,** 401-6.

Rugg-Gunn, P. J., Ferguson-Smith, A. C., and Pedersen, R. A. (2005). Epigenetic status of human embryonic stem cells. *Nat Genet* **37,** 585-7.

Sabapathy, K., Klemm, M., Jaenisch, R., and Wagner, E. F. (1997). Regulation of ES cell differentiation by functional and conformational modulation of p53. *Embo J* **16,** 6217-29.

Sah, N. K., Khan, Z., Khan, G. J., and Bisen, P. S. (2006). Structural, functional and therapeutic biology of survivin. *Cancer Lett* **244,** 164-71.

Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T., and Jacks, T. (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* **10,** 175-80.

Sakamuro, D., Elliott, K. J., Wechsler-Reya, R., and Prendergast, G. C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. *Nat Genet* **14,** 69-77.

Santiago-Lomeli, M., Gomez-Quiroz, L. E., Ortiz-Ortega, V. M., Kershenobich, D., and Gutierrez-Ruiz, M. C. (2005). Differential effect of interleukin-10 on hepatocyte apoptosis. *Life Sci* **76,** 2569-79.

Satin, J., Kehat, I., Caspi, O., Huber, I., Arbel, G., Itzhaki, I., Magyar, J., Schroder, E. A., Perlman, I., and Gepstein, L. (2004). Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. *J Physiol* **559,** 479-96.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* **10,** 55-63.

Savill, J. (1997). Apoptosis in resolution of inflammation. *J Leukoc Biol* **61,** 375-80.

Sbisa, E., Catalano, D., Grillo, G., Licciulli, F., Turi, A., Liuni, S., Pesole, G., De Grassi, A., Caratozzolo, M. F., D'Erchia, A. M., Navarro, B., Tullo, A., Saccone, C., and Gisel, A. (2007). p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data. *BMC Bioinformatics* **8 Suppl 1,** S20.

Scarlett, J. L., Sheard, P. W., Hughes, G., Ledgerwood, E. C., Ku, H. H., and Murphy, M. P. (2000). Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jurkat cells. *FEBS Lett* **475,** 267-72.

Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002). Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell* **1,** 289-98.

Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R. S., and Benvenisty, N. (2001). Induced neuronal differentiation of human embryonic stem cells. *Brain Res* **913,** 201-5.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A., and Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **97,** 11307-12.

Schulz, T. C., Noggle, S. A., Palmarini, G. M., Weiler, D. A., Lyons, I. G., Pensa, K. A., Meedeniya, A. C., Davidson, B. P., Lambert, N. A., and Condie, B. G. (2004). Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem Cells* **22,** 1218-38.

Schulz, T. C., Palmarini, G. M., Noggle, S. A., Weiler, D. A., Mitalipova, M. M., and Condie, B. G. (2003). Directed neuronal differentiation of human embryonic stem cells. *BMC Neurosci* **4,** 27.

Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1992). Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J Biol Chem* **267,** 5317-23.

Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G., and Fiers, W. (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *Embo J* **12,** 3095-104.

Segev, H., Fishman, B., Ziskind, A., Shulman, M., and Itskovitz-Eldor, J. (2004). Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* **22,** 265-74.

Segui, B., Cuvillier, O., Adam-Klages, S., Garcia, V., Malagarie-Cazenave, S., Leveque, S., Caspar-Bauguil, S., Coudert, J., Salvayre, R., Kronke, M., and Levade, T. (2001). Involvement of FAN in TNF-induced apoptosis. *J Clin Invest* **108,** 143-51.

Shemetov, A. A., Seit-Nebi, A. S., and Gusev, N. B. (2007). Structure, properties, and functions of the human small heat-shock protein HSP22 (HspB8, H11, E2IG1): A critical review. *J Neurosci Res*.

Shimizu, S., and Tsujimoto, Y. (2000). Proapoptotic BH3-only Bcl-2 family members induce cytochrome c release, but not mitochondrial membrane potential loss, and do not directly modulate voltage-dependent anion channel activity. *Proc Natl Acad Sci* **97,** 577-82.

Shinmura, K., Bennett, R. A., Tarapore, P., and Fukasawa, K. (2007). Direct evidence for the role of centrosomally localized p53 in the regulation of centrosome duplication. *Oncogene* **26,** 2939-44.

Skaar, J. R., Florens, L., Tsutsumi, T., Arai, T., Tron, A., Swanson, S. K., Washburn, M. P., and DeCaprio, J. A. (2007). PARC and CUL7 form atypical cullin RING ligase complexes. *Cancer Res* **67,** 2006-14.

Slavin, J. (1995). Fibroblast growth factors: at the heart of angiogenesis. *Cell Biol Int* **19,** 431-44.

Slee, E. A., Adrain, C., and Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* **276,** 7320-6.

Sleeper, E., Tamm, C., Frisen, J., Zhivotovsky, B., Orrenius, S., and Ceccatelli, S. (2002). Cell death in adult neural stem cells. *Cell Death Differ* **9,** 1377-8.

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3,** Article3.

Snir, M., Kehat, I., Gepstein, A., Coleman, R., Itskovitz-Eldor, J., Livne, E., and Gepstein, L. (2003). Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. *Am J Physiol Heart Circ Physiol* **285,** H2355-63.

Soddu, S., Blandino, G., Citro, G., Scardigli, R., Piaggio, G., Ferber, A., Calabretta, B., and Sacchi, A. (1994). Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood* **83,** 2230-7.

Soddu, S., Blandino, G., Scardigli, R., Coen, S., Marchetti, A., Rizzo, M. G., Bossi, G., Cimino, L., Crescenzi, M., and Sacchi, A. (1996). Interference with p53 protein inhibits hematopoietic and muscle differentiation. *J Cell Biol* **134,** 193-204.

Spivakov, M., and Fisher, A. G. (2007). Epigenetic signatures of stem-cell identity. *Nat Rev Genet* **8,** 263-71.

Stachowiak, M. K., Moffett, J., Maher, P., Tucholski, J., and Stachowiak, E. K. (1997). Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism. *Mol Neurobiol* **15,** 257-83.

Stewart, Z. A., and Pietenpol, J. A. (2001). p53 Signaling and cell cycle checkpoints. *Chem Res Toxicol* **14,** 243-63.

Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2007). Apoptosis and differentiation of human embryonic stem cells induced by sustained activation of c-Myc. *Oncogene*.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka , Tomoda K, Yamanaka S (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*. doi:10.1016/j.cell.2007.11.019

Takekawa, M., Adachi, M., Nakahata, A., Nakayama, I., Itoh, F., Tsukuda, H., Taya, Y., and Imai, K. (2000). p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. *Embo J* **19,** 6517-26.

Taupin, D. R., Kinoshita, K., and Podolsky, D. K. (2000). Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci U S A* **97,** 799-804.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145-7.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281,** 1312-6.

Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P. C., and Croce, C. M. (1985). Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. *Nature* **315,** 340-3.

Vacca, A., Ribatti, D., Roccaro, A. M., Frigeri, A., and Dammacco, F. (2001). Bone marrow angiogenesis in patients with active multiple myeloma. *Semin Oncol* **28,** 543-50.

Valdimarsdottir, G., and Mummery, C. (2005). Functions of the TGFbeta superfamily in human embryonic stem cells. *Apmis* **113,** 773-89.

Vallier, L., Alexander, M., and Pedersen, R. A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* **118,** 4495-509.

Vallier, L., Reynolds, D., and Pedersen, R. A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol* **275,** 403-21.

Vaux, D. L., Cory, S., and Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335,** 440-2.

Vaux, D. L., and Korsmeyer, S. J. (1999). Cell death in development. *Cell* **96,** 245-54.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102,** 43-53.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* **184,** 39-51.

Vodyanik, M. A., Bork, J. A., Thomson, J. A., and Slukvin, II. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* **105,** 617-26.

Wadhwa, R., Yaguchi, T., Hasan, M. K., Mitsui, Y., Reddel, R. R., and Kaul, S. C. (2002). Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. *Exp Cell Res* **274,** 246-53.

Wang, G., Zhang, H., Zhao, Y., Li, J., Cai, J., Wang, P., Meng, S., Feng, J., Miao, C., Ding, M., Li, D., and Deng, H. (2005). Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem Biophys Res Commun* **330,** 934-42.

Wang, L., Li, L., Shojaei, F., Levac, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A., and Bhatia, M. (2004). Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* **21,** 31-41.

Wang, Q., and Beck, W. T. (1998). Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53. *Cancer Res* **58,** 5762-9.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J. B., Nishikawa, S., Muguruma, K., and Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* **25,** 681-6.

Wei, C. L., Wu, Q., Vega, V. B., Chiu, K. P., Ng, P., Zhang, T., Shahab, A., Yong, H. C., Fu, Y., Weng, Z., Liu, J., Zhao, X. D., Chew, J. L., Lee, Y. L., Kuznetsov, V. A., Sung, W. K., Miller, L. D., Lim, B., Liu, E. T., Yu, Q., Ng, H. H., and Ruan, Y. (2006). A global map of p53 transcription-factor binding sites in the human genome. *Cell* **124,** 207-19.

Wei, L., Cui, L., Snider, B. J., Rivkin, M., Yu, S. S., Lee, C. S., Adams, L. D., Gottlieb, D. I., Johnson, E. M., Jr., Yu, S. P., and Choi, D. W. (2005). Transplantation of embryonic stem cells overexpressing Bcl-2 promotes functional recovery after transient cerebral ischemia. *Neurobiol Dis* **19,** 183-93.

Weinberg, W. C., Azzoli, C. G., Chapman, K., Levine, A. J., and Yuspa, S. H. (1995). p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein. *Oncogene* **10,** 2271-9.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature*.

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* **2,** 70-5.

Wolff, B., Sanglier, J. J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* **4,** 139-47.

Wong, G. H., Tartaglia, L. A., Lee, M. S., and Goeddel, D. V. (1992). Antiviral activity of tumor necrosis factor is signaled through the 55-kDa type I TNF receptor [corrected]. *J Immunol* **149,** 3350-3.

Wood, D. E., and Newcomb, E. W. (2000). Cleavage of Bax enhances its cell death function. *Exp Cell Res* **256,** 375-82.

Wu, G., Nomoto, S., Hoque, M. O., Dracheva, T., Osada, M., Lee, C. C., Dong, S. M., Guo, Z., Benoit, N., Cohen, Y., Rechthand, P., Califano, J., Moon, C. S., Ratovitski, E., Jen, J., Sidransky, D., and Trink, B. (2003). DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. *Cancer Res* **63,** 2351-7.

Xia, X., Zhang, Y., Zieth, C. R., and Zhang, S. C. (2007). Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner. *Stem Cells Dev* **16,** 167-76.

Xiao, L., Yuan, X., and Sharkis, S. J. (2006). Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells* **24,** 1476-86.

Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., and Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* **19,** 971-4.

Xu, C., Police, S., Rao, N., and Carpenter, M. K. (2002a). Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* **91,** 501-8.

Xu, C., Rosler, E., Jiang, J., Lebkowski, J. S., Gold, J. D., O'Sullivan, C., Delavan-Boorsma, K., Mok, M., Bronstein, A., and Carpenter, M. K. (2005a). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* **23,** 315-23.

Xu, D., Wilson, T. J., Chan, D., De Luca, E., Zhou, J., Hertzog, P. J., and Kola, I. (2002b). Ets1 is required for p53 transcriptional activity in UV-induced apoptosis in embryonic stem cells. *EMBO Journal* **21,** 4081-93.

Xu, R. H., Chen, X., Li, D. S., Li, R., Addicks, G. C., Glennon, C., Zwaka, T. P., and Thomson, J. A. (2002c). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* **20,** 1261-4.

Xu, R. H., Peck, R. M., Li, D. S., Feng, X., Ludwig, T., and Thomson, J. A. (2005b). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* **2,** 185-90.

Xue, T., Cho, H. C., Akar, F. G., Tsang, S. Y., Jones, S. P., Marban, E., Tomaselli, G. F., and Li, R. A. (2005). Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers. *Circulation* **111,** 11-20.

Yamane, T., Dylla, S. J., Muijtjens, M., and Weissman, I. L. (2005). Enforced Bcl-2 expression overrides serum and feeder cell requirements for mouse embryonic stem cell self-renewal. *Proc Natl Acad Sci U S A* **102,** 3312-7.

Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000). p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404,** 99-103.

Yang, S., Tutton, S., Pierce, E., and Yoon, K. (2001). Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol Cell Biol* **21,** 7807-16.

Yi, C., Li, S., Chen, X., Wiemer, E. A., Wang, J., Wei, N., and Deng, X. W. (2005). Major vault protein, in concert with constitutively photomorphogenic 1, negatively regulates c-Jun-mediated activator protein 1 transcription in mammalian cells. *Cancer Res* **65,** 5835-40.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II, and Thomson, J. A. (2007). Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science*.

Zaehres, H., and Daley, G. Q. (2006). Transgene expression and RNA interference in embryonic stem cells. *Methods Enzymol* **420,** 49-64.

Zeng, X., Cai, J., Chen, J., Luo, Y., You, Z. B., Fotter, E., Wang, Y., Harvey, B., Miura, T., Backman, C., Chen, G. J., Rao, M. S., and Freed, W. J. (2004). Dopaminergic differentiation of human embryonic stem cells. *Stem Cells* **22,** 925-40.

Zhan, X., Dravid, G., Ye, Z., Hammond, H., Shamblott, M., Gearhart, J., and Cheng, L. (2004). Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro. *Lancet* **364,** 163-71.

Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O., and Thomson, J. A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* **19,** 1129-33.

Zhang, X. N., Xiong, W., Wang, J. D., Hu, Y. W., Xiang, L., and Yuan, Z. H. (2004). siRNA-mediated inhibition of HBV replication and expression. *World J Gastroenterol* **10,** 2967-71.

Zheng, T. S., Hunot, S., Kuida, K., Momoi, T., Srinivasan, A., Nicholson, D. W., Lazebnik, Y., and Flavell, R. A. (2000). Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. *Nat Med* **6,** 1241-7.

APPENDIX I

Formulas for commonly used reagents

10% SDS
Dissolve 100 g of SDS (Sigma-Aldrich) [Cat No: L4390] in 900 ml ddH_2O, heating a 65 °C water bath to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl (Lab-Scan) [Cat No: A8601]. Make up to 1 liter with ddH_2O and store at RT.

10% Tween 20
Dissolve 10 g of Tween 20 (Sigma-Aldrich) [Cat No: 1379] in 90 ml ddH_2O. Store at RT.

5 M NaOH
Dissolve 200g of NaOH pellets (AnalaR) [Cat No: 10252.4x] in 800 ml of ddH_2O, taking care with generated heat. Make up to 1 liter in ddH_2O and store at RT.

1.5 M Tris-HCl pH 8.8
Dissolve 90.75 g of Tris base (Merk) [Cat No: 108382] in 350 ml of ddH_2O. Adjust to pH with HCl (Lab-Scan) [Cat No: A8601] and make up to 400 ml with ddH_2O.

0.5 M Tris-HCl pH 6.8
Dissolve 15 g of Tris base (Merk) [Cat No: 108382] in 350 ml of ddH_2O. Adjust to pH with HCl (Lab-Scan) [Cat No: A8601] and make up to 250 ml with ddH_2O.

0.5 M EDTA pH 8.0
Add 186.1 g of disodium ethylenediaminetetra-acetate.$2H_2O$ (EDTA salt) (AppliChem GmbH) [Cat No: A1104] to 800 ml of ddH_2O and stir vigorously on a magnetic stirrer. Adjust to pH 8.0 with NaOH (~20 g of NaOH pellets) (AnalaR) [Cat No: 10252.4x], bringing the EDTA salt into solution. Autoclave and store at RT.

50x TAE
484 g Tris-base (Merk) [Cat No: 108382]
114.2 g Glacial acetic acid (Lab-Scan) [Cat No: A8401]
200 ml 0.5 M EDTA (AppliChem GmbH) [Cat No: A1104]

Combine all reagents and bring to final volume of 2 liters with ddH_2O. For 1x TAE working solution, dilute 200 ml of 50x TAE stock to 10 liters of ddH_2O and store at RT.

DNA electrophoresis loading dye
For 10x DNA gel-loading dye, the following mix is made up in ddH_2O and stored at -20 °C:
0.25% (w/v) Orange G (Sigma-Aldrich) [Cat No: O7252]
15% (v/v) Ficoll ® 400 (Sigma-Aldrich) [Cat No: F4375]

5 M NaOH
Dissolve 200g of NaOH pellets (AnalaR) [Cat No: 10252.4x] in 800 ml of ddH_2O, taking care with generated heat. Make up to 1 liter in ddH_2O and store at RT.

1.5 M Tris-HCl pH 8.8
Dissolve 90.75 g of Tris base (Merk) [Cat No: 108382] in 350 ml of ddH_2O. Adjust to pH with HCl (Lab-Scan) [Cat No: A8601] and make up to 400 ml with ddH_2O.

0.5 M Tris-HCl pH 6.8
Dissolve 15 g of Tris base (Merk) [Cat No: 108382] in 350 ml of ddH_2O. Adjust to pH with HCl (Lab-Scan) [Cat No: A8601] and make up to 250 ml with ddH_2O.

0.5 M EDTA pH 8.0
Add 186.1 g of disodium ethylenediaminetetra-acetate.$2H_2O$ (EDTA salt) (AppliChem) [Cat No: A1104] to 800 ml of ddH_2O and stir vigorously on a magnetic stirrer. Adjust to pH 8.0 with NaOH (~20 g of NaOH pellets) (AnalaR) [Cat No: 10252.4x], bringing the EDTA salt into solution. Autoclave and store at RT.

4% Paraformaldehyde (PFA)
Add 2 g of PFA (Merk) [Cat No:294474L] to 45 ml of PBS and heat approximately at 50 °C. Add 20-40 μl of 5 M NaOH and stir to dissolve. Adjust to pH to 7.4 with HCl (Lab-Scan) [Cat No: A8601] and make up to 50 ml with PBS.

10% Ammonium persulfate (APS)
Make fresh just before use. Weigh 0.1g of APS (Sigma-Aldrich) [Cat No: A3678] into a polypropylene 1.7 ml microtubes (Axygen, Union City CA, USA) [Cat No: MCT175-C]. Add 1ml of ddH_2O and discard after use.

Running buffer (2x)
12.2 g of Tris base (Merk) [Cat No: 108382]
57.65g of Glycine (Merk) [Cat No: 104201]
20 ml of 10% SDS

Dissolve 12.2 g of Tris base and 57.65g of Glycine in 800 ml of ddH_2O. Add 20 ml of 10% SDS and make up to 2 liters with ddH_2O. Store at RT.

3x Laemmli Loading Buffer (Reducing) (50ml)
9.375 ml 1M Tris pH 6.8
3.0 g SDS (Sigma-Aldrich) [Cat No: L4390]
15 ml Glycerol (Sigma-Aldrich) [Cat No: G2025]
15 mg Bromophenol blue (Sigma-Aldrich) [Cat No: B6131] (add 1ml of a 15mg/ml solution of bromophenol blue in ddH_2O)

Dissolve 3g SDS in 10 ml ddH_2O. Add 9.375ml 1M Tris to 26.5ml with ddH_2O. Add 1ml of bromophenol blue solution. Combine with 15 ml glycerol and mix well. Total volume should be 42.5 ml. Prepare 850 μl aliquots. Before use, add 150 μl of β-Mercaptoethanol (Sigma-Aldrich) [Cat No: M7154] and dilute to 1x.

APPENDIX II

TABLE A 2.1- List of genes upregulated in p53 shRNA transduced hESC (Ingenuity Pathways Analysis)

Common name	Fold Change	B-stat	Genbank	Biological process/role	p53 RE
	30.40	9.68	AI810049		
HTR7	8.25	9.25	NM_000872	G-protein coupled receptor protein signaling pathway	
PLCD1	2.84	5.75	NM_006225	Lipid metabolic process, Intracellular signaling cascade	6
APOBEC3B	1.87	5.39	NM_004900	Growth or cell cycle control	2
ATP6V1B1	2.99	5.34	NM_001692	Ion transport, regulation of pH, ATP synthesis coupled proton transport	5
SLC7A14	3.82	4.49	NM_020949	Transport, amino acid transport	4
CXXC4	3.16	4.47	NM_025212	Negative regulation of Wnt receptor signaling pathway	3
VASH1	1.93	4.45	NM_014909	Cell cycle arrest, anti-angiogenesis	5
SDSL	2.25	4.33	NM_138432	Amino acid metabolic process	3
TFF3	1.99	4.15	NM_003226	Defense response, digestion, resistance to apoptosis	2
CAPN11	1.83	3.39	NM_007058	Proteolysis	5
PCDH11Y	2.48	3.05	NM_032971	Cell adhesion	
C1QL4	2.68	2.87	NM_001008223	Phosphate transport	
PCDH11X	3.38	2.72	NM_032967	Cell adhesion	
KIAA1509	1.72	2.58	BX640973		
C11orf11	2.27	2.50	NM_006133	Lipid catabolic process	3
NRGN	2.73	2.37	NM_006176	Signal transduction, nervous system development, apoptosis	
LHFP	1.76	2.24	NM_005780		3
VSIG2	2.95	2.10	NM_014312		
OLFML3	1.65	1.97	NM_020190		
TFPI	1.83	1.94	NM_006287	Blood coagulation	3
EDNRB	2.01	1.91	NM_003991	Migration; regulation of pH, G-protein signaling, differentiation	
ZNF467	1.97	1.84	NM_207336	Regulation of transcription, DNA-dependent	1
ASB9	1.74	1.83	NM_024087	Intracellular signaling cascade	10
FOLR1	2.20	1.81	NM_016724	Receptor-mediated endocytosis, folic acid metabolic process, proliferation, cell viability	5

LIX1L	1.80	1.68	NM_153713		2
BIN1	1.83	1.64	NM_139348	Negative regulation of progression through cell cycle, development, cell differentiation	
GLDN	1.65	1.57	NM_181789	Phosphate transport, nervous system development, cell differentiation	4
TFPI	2.02	1.56	NM_001032281	Blood coagulation	
DYRK3	1.55	1.48	NM_003582	Protein amino acid phosphorylation, erythrocyte differentiation	1
FBXL2	1.52	1.35	NM_012157	Protein modification process, proteolysis, ubiquitin cycle	3
MFNG	1.72	1.31	NM_002405	Pattern specification process	7
SLC25A34	2.20	1.30	NM_207348	Transport	5
LOC401074	1.78	1.28	XM_942831		
C2orf11	2.36	1.27	NM_144629		6
HGC6.3	2.03	1.25	AB016902		
CYTL1	1.80	1.25	NM_018659	Signal transduction	2
EPDR1	1.51	1.24	NM_017549	Cell-matrix adhesion	3
HLA-DPA1	1.91	1.23	NM_033554	Immune response, antigen processing and presentation	
DLGAP2	2.08	1.22	NM_004745	Cell-cell signaling, nerve-nerve synaptic transmission	
PTGS1	1.92	1.21	NM_080591	Lipid metabolic process, blood pressure regulation, proliferation, apoptosis	7
VIM	1.62	1.17	NM_003380	Cell motility	2
PRICKLE1	1.90	1.11	NM_153026		5
KCNK12	1.80	1.07	NM_022055	Ion transport, potassium ion transport	
OSAP	1.51	1.04	NM_032623		
PHF7	1.51	1.00	NM_016483		3
SYNE2	1.71	0.99	NM_015180		4
CAMP	2.90	0.90	NM_004345	Defense response to bacterium	
HTR7	2.28	0.89	NM_019859	G-protein coupled receptor protein signaling pathway, circadian rhythm, circulation	2
FAM46C	2.18	0.89	NM_017709		1
EDNRB	2.11	0.86	NM_003991		
UTS2	1.77	0.84	NM_021995	Muscle contraction, synaptic transmission, blood pressure regulation, proliferation	1
RAB3GAP1	1.54	0.78	AI872635		
KEL	1.91	0.77	NM_000420	Protein amino acid N-linked glycosylation, proteolysis, vasoconstriction	7

PMP22	1.76	0.70	NM_153322	Synaptic transmission, peripheral nervous system development, mechanosensory behavior, negative regulation of cell proliferation	7
PDE6G	1.83	0.69	NM_002602	Activation of MAPK, visual perception, EGF receptor signaling pathway and G-protein coupled receptor protein signaling pathway	2
LGALS3BP	1.53	0.68	NM_005567	Cellular defense response; cell adhesion; signal transduction	3
SELENBP1	1.47	0.68	NM_003944	Protein transport	2
IL11RA	1.48	0.63	NM_004512		4
MLLT4	1.74	0.62	NM_005936	Cell adhesion, signal transduction, cell-cell signaling	
SERPINA5	1.78	0.56	NM_000624	Spermatogenesis, fusion of sperm to egg plasma membrane	2
SHMT1	1.69	0.56	NM_004169	Glycine and L-serine metabolic processes, one-carbon compound metabolic process	8
LOC647534	1.49	0.51	XM_942883		
LOC143381	2.56	0.50	BX648964		
CLDN19	1.83	0.48	NM_148960	Visual perception, calcium-independent cell-cell adhesion, response to stimulus	4
WFIKKN1	1.86	0.46	NM_053284		2
FAM43B	1.68	0.45	NM_207334		
STARD8	1.45	0.44	NM_014725	Signal transduction	3
RAB36	2.17	0.44	NM_004914	Small GTPase mediated signal transduction, protein transport	5
ACTN3	1.74	0.41	NM_001104	Muscle contraction, regulation of apoptosis, focal adhesion formation	
GEFT	1.66	0.41	NM_182947	Regulation of Rho protein signal transduction	1
MVP	1.52	0.41	NM_005115	Response to drug (overexpressed in multidrug-resistant cancer cells)	5
KIAA1086	1.67	0.41	XM_940014		
ANKRD19	1.91	0.40	NM_001010925		5
MB	1.65	0.39	NM_005368	Response to hypoxia, oxygen transport, enucleate erythrocyte differentiation	5
HSPB8	1.95	0.37	NM_014365	Protein folding, apoptosis and growth	9
	1.51	0.36	CA407939		
TXNDC13	1.77	0.36	NM_021156	Electron transport, cell redox homeostasis	2
LOC645550	2.06	0.34	XM_928570		
ATP1A2	1.62	0.34	NM_000702	Potassium and sodium ion transport; regulation of striated muscle contraction, sperm motility, cellular hydrogen ion homeostasis	7
LOC641860	2.09	0.34	XM_935765		
TUBAL3	1.61	0.23	NM_024803	Microtubule-based movement, protein polymerization	
PCBD1	1.59	0.21	NM_001001939	Tetrahydrobiopterin biosynthetic process; protein homotetramerization and hetero-oligomerization	

IL12RB2	2.02	0.18	NM_001559	Cell surface receptor linked signal transduction, positive regulation of cell proliferation	
CD79B	1.96	0.18	NM_000626	Immune response, cell surface receptor linked signal transduction, proliferation, apoptosis, survival, adhesion, development, cell cycle progression	
CBX4	1.45	0.15	NM_003655	Chromatin modification, assembly or disassembly; regulation of transcription, DNA-dependent, ubiquitin cycle; anti-apoptosis	3
ARHGEF6	1.50	0.13	NM_004840	Apoptosis, JNK cascade, regulation of Rho protein signal transduction	5
TMEM88	1.90	0.11	NM_203411		5
CPXM	1.45	0.11	NM_019609	Proteolysis, cell adhesion	3
FZD2	1.44	0.06	NM_001466	Establishment of tissue polarity, Wnt receptor and G-protein coupled receptor protein signaling pathways, cell-cell signaling, development	2
IL11RA	1.94	0.06	NM_147162	Proliferation, response, hematopoiesis, differentiation, formation, accumulation	4
SC5DL	1.44	0.04	NM_006918	Lipid metabolic process, sterol biosynthetic process	2
LOC440928	2.07	0.04	XM_942885		
CTSK	1.67	0.00	NM_000396	Proteolysis	

TABLE A 2.2- List of functions of upregulated genes in p53 shRNA transduced hESC (Ingenuity Pathways Analysis)

Function	Molecules
Cell Signaling	PDE6G,CAPN11,CD79B,EDNRB,MFNG,CLDN19,UTS2,PTGS1,HSPB8,MLLT4,IL12RB2,HLA-DPA1,NRGN,PLCD1,CYTL1,CAMP,SERPINA5,LGALS3BP,ARHGEF6,HTR7,TFPI,FZD2,CXXC4,ACTN3
Cardiovascular System Development and Function	PLCD1,VASH1,MB,EDNRB,UTS2,ATP1A2,PTGS1,VIM,KEL,TFPI
Cellular Movement	VASH1,TFF3,CAMP,EDNRB,SERPINA5,ARHGEF6,UTS2,MLLT4,VIM,TFPI,BIN1
Cardiovascular Disease	MB,EDNRB,SERPINA5,PTGS1,ATP1A2,UTS2,TFPI,BIN1
Cancer	TFF3,EDNRB,CD79B,PTGS1,HSPB8,OLFML3,VIM,FOLR1,PLCD1,VASH1,LGALS3BP,ATP1A2,TFPI,ATP6V1B1,ACTN3
Molecular Transport	MB,EDNRB,CD79B,PTGS1,UTS2,VIM,NRGN,FOLR1,PLCD1,CAMP,HTR7,ATP1A2,FZD2,MVP,IL11RA
Vitamin and Mineral Metabolism	PLCD1,CAMP,EDNRB,CD79B,HTR7,UTS2,FZD2,NRGN,FOLR1
Tissue Morphology	MB,EDNRB,ATP1A2,UTS2,PTGS1,MLLT4,VIM,GEFT,ACTN3
Immunological Disease	CAMP,PTGS1,ATP1A2,HSPB8,VIM,IL12RB2,SELENBP1
Inflammatory Disease	CAMP,CD79B,ATP1A2,PTGS1,HSPB8,VIM,IL12RB2,IL11RA
Cell Death	TFF3,EDNRB,CD79B,PTGS1,HSPB8,IL12RB2,BIN1,NRGN,PMP22,CAMP,LGALS3BP,ATP1A2,MVP
Cellular Growth and Proliferation	TFF3,EDNRB,UTS2,PTGS1,HSPB8,VIM,IL12RB2,BIN1,FOLR1,PLCD1,PMP22,SHMT1,TFPI,MVP
Protein Degradation	CTSK,SERPINA5,FBXL2,CPXM1
Carbohydrate Metabolism	EDNRB,MFNG,TFPI,MVP,IL11RA
Hematological Disease	CAMP,MB,PTGS1,TFPI,FOLR1,SELENBP1,IL11RA
Dermatological Diseases and Conditions	PLCD1,PMP22,CAMP,CD79B,PTGS1,ATP1A2,HSPB8,NRGN,IL11RA,FOLR1
Neurological Disease	PMP22,EDNRB,HTR7,ARHGEF6,ATP1A2,HSPB8,VIM,IL12RB2
Skeletal and Muscular Disorders	CTSK,CD79B,PTGS1,HSPB8,VIM,BIN1,IL11RA
Cell-To-Cell Signaling and Interaction	PMP22,TFF3,CAMP,LGALS3BP,HTR7,UTS2,MLLT4,VIM,TFPI,NRGN
Nervous System Development and Function	PMP22,EDNRB,HTR7,UTS2,VIM,GEFT,BIN1,NRGN
Metabolic Disease	ATP1A2,HTR7,PTGS1,ATP6V1B1
Organismal Injury and Abnormalities	ATP1A2,HTR7,PTGS1,UTS2,VIM,IL11RA
Respiratory Disease	MB,EDNRB,PTGS1,SELENBP1
Connective Tissue Disorders	CD79B,PTGS1,HSPB8,VIM,IL11RA
Cellular Development	PMP22,CD79B,EDNRB,PTGS1,IL12RB2,GEFT,FZD2,BIN1,DYRK3,IL11RA
Organismal Development	PLCD1,MB,PTGS1
Organ Development	PLCD1,MB,EDNRB,IL11RA
Cell Morphology	PMP22,TFF3,CAMP,EDNRB,SERPINA5,HTR7,MLLT4,IL12RB2,ATP6V1B1
Drug Metabolism	PTGS1,VIM,TFPI,MVP,IL11RA,FOLR1
Lipid Metabolism	PLCD1,PTGS1,VIM,SC5DL
Small Molecule Biochemistry	EDNRB,MFNG,UTS2,PTGS1,VIM,MLLT4,SC5DL,FOLR1,PLCD1,CAMP,HTR7,ATP1A2,SHMT1,TFPI,MVP,IL11RA
Skeletal and Muscular System	CTSK,MB,ATP1A2,UTS2,GEFT,ACTN3,IL11RA

Development and Function	
Immune Response	CAMP,CD79B,CLDN19,PTGS1,ARHGEF6,UTS2,MLLT4,IL12RB2,ACTN3
Cell Cycle	VIM
Cellular Compromise	VIM,BIN1
Cellular Function and Maintenance	CD79B,VIM,BIN1,FOLR1
Connective Tissue Development and Function	HTR7,GEFT,FZD2
DNA Replication, Recombination, and Repair	TFF3,CAMP,ATP1A2,VIM,BIN1,FOLR1
Developmental Disorder	CTSK,ARHGEF6
Digestive System Development and Function	TFF3,EDNRB,PTGS1,FOLR1
Embryonic Development	PMP22,EDNRB,PTGS1,MLLT4,FZD2,CXXC4,NRGN,IL11RA
Gene Expression	CAMP,ZNF467
Genetic Disorder	PMP22,CTSK,ARHGEF6,HSPB8
Hair and Skin Development and Function	PLCD1,MB,EDNRB
Hematological System Development and Function	CAMP,CD79B,SERPINA5,PTGS1,ARHGEF6,UTS2,IL12RB2,TFPI,DYRK3,IL11RA
Hepatic System Development and Function	PTGS1
Nucleic Acid Metabolism	CAMP,HTR7,UTS2,ATP1A2,SHMT1,MLLT4,FOLR1
Organ Morphology	EDNRB,SERPINA5,PTGS1,FOLR1,IL11RA
Renal and Urological Disease	EDNRB,PTGS1,ATP6V1B1
Reproductive System Disease	TFF3,EDNRB,SERPINA5,PTGS1,ATP6V1B1,FOLR1
Respiratory System Development and Function	ATP1A2,PTGS1,IL11RA
Amino Acid Metabolism	EDNRB,SHMT1,SDSL,FOLR1
Tissue Development	TFF3,CTSK,LGALS3BP,PTGS1,MLLT4,GEFT,TFPI,FZD2,IL11RA
Organismal Functions	TFF3,PTGS1
Auditory Disease	CAMP
Behavior	CLDN19
Cellular Assembly and Organization	PMP22,CTSK,CAMP,VIM,GEFT,BIN1
Gastrointestinal Disease	TFF3,ATP1A2,PTGS1
Immune and Lymphatic System Development and Function	IL12RB2
Infectious Disease	CAMP,PTGS1,FOLR1
Renal and Urological System Development and Function	PMP22,TFF3,CAMP,EDNRB,ATP6V1B1
Reproductive System Development and Function	PLCD1,SERPINA5,ATP1A2,PTGS1,MLLT4
Tumor Morphology	TFF3

Protein Synthesis	CTSK,SERPINA5,FBXL2,CPXM1,FOLR1
Ophthalmic Disease	PTGS1
Nutritional Disease	IL11RA
Psychological Disorders	IL11RA
Energy Production	CAMP,ATP1A2,SHMT1
Protein Trafficking	EDNRB,FOLR1
Post-Translational Modification	CD79B,EDNRB,FBXL2,KEL
Organismal Survival	PLCD1,PTGS1,IL11RA,FOLR1

TABLE A 2.3- List of genes downregulated in p53 shRNA transduced hESC (Ingenuity Pathways Analysis)

Common name	Fold Change	B-stat	Genbank	Biological process/role	p53 RE
TP53	7.72	5.961	NM_000546	Nucleotide-excision repair, regulation of transcription, protein complex assembly, response to DNA damage stimulus, apoptosis, cell cycle, development, cell aging	
DPEP3	9.26	5.020	NM_022357	Proteolysis	4
MCFD2	2.03	4.837	NM_139279	ER to Golgi vesicle-mediated transport; protein transport and pluripotency, survival of rat neural stem cells	3
TRIM22	2.16	4.501	NM_006074	Regulation of transcription, DNA-dependent, immune response, response to virus	3
LOC651997	2.02	4.280	XM_941292		
KCNAB1	2.94	4.036	NM_172159	Ion transport; potassium ion transport	4
	1.81	3.012	AW445083		
DDX6	3.08	2.751	NM_004397	May contribute to the cell proliferation and carcinogenesis.	5
ADFP	1.70	2.145	NM_001122		3
LOC647784	2.25	2.131	XM_943395		
ZNF676	1.67	2.043	NM_001001411	Regulation of transcription, DNA-dependent	
NTRK3	1.67	1.897	NM_002530	Protein amino acid phosphorylation, transmembrane receptor protein tyrosine kinase signaling pathway, multicellular organismal development, cell differentiation	3
AP1M2	1.76	1.695	NM_005498	Protein complex assembly, protein targeting, vesicle targeting	4
CST6	2.07	1.681	NM_001323	Epidermis development; anatomical structure morphogenesis	2
C20orf23	2.26	1.628	NM_024704	Microtubule-based movement, cell communication	7
DDX19B	1.79	1.361	NM_001014451	mRNA export from nucleus	8
TMEM99	1.60	1.286	NM_145274		3
NALP7	1.75	1.268	NM_139176		3
RCN3	1.75	1.242	NM_020650		3
HBA1	1.83	1,239	NM_000558	Hemolysis, erythropoiesis, cell viability, apoptosis, formation, cell death	7
AK2	1.49	1.208	NM_001625	Nucleic acid metabolic process and may play a role in apoptosis.	1
SNAI3	1.56	1.154	NM_178310		
NME1	1.61	1.060	NM_198175	Negative regulation of cell proliferation and progression through cell cycle, nucleotide metabolic process, regulation of apoptosis, negative regulation	
CDKN1A	2.47	1.013	NM_078467	Response to DNA damage stimulus, cell cycle arrest, regulation of cyclin-dependent protein kinase activity, apoptosis, non-apoptotic programmed cell death	7
GALNT13	2.16	1.010	NM_052917	Protein amino acid O-linked glycosylation	1
ANKRD41	1.55	0.950	NM_152363		5
TCERG1L	1.69	0.909	NM_174937		3
	1.69	0.907	BU621341		
ANXA2	1.63	0.898	NM_001002857	Skeletal development and phospholipase inhibitor activity	4

	1.73	0.897	CN292254		
LOC651029	1.73	0.879	XM_944471		
SAPS1	1.73	0.794	NM_014931	Regulation of phosphoprotein phosphatase activity	9
ATP2A1	1.76	0.583	CB850608	Cation and proton transport; regulation of striated muscle contraction	
	1.48	0.581	XM_373970		
ISG20L1	1.38	0.515	NM_022767		2
LOC653203	1.52	0.491	XM_927850		
FAM48B1	1.67	0.483	XM_093087		
MPZL1	1.48	0.407	NM_003953	Transmembrane receptor protein tyrosine kinase signaling pathway, cell-cell signaling	8
XKRY	1.39	0.394	NM_004677		
HIST1H2BH	2.56	0.351	NM_003524	Nucleosome assembly, chromosome organization and biogenesis	
NOXO1	1.64	0.329	NM_144603	Superoxide metabolic process, cell communication	1
	1.76	0.328	BG217697		
	1.88	0.273	CA391986		
TMEM43	1.38	0.209	NM_024334		4
	1.66	0.188	DA236664		
NALP7	1.64	0.182	NM_206828	Defense response	
MARVELD3	1.44	0.175	NM_001017967		2
CCL2	1.75	0.171	NM_002982	Protein phosphorylation, cellular calcium ion homeostasis; anti-apoptosis; chemotaxis; cell adhesion; G-protein signaling, JAK-STAT cascade, cell-cell signaling, morphogenesis	4
CST6	3.60	0.164	NM_001323		2
OR4F21	1.56	0.110	NM_001005504		
BRP44L	1.42	0.070	NM_016098		5
C1orf24	1.53	0.020	NM_052966		
TEK	1.77	0.013	NM_000459	Protein phosphorylation, cell-matrix adhesion, transmembrane receptor protein tyrosine kinase signaling pathway; cell-cell signaling and adhesion, regulation cell migration and angiogenesis	3
LOC196264	1.47	0.008	NM_198275		3

TABLE A 2.4- List of functions of downregulated genes in p53 shRNA transduced hESC (Ingenuity Pathways Analysis)

Function	Molecules
Cellular Movement	TP53,NME1,CCL2,CDKN1A,ANXA2,CST6,TEK
Cellular Growth and Proliferation	TP53,NME1,CCL2,NTRK3,ADFP,CDKN1A,ANXA2,TRIM22,AK2,CST6,TEK
Cancer	TP53,NME1,CCL2,NTRK3,CDKN1A,ANXA2,TRIM22,CST6,TEK
Cell Cycle	TP53,NTRK3,CDKN1A
Cell-To-Cell Signaling and Interaction	TP53,NME1,CCL2,CDKN1A,ANXA2
DNA Replication, Recombination, and Repair	TP53,NME1,CDKN1A
Nucleic Acid Metabolism	TP53,NME1,AK2,DDX19B
Renal and Urological Disease	TP53,CCL2,CDKN1A
Small Molecule Biochemistry	TP53,NME1,CDKN1A
Hematological Disease	TP53,NME1,CCL2,NTRK3,CDKN1A,HBA1,MCFD2,ANXA2
Tumor Morphology	TP53,NME1,CCL2,CDKN1A,TEK
Hepatic System Development and Function	TP53,CDKN1A
Neurological Disease	TP53,CCL2,NTRK3,CDKN1A
Cellular Function and Maintenance	TP53,NME1,CDKN1A
Cell Death	TP53,NTRK3,CDKN1A,MCFD2
Cellular Development	TP53,NME1,CCL2,NTRK3,CDKN1A,ANXA2,TEK
Gastrointestinal Disease	TP53,CDKN1A
Hematological System Development and Function	TP53,CCL2,CDKN1A,ANXA2,TEK
Immune and Lymphatic System Development and Function	TP53,CCL2,CDKN1A,ANXA2
Skeletal and Muscular Disorders	TP53,CCL2,CDKN1A
Cell Signaling	TP53,MPZL1,CCL2,NTRK3,CDKN1A,TEK
Respiratory Disease	TP53,CDKN1A
Endocrine System Disorders	TP53,CDKN1A,ANXA2
Cellular Assembly and Organization	TP53,CDKN1A
Hair and Skin Development and Function	TP53,CDKN1A
Tissue Morphology	TP53,CDKN1A
Immune Response	TP53,CCL2,CDKN1A,TRIM22
Connective Tissue Disorders	TP53,CCL2,NTRK3,CDKN1A
Inflammatory Disease	TP53,CCL2,CDKN1A
Organismal Survival	TP53,CCL2,NTRK3,CDKN1A,TEK
Cardiovascular System Development and Function	TP53,CCL2,ANXA2,TEK
Immunological Disease	TP53,NME1,CCL2,CDKN1A
Skeletal and Muscular System Development and Function	TP53,CCL2,CDKN1A
Cell Morphology	TP53,CCL2,NTRK3,CDKN1A,ANXA2,TEK

Reproductive System Disease	TP53,NME1,CDKN1A,ANXA2,CST6,TEK
Cellular Compromise	TP53,CDKN1A
Connective Tissue Development and Function	TP53,CCL2,NTRK3,CDKN1A
Organismal Development	TP53,CCL2,ANXA2,TEK
Dermatological Diseases and Conditions	TP53,NME1,CDKN1A
Lipid Metabolism	TP53,CDKN1A
Tissue Development	TP53,CCL2,CDKN1A,TEK
Organ Morphology	TP53,CDKN1A
Gene Expression	TP53,CDKN1A
Free Radical Scavenging	TP53,CDKN1A
Hepatic System Disease	TP53,CDKN1A
Molecular Transport	TP53,CDKN1A
Renal and Urological System Development and Function	TP53,CDKN1A
Digestive System Development and Function	TP53
Nervous System Development and Function	CDKN1A
RNA Post-Transcriptional Modification	TP53
Reproductive System Development and Function	TP53