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# Analysis of stem cell genome stability and cancer-associated developmental programmes

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# Analysis of stem cell genome stability and cancer-associated developmental programmes

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Khalid Ibrahim AlZEER

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**Abstract**

During the developmental programme, post-meiotic cells, gametes, fuse to form a zygote, which divides and forms embryo stem cells. In complex metazoans, such as humans, these cells proliferate and ultimately differentiate to form the distinct organs of a functioning adult. Genomic instabilities in stem cells, germ line cells and somatic cells can result in cells becoming cancerous and one of the primary oncogenic factors is DNA replication stress. In cancer tissues developmental programmes become deregulated with cells loosing cellular identity and potentially disconnecting from their micro environment and undergoing metastasis to other regions of the body, causing extensive organ failure. Within tumours, there are so called cancer stem cells, cells which may have been the original progenitor cells for the tumour formation, but which are also proposed to be capable of tumour self renewal and may be responsible for therapeutic resistance. The relationship between cancer stem cells, embryo stem cells and how their developmental programmes are regulated and linked to genomic instabilities is very poorly understood.

This current study explores the links between genome instability pathways and developmental programmes. Firstly, it is known that some meiosis-specific genes which function to drive meiotic chromosomal rearrangements become aberrantly activated in somatic, cancerous tissues. This resulted in the hypothesis that activation of meiotic recombination hotspots might generate regions which become refractory to somatic DNA replication and result in genomic loci with the potential to drive oncogenic rearrangements. Due to the technical difficulties of using mammalian cells, we addressed this hypothesis in a widely used experimental model eukaryote, the fission yeast. From this work no evidence for the activation of eukaryotic meiotic recombination hotspots under replicative stress conditions could be found.

Prior to this work a preliminary study had found that mouse embryo stem cells exhibited a significant sensitivity to the topoisomerase poison camptothesin, relative to full differentiated mouse embryonic fibroblasts. This difference in sensitivity was not observed using other DNA damaging agents tested. Given that camptothecin generates DNA double-strand breaks in a replication-dependent fashion this current study set out to determine whether cells with stem cell characteristic have distinct responses to oncogenic insult which perturbs DNA replication. To explore this in humans the teratocarcinoma cell line NTERA2 was employed as it expresses many of the markers of stem cells / cancer stem cells and can be differentiated *in vitro* to provide isogenic differentiated and undifferentiated human cells. Studies of sensitivities of human NTERA2 (differentiated *vs.* undifferentiated) and preliminary analyses of mouse stem cells provides evidence that there are inter-species distinctions and possible subtle mechanistic changes to cancer-suppressing genome stability mechanisms upon cellular differentiation in human cells away from the stem cell state.

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**Abbreviations**

| | |
|---|---|
| AEBSF | (2-aminoethyl) benzenesulfonyl fluoride |
| APH | Aphidicolin |
| ARS | Autonomously replicating sequence |
| ATM | Ataxia telangiectasia mutated |
| ATR | Ataxia telangiectasia and Rad3 |
| BFB | Breakage-fusion-bridge |
| BMI -1 | B lymphoma Mo-MLV insertion region 1 |
| CIN | Chromosome instability number |
| CPT | Camptothecin |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMs | Double minutes |
| DMSO | Dimethlysulphoxide |
| DNA | Deoxyribonucleic Acid |
| dNTPs | Deoxyribonucleotide triphosphates |
| DSBs | Double-strand breaks |
| EC | Embryonal Carcinoma |
| ECACC | European Collection of Cell Cultures |
| EDTA | (Ethylenedinitrilo) tetra acetic acid |
| EMT | Epithelial-mesenchymal transition |
| ESC | Embryonic stem cell |
| FBS | Foetal Bovine Serum |
| GCRs | Gross chromosomal rearrangements |
| HMBA | Hexamethylene Bisacetamide |
| HNSCCs | Head and neck squamous cell carcinomas |
| HR | Homologous recombination |

| | |
|---|---|
| HSC | Haematopoietic stem cell |
| HSRs | Homogeneously staining regions |
| HU | Hydroxyurea |
| Ihh | Indian hedgehog |
| *iPSCs* | *Induced pluripotent stem cells* |
| IR | Ionising radiation |
| LEF | Lymphocyte Enhancer Factor |
| LOH | Loss of heterozygosity |
| LRP | LDL (Low Density Lipoprotein) Receptor Related Protein |
| MAPK | Mitogen activated protein kinase |
| MCM | Mini-chromosome maintenance |
| MEFs | Mouse embryonic fibroblasts |
| MEM | Minimum Eagle's Medium |
| MET | Mesenchymal to epithelial transition |
| MMP | Matrix Metalloproteinase |
| MMR | Mismatch Repair |
| mRNA | Messanger Ribonucleic Acid |
| NES | Nuclear Export Sequence |
| NF κB | Nuclear factor κB |
| NHEJ | Non-homologous end joining |
| NLS | Nuclear Localisation Sequence |
| NOD/SCID | Nonobese diabetes/severe combined immunodeficiency |
| Notch1 | Notch homolog 1, translocation-associated |
| Oct-4 | octamer-binding transcription factor 4 |
| ORC | Origin recognition complex |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PHL | Phleomycin |
| PI3K | Phosphoinositide 3-kinase |
| PP2A | Protein Phosphatase 2A |
| RAG | Recombination activating proteins |
| RFBs | replication fork barriers |
| RFPs | replication fork pauses |
| RNA | Ribonucleic Acid |
| RT | Reverse Transcription |
| RTS1 | Replication Termination Sequence 1 |
| SCF | SKP-1, Cdc53, F-box |
| SDS- PAGE | Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis |
| Ser | Serine |
| Shh | Sonic hedgehog |
| TAD | Transcriptional activation domain |
| TCF | T-Cell Factor |
| TGFβ | Transforming growth factor beta |
| TLE | Transducing-Like Enhancer of split |
| UV | Ultra violet |
| XRCC4 | X-ray repair cross-complementing protein 4 |

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# Chapter 1

## 1.1 Introduction

For more than 100 years, human cancers have been identified as a morphologically heterogeneous population of cells (Hope, Jin & Dick 2004a). In the past ten years, however, it has become clear that functional heterogeneity exists within the cells that constitute various human cancers of the blood, breast, brain, skin, bone, and prostate (Bonnet, Dick 1997a, Gibbs et al. 2005). Cancer defines a group of disorders that involve uncontrolled cellular growth, cellular invasion of tissues and the probability of metastasis if not treated at a timely stage. These cellular abnormalities arise from collected genetic modifications, either through changes in primary genetic sequence or through epigenetic changes (Feinberg, Ohlsson & Henikoff 2006, Jones, Baylin 2007).

Cancer is a genetic disease that modifies three kinds of genes, affecting tumour progression, termed stability genes, oncogenes and tumour suppressor genes (Vogelstein, Kinzler 2004). In the multi-step carcinogenesis model, cancer develops through several stages during the accumulation of molecular alterations, progressing from a pre-invasive to an invasive disease (Hong, Sporn 1997).

## 1.2 Cancer stem cells

It has been recently proven that just a small subpopulation of cancer cells can form new tumours in haematological malignancies (Pardal, Clarke & Morrison 2003a). In spite of the clonal source of many cancers, research into leukaemia and, more recently, solid cancers proposes that tumour cell populations are heterogeneous with regard to proliferation and differentiation. This characteristic is described by the cancer stem cell hypothesis. Supposed ‘cancer stem cells’ have been identified and characterized in myeloid leukaemia, breast, brain and lung cancers (Jordan 2004).

The theory of a cancer stem cell originated based on the observation of correspondences between the self-renewal mechanisms of normal organs and successive proliferation in cancers (Reya et al. 2001a). The cancer stem cell hypothesis suggests that not all the cells in a tumour have the capability to proliferate and maintain the growth of the tumour, and that only a small subpopulation of cells in the tumour, named cancer stem cells, are primed to proliferate and self-renew. These unusual cells form tumours and maintain their growth, and have been separated in haematological cancers such as leukaemia (Bonnet, Dick 1997b) and

multiple myeloma, and in solid tumours such as breast tumours (Al-Hajj et al. 2003a) and brain tumours (Singh et al. 2003a).

Stem cells are undifferentiated and unspecialized cells (Sell 2004). There are three classes of stem cells: embryonal, germinal and somatic (or adult) stem cells (Blagosklonny 2005). Embryonal stem cells are created during the first divisions of a fertilised egg. These stem cells have the ability to develop into all of the cells in adult organs. Through the embryogenic system, the progeny of embryonal stem cells lose this potential and develop differentiated properties through a practice named determination. Germinal stem cells in adults make eggs and sperm. Somatic stem cells have "limited" differentiation potential, and are responsible for cells that differentiate inside mature tissue. Different kinds of stem cells have different types of potential and reproduce differently. By definition, an adult stem cell is a cell that advances from a given organ, and which has long-term replicative potential and the capability to both self-renew and differentiate inside the environment of the organ. Stem cells in adult tissues are primarily quiescent, and have the ability for self-renewal and differentiation in all tissue types. Through unbalanced division, each stem cell generates one daughter cell that then continues as a stem cell and a daughter cell "more differentiated", and so on. This initiates the process of determination and generates quickly proliferating progenitor cells, which are devoted to differentiation. Progenitor cells go through a limited number of cell divisions and then differentiate or die (Al-Hajj, Clarke 2004a). Thus, in normal tissue, three differing types of subdivision can be described: self-renewal subdivision involving quiescent stem cells, proliferating subdivision involving proliferating progenitors with the express potential for self-renewal and terminal subdivision involving differentiated cells or apoptotic cells.

As mentioned above, the idea of the cancer stem cell originated when correspondences were observed between the self-renewal mechanisms of stem cells and those of cancer cells (Reya et al. 2001b). Both kinds of cells self-renew and differentiate into other cells. Tumours are heterogeneous in terms of cell phenotype and proliferative potential, and the concept of progressing mutations only partially describes this heterogeneity. Cancer stem cells are a minute population of tumour cells that are able to self-renew and develop into all the constituents of a heterogeneous tumour.

Previous models of cancer attributed the unregulated growth of tumours to additional genetic changes that resulted in the activation of genes promoting proliferation, the silencing of genes

implicated in inhibiting proliferation and the circumvention of genes involved in programmed cell death (Al-Hajj, Clarke 2004b). In the cancer stem cell hypothesis, another crucial occurrence in tumour progression is the change of genes implicated in the regulation of stem cell renewal. It is thus not surprising that various genes originally identified as playing a role in tumour progression were subsequently implicated in normal stem cell self-renewal (Bjerkvig et al. 2005). Cancer stem cells and normal stem cells may participate in the same self-renewal and proliferation mechanisms. Alterations that deregulate the pathways regulating normal stem cell self-renewal have also been noticed in various cancers, suggesting that the deregulation of self-renewal pathways could be necessary for cancers to develop.

### 1.3 Genomic instability

It is widely accepted that cancer cells may be genetically unstable (Lengauer, Kinzler & Vogelstein 1998), and that the genomic instability is a mechanism of both tumour progression and heterogeneity. The tumour instability is explained by a more enhanced accumulation of genetic changes in tumours contrasted with normal cells, and is sorted into four various types, (1) subtle sequence alterations; (2) changes of chromosome numbers; (3) chromosome translocations; and (4) gene amplifications (Lengauer, Kinzler & Vogelstein 1998).

### 1.4 Alterations in chromosome number

Chromosome number instability (CIN), occurs in the majority of human malignancies (Mitelman et. al., 1994; 1997). As inspected by Lengauer et. al., (1998) in more detail, genes that, when mutated, can lead to CIN contain those concerned in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function and centrosome-microtube formation, as well as checkpoint genes that observe the proper progression of the cell cycle (Hartwell, Smith 1985a, Hartwell 1992, Murray 1995, Fukasawa et al. 1996, Elledge 1996, Nasmyth 1996, Paulovich, Toczyski & Hartwell 1997, Taylor, McKeon 1997, Doxsey 1998, Jin, Spencer & Jeang 1998, Lane 1998, Rotman, Shiloh 1998). The fact that genetic defects of so many genes can lead to CIN proposes a heterogeneous basis for CIN in cancers, with many genes each playing a role in a distinc part of the tumour progression (Lengauer, Kinzler & Vogelstein 1998). In DNA-damage checkpoint deficiency, chromosomes comprising damaged DNA could separate inappropriately, resulting in CIN because sister chromatids remain linked by DNA or DNA-protein links, chromosomes which and are also susceptible to

gross structural changes due to single-stranded gaps or double-stranded breaks. Genomic deletion produced by double-stranded breaks can generate loss-of-heterozygosity (LOH), a major oncogene factor (Shen et al. 2000). Chromosome instability appearing from missed DNA damage checkpoints is often related to enhanced mitotic recombination as well as with irregular chromosome segregation (Hartwell, Smith 1985b), thus, tumourogenesis is eventually associated with the accumulation of gross chromosomal rearrangements (GCRs), such as translocations, deletions of chromosome arms, interstitial deletions or inversions (Gauwerky, Croce 1993, Shikano et al. 1993, Mitelman, Mertens & Johansson 1997, Chen, Kolodner 1999). In many instances, GCRs inactivate tumor-suppressor genes or give rise to novel fusion protein that initiates carcinogenesis.

**1.5 Chromosome translocations**

Translocations are rearrangements of chromosomes in which a chromosome portion is transplaced from one chromosome to another. Faults in chromosome replication can result in translocations (Mirkin and Mirkin, 2007). The system of chromosomal translocation brings two formerly unlinked parts of the genome the juxtaposition of elements that can interrupt the normal expression of the gene next to the breakpoint. These events are especially important when the breakpoint of the translocation cause in unacceptable expression of an oncogene or synthesis of an unknown oncogenic fusion protein (Agarwal et. al., 2006). Such chromosome alterations and mutations can activate cellular oncogenes in a dominant pattern. Chromosomal translocations are frequently associated with several cancers especially haematologic malignancies and childhood sarcomas (Aplan, 2006). In fact, the initial translocation identified in a human neoplasia was t (9;22) (q34;q11), causing the Philadelphia Chromosome (Novell and Hungerford, 1960). The symbols t (9;22) (q34;q11) indicate a translocation between chromosomes 9 and 22 with breakpoints in bands 9q34 and 22q11, respectively (Mitelman et. al., 2007). Chromosomal translocations are increasingly being used in the clinic to guide therapeutic outcomes (Agarwal, Tafel & Kanaar 2006, Felix, Kolaris & Osheroff 2006, Aplan 2006). Even so, the processes that cause these translocations are still poorly understood (Aplan, 2006). These modifications can be discovered cytogenetically as fusions of various chromosomes or of normally non-contiguous parts of single chromosomes. Large parts of chromosomal arms are frequently removed through recombination that leads to translocations, and these deletions are viewed as the defeats of heterozygosity. Translocations can arise in cells that enter mitosis before recombination-

promoting double-stranded DNA breaks are repaired. Alterations of genes implicated in double-stranded break repair or DNA damage checkpoints therefore, can cause the translocation instability in human cancers (Lengauer, Kinzler & Vogelstein 1998).

### 1.6 Gene amplification

Gene amplification is a well defined origin of oncogene activation throughout tumour development, and some genomic areas are more frequently amplified than others (Luo et al. 2006). For example, amplification of chromosome locus 11q13 happens at high frequencies in defined human cancers, involving lung, bladder, breast and ovarian carcinomas, in addition in head and neck squamous cell carcinomas (HNSCCs) (Hui et al. 1997, Zaharieva et al. 2003).

Gene amplification, the addition in the copy number of a part of the genome, is a overall concept of genome instability in tumour cells and a key mechanism of oncogene activation in addition to drug resistance, because it leads to over-expression of related genes. Amplification of DNA sequences including cancer genes has been observed in various types of solid tumours and lymphomas (Futreal et al. 2004, Santarius et al. 2010). The fact that gene amplification has never been discovered in cells of normal origin (Wright et al. 1990, Tlsty 1990) suggests that either control mechanisms that preventing of gene amplification are active (such as the p53-mediated damage-sensing pathway), or cells carrying gene amplifications do not survive. Cytogenetic displays of amplified DNA include self-replicating extrachromosomal elements termed “double minutes” (DMs), amplified areas on a single chromosome (homogeneously staining regions, HSRs) or amplified areas distributed during the genome (Albertson 2006). The existence of specific regions of the genome that are hotspots for amplification in cancers with similar cell of origin suggests that they contain genes relevant for tumour formation and progression (Lockwood et al. 2008, Myllykangas et al. 2006). In addition, the genomic context where the amplified DNA is embedded (Gajduskova et al. 2007) and its proneness to breakage (Ciullo et al. 2002) seem to contribute to the propensity to amplify of specific genomic territories. Moreover, the instability of amplified DNA further increases the extent of amplification. A large body of evidence indicates that DNA double-strand breaks (DSBs) can promote gene amplification through different processes such as successive breakage-fusion-bridge (BFB) cycles, unequal sister

chromatid exchange, rolling circle replication or fold-back priming (Mondello, Smirnova & Giulotto 2010).

### 1.7 Gross chromosomal Rearrangements

Genetic instability is a transient or a persistent stage that resulted from a sequence of mutational events causing gross genetic changes (Jefford and Irminger-Finger, 2006; Venkatesan et. al., 2006). High fidelity DNA replication is intrinsic to avoid eukaryotic genomes acquiring mutations. DNA replication is continuously challenged by inherent conditions in cells such as damaged DNA templates, protein complexes bound to DNA and insufficient supplies of deoxyribonucleotide triphosphates (dNTPs) (Noguchi et al. 2004). In addition to intra cellular events causing DNA damage, it can also be generated by external or environmental factors, for instance, UV light, and gamma radiation (Venkatesan *et. al*., 2006). Cells have processes to detect and repair DNA damage. However, incorrect repair can result in gross chromosomal rearrangements (GCRs) (Banerjee et. al., 2007). GCRs include the structural alterations that may happen either at the chromosome level, leading to a decrease or increase of great portions of chromosomes such as, translocations, deletions, inversions, amplifications, chromosome termination to end fusions and ploidy changes or at the nucleotide level affecting gene structures or expression; for instance, mutations, deletions, gene amplifications, microsattellite amplification and gene silencing by epigenetic effects (Kolodner et. al., 2002; Aplan, 2006; Banerjee et. al., 2007)

GCRs can be caused by primary mutations affecting DNA replication (Venkatesan et. al., 2006; Mirkin and Mirkin, 2007). Throughout DNA replication, DNA replication forks can stall at broken DNA, at naturally occurring sequences such as replication fork barriers or when the collision happens with different proteins accompanying with DNA metabolism such as RNA polymerase II (Prado and Aguilera, 2005). In vertebrates, cancer is frequently linked to large genome rearrangements appearing from incorrect repair of DSBs (Hasty et. al., 2003; Aplan, 2006).

### 1.8 Loss of heterozygosity (LOH)

Human cells generally contain two distinct alleles for a given gene. This provides an additional functional gene, should one become inactive. Loss of heterozygosity (LOH) can result in the loss of the active gene and, if this is a tumor suppressor gene, this can result in

carcinogenesis. LOH can arise as a result of several processes, including chromosome breakage, translocation, deletion, gene conversion, mitotic recombination and loss, chromosomal fusion or entire chromosomal loss (Thiagalingam et al. 2002). A high frequency of chromosomal breakage can cause LOH and is a hallmark of genetic instability associated with oncogenesis (Thiagalingam et al. 2002).

### 1.9 Repair of chromosomal breaks

Non-homologous end joining (NHEJ) and homologous recombination (HR) are pathways for the repair of chromosome breaks, such as DSBs. DSBs are a type of DNA damage that can be generated by endogenous or exogenous agents. For instance, when the cell is exposed to ionising radiation (IR), this can cause complex, clustered kinds of DSB damages via the random deposition of energy. IR can also cause DBSs indirectly by the production of reactive oxygen species (O'Driscoll, Jackson & Jeggo 2006). Closely packed single strand-breaks can also cause DBSs. In certain recombination processes, like V(D)J recombination in the immunoglobulin genes of the immune mechanism (i.e. the generation of diversity in the development of T-Cells and B-Cells), DSBs are also produced by recombination activating proteins (RAG) (O'Driscoll, Jackson & Jeggo 2006). Meiosis division of a gamete-producing cell includes the programmed production of a location or site-specific of DSB (O'Driscoll, Jackson & Jeggo 2006). Telomere shortening can also activate DSB repair and the spread of apoptosis/senescence via p53 activation (Smith, de Lange 2000). However, there is significant variation in the events which cause DSBs and the activation of related repair pathways. For instance, the exogenous production of DSBs by IR during G1 of the cell cycle causes the activation of NHEJ and ATM signalling. Conversely, replication fork stalling activates initial HR and ATR signalling. The repair pathway choice is heavily influenced by the cell cycle period in which the damage occurs with NHEJ being more prevalent in G1and HR in S-phase and G2. The level of DSBs damage also influences the activation of the pertinent rejoining process (O'Driscoll, Jackson & Jeggo 2006).

### 1.10 Non-homologous End Joining Pathway

Most DSBs in eukaryotic cells are repaired by either NHEJ or HR. This is in spite of the fact that current works has established that a third of DBSs are repaired by lesser characterised repair mechanisms known as micro homology-mediated end joining (MMEJ) (McVey, Lee 2008). NHEJ is the dominant pathway of DSB repair in mammalian cells , and even though

NHEJ-defective cell lines display marked defects in DSB repair and sensitivity to IR, cells defective in ATM , a crucial protein kinase participating in NHEJ repair and cell cycle arrest, repair the majority DSBs usually (Goodarzi, Noon & Jeggo 2009). Despite this, 10-20% of visible IR-induced DSBs (repair foci) is repaired with slow kinetics and requires ATM and a target nuclease, Artemis, implicated in ends processing (Riballo et al. 2004). HR occurs in the late S-G2 phases, while NHEJ happens mostly in the G1 phase (O'Driscoll, Jeggo 2006, Lieber 2010). Central proteins involved in the NHEJ mechanism are Ku dimers (Ku70-Ku80), DNA-PKcs (the catalytic subunit of DNA-PK), XRCC4, Ligase IV, Artemis and cernunnos-XLF (Buck et al. 2006, Ahnesorg, Smith & Jackson 2006a). The Ku complex (Ku80/Ku70) is involved in the early recognition of DNA DSBs by its elevated attraction towards DNA ends. The addition the Ku heterodimer to the end of a DSB recruits DNA-PKcs, a serine/threonine protein kinase. The regulatory Ku70/80/DNA-PKcs complex works as a DNA damage sensor. Purified Artemis protein has been shown to have single-stranded 5′ to 3′ exonuclease activity. However, when the Artemis protein creates a complex in the presence of DNA-PKcs, Artemis is phosphorylated and obtains endonucleotytic activity on 5′ and 3′ overhangs, and also hairpins (Ma et al. 2002). Artemis supplies a significant nucleolytic processing activity to organise DNA ends for re-ligation (Sekiguchi, Ferguson 2006a). A member of the NHEJ protein family, Cernunnos-XLF, is believed to participate in DSB end joining alongside XRCC4 and Ligase IV, the precise function of this protein is not yet known. It has been proposed that Cernunnos-XLF might function as a link between XRCC4 and Ligase IV and the different NHEJ elements to assist in the enrolment of the other factors to the ends of DSBs (Ahnesorg, Smith & Jackson 2006b, Ahnesorg, Smith & Jackson 2006b). Alternatively, it might be implicated in the regulation of XRCC4-Ligase IV activity through the variation of active and inactive multimeric stages of XRCC4 (Sekiguchi, Ferguson 2006b). DNA-PKcs and Ku70/80 have been shown to be required for telomere maintenance (d'Adda di Fagagna et al. 2001). Analogous mechanisms were observed in mice deficient in Ku86 which had raised chromosome end-to-end fusion with strong telomeric signals at a site of fusion. This, therefore, shows the significant function of Ku80/Ku86 in the telomere end capping process in mice (Samper et al. 2000, Espejel et al. 2002, Espejel, Blasco 2002). In the same way, DNA-PKcs were shown to be necessary in conserving mammalian telomeres (Bailey et al. 2004) and that repression of DNA-PKcs caused raised levels of chromatid fusions (Bailey et al. 2001, Bailey, Goodwin 2004). Analogous research into DNA-PKcs defective mice showed increased levels of telomere fusions showing telomere dysfunction during the process of a telomere end capping (Samper et al. 2000,

Hande et al. 1999, Goytisolo et al. 2001). DNA - PK is characteristically participating in the repair of DSBs by assisting processing of damaged ends, however in the event of apoptotic conditions, it appears that the function of DNA-PK and Artemis changes to that of final executioners (Britton et al. 2009).

### 1.11 Homologous Recombination (HR)

Homologous recombination is another type of DSB repair engaging primarily in late S and G2 phases of a cell cycle. HR is often more precise than NHEJ because it uses a sister chromatid as a template when repairing DSBs. HR is mainly responsible for repairing DSBs that appear because of replication fork stalling (in late S phase). The HR pathway includes a nucleolytic mechanism, strand invasion, Holliday junction formation and branch migration (see figure 1.1). Many proteins are implicated in HR, including RAD51, RAD52, RPA, BRCA1, BRCA2, XRCC2, XRCC3, RAD54, DNA polymerases and DNA ligases. HR repair can be mediated two mechanisms, RAD51-dependent pathway, which is an error-free and RAD51-independent pathway, which can cause errors in the DNA sequence (Griffin, Thacker 2004). The RAD51-dependent pathway includes a homology search and strand invasion to permit the recondition of the primary DNA sequence, dependent on the undamaged homologous sequence (Figure 1.1). Rad51 forms a filament on ssDNA and this structure mediates the invasion of the homologous duplex in an ATP-dependent reaction (Holthausen, Wyman & Kanaar 2010). Rad52 has an essential role in break-induced replication and single-strand annealing (SSA) (McEachern, Haber 2006, Llorente, Smith & Symington 2008).The RAD52 protein identifies the broken DNA ends and binds to the DNA ends with a 3' single strand generated by the nucleolytic activity of the Mre11- Rad50- Nbs1 complex. Rad52 assist the loading of Rad51 onto ssDNA. Subsequently, the creation of a nucleoprotein filament across the 3' single-strand DNA is achieved via RAD51 polymerization and with the assistance of a single-strand DNA binding protein, RAD52 and replication protein A (RPA). The RAD51 nucleoprotein filament inspects for a homologous duplex, after which the DNA strand exchange gives rise to a linked molecule between the homologous damaged and undamaged duplexes. BRCA2 helps to load RAD51 onto the ssDNA molecule, while BRCA1 is needed as a regulatory protein. After branch migration and Holliday junction formation, DNA synthesis obtains the position where DNA polymerases and accessory elements fill the gap and DNA Ligase IV and XRCC4 relegate the remaining breaks. A BRCA1-defect leads to sensitivity to ionising radiation and sensitivity to DNA cross linking

agents like mitomycin C (Powell, Kachnic 2003). BRAC1 function has also been linked to heterochromatin formation (Zhu et al. 2011). In addition, a BRCA1-deficient human lymphoblastoid cell line has an elevated level of chromosome end-to-end fusion that proposes a function of BRCA1in telomere capping (Al-Wahiby, Slijepcevic 2005).

### 1.12 DNA Replication

Cells duplicate at cellular division, which requires the prior duplication of the genetic material via DNA replication (Karp , Branzei, Foiani 2007). Faults in chromosome replication can cause translocation that appears as a result of a recombination event next to stalled replication forks, and DNA replication pause locations can be hotspots for recombination (Labib, Hodgson 2007).

DNA replication is semi-conservative because each daughter duplex includes one strand from the progenitor molecule. The occurrences included in the initiation of chromosomal replication are analogous in the eukaryotes Archae and Eubacteria. As a result of this, replication is initiated by association of specific initiator protein(s) to DNA sites, called replication origins, and this causes the localised unwinding of the DNA duplex followed by the formation of replication forks (Zakrzewska-Czerwińska et al. 2007).

### 1.13 Replication initiation

Even though DNA replication is a necessary characteristic of cellular proliferation, the processes of its regulation in mammalian cells are poorly understood (Goldman 1988). There is significant uncertainty about the DNA sequences responsible for the initiation of replication along mammalian chromosomes (DePamphilis 1993, Dijkwel, Hamlin 1995, Huberman 1995).

The molecular process controlling replication initiation in eukaryotes ensures that the numerous origins of replication fire just once per cell cycle (Kawasaki et al. 2006). The origin recognition complex (ORC) marks the location of replication origins in the genome and functions as the ‘landing pad’ for the accumulation of a multiprotein, pre-replicative complex (pre-RC) at the origins. In the model organism *Schizosaccharomyces pombe*, the pre-RC contains Cdc18, ORC, mini-chromosome maintenance (MCM) and Cdc10-dependent

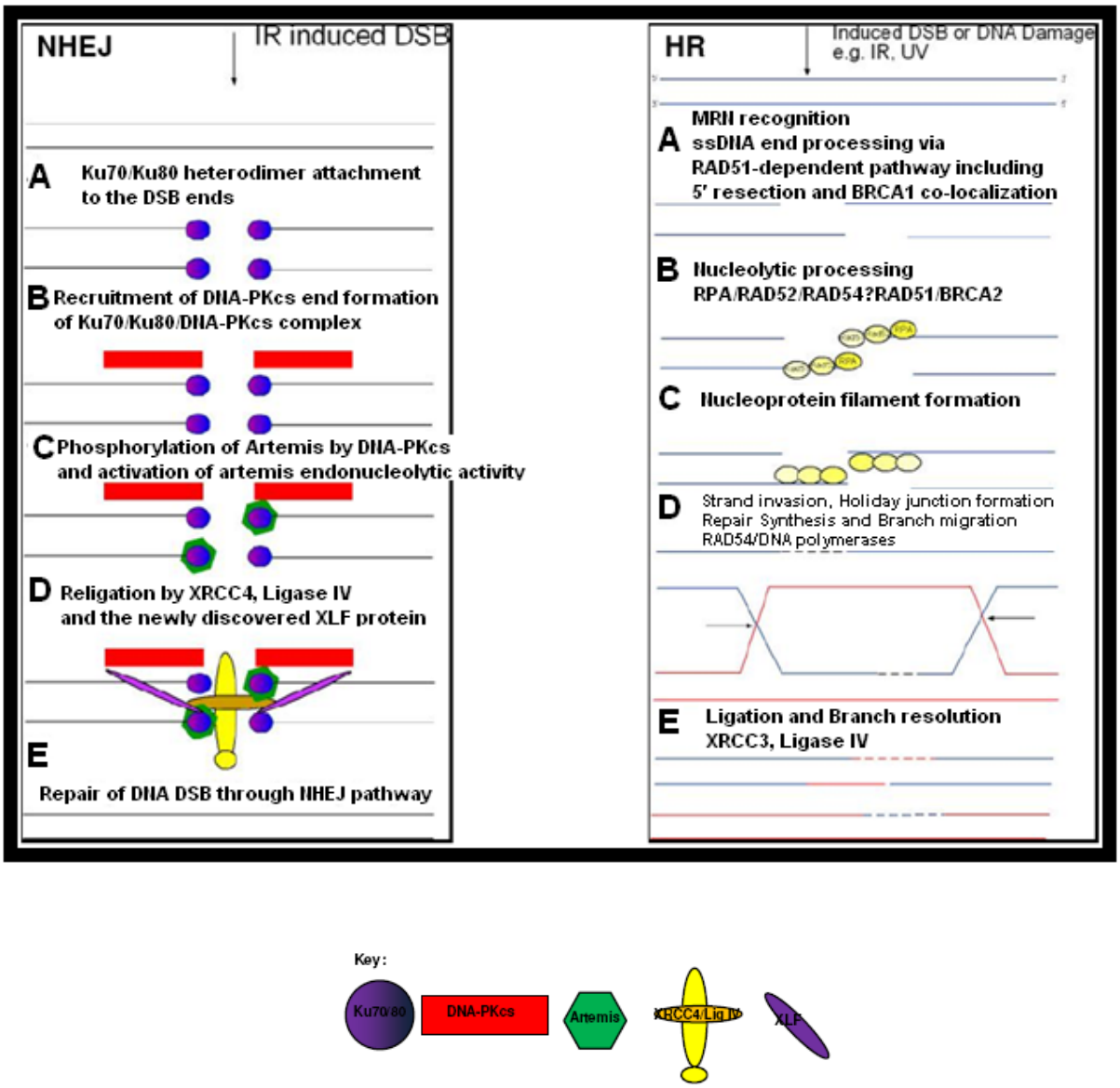



**Figure1.1Graphic representation of NHEJ and HR**. NHEJ A: DSB is discovered in a mammalian cell through sensor molecules MRN complex and ATM, which initiators Ku70/Ku80 heterodimmers that are bound to the ends of the broken DNA molecule. The doughnut shape of the Ku heterodimer is exactly matched to its DNA double helix shape and is inserted at the ends of the broken DNA molecule. B: The dimerization of Ku70/Ku80 recruits DNA-PKcs to the ends of the broken DNA molecule, forming a Ku70/Ku80/DNA-Pkcs complex. C: One of the functions of DNA-PKcs is to phosphorylate the Artemis molecule to activate its endonucleolytic properties to "chew" any overhangs at the end of the DNA molecule, a process that is necessary for the proper re-ligation step. D: Ligation occurs in the presence of XRCC4 (known as the x-ray cross linking protein), Ligase IV protein and the newly discovered Cernunnos-XLF proteins. XLF protein may be used to bridge XRCC4 and Ligase IV proteins (Yasaei 2009).

transcript protein (Cdt1). The MCM proteins function as crucial members in the process that limit eukaryotic DNA replication to once per cell cycle.

DNA replication starts at multiple origins on eukaryotic chromosomes (Bell, Dutta 2002, Gilbert 2001) (Figure 1.2). *Saccharomyces cerevisiae* is noted to have about 400 origins of replication, located around every 40-150 kb. These are responsible for the replication of the 14 Mb genome (Branzei, Foiani 2007).

The origins of DNA replication in *S. pombe* contain a specific consensus sequence similar to the autonomously replicating sequence (ARS) consensus of *S. cerevisiae* (Clyne, Kelly 1995). However, the origins of DNA replication in *S. pombe* are greater (> 500 bp), are AT rich and are mainly detected in intergenic regions (Dai, Chuang & Kelly 2005, Hayashi et al. 2007).

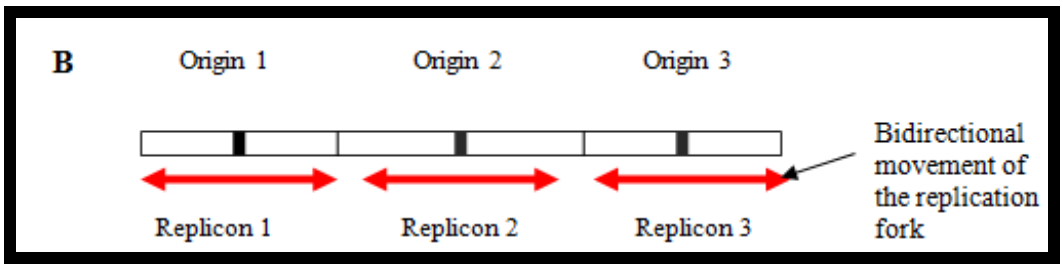



**Figure1.2 Origin of replication and termination in eukaryotes**. Linear eukaryotic chromosome replicates as many individual replicons moving bidirectionally Adapted from (Rothstein et al., 2000).

### 1.14 Dynamics of the Replication Fork

The replication fork is the point at which new DNA daughter strands are formed from the parental strands in a template-directed fashion. Because of its Y-shaped form, this reactive region is called a replication fork (Figure 1.3). Origin firing is accompanied by replication fork formation after the origins are permitted (Branzei, Foiani 2007). At a replication fork, the DNA of new daughter strands is created via a multi-enzyme complex that comprises DNA polymerases and numerous other proteins. The replication mechanism needs different activities and elements for each protein for the elongation, initiation and termination steps.

In eukaryotes, before the start of the S-phase of the cell cycle, protein kinases are activated, directing the initiation of replication. The unwinding of the DNA duplex and disconnecting of the strands requires the assistance of two kinds of proteins that join to the DNA. Firstly, a helicase is requiring unwind the duplex DNA and second, a "primase" produces short RNA primers that are needed to start DNA synthesis. DNA helicases unwind the DNA duplex in a reaction that utilises energy from ATP hydrolysis to break the hydrogen bonds binding strands, exposing the single-stranded DNA templates. Associated with these are the multiple copies of the heterotrimeric single-stranded binding protein A (replication protein A; RPA), which preserve the DNA in a single-strand state. In one model of DNA polymerases ε and δ are involved in the replication of both leading and lagging strands, working with their accessory proteins, like replicating factor C (RFC) and proliferating cell nuclear antigen (PCNA). Together these proteins form a large complex termed the 'replisome' (Figure 1.3) (Waga, Stillman 1998, Baker, Bell 1998, Johnson, O'Donnell 2005).

The creation of a function of DNA replication requires many supplemental elements (Mcm10, Cdc45, Dpb11, Sld2, Sld3 and the GINS complex in *S. cerevisiae*) and activation of S-phase cyclin-depedent kinases (CDKs) and Cdc7- Dbf4 kinases (DDK), which both phosphorylate proteins of the repliosome (e.g. Mcm proteins, Sld2, Sld3) (Moldovan, Pfander & Jentsch 2007). These reactions function by assembling the replicative helicase, which is comprised of the Mcm2-7 complex with joined elements, and include the DNA polymerases and other elements essential for DNA synthesis (Figure 1.3).

The two DNA strands are created via various processes. Leading strands can be replicated successively during the 5'- to - 3' polymerase activity of the DNA polymerase δ (Pol δ) (Figure 1.3). The lagging strand is simultaneously replicated in an "interrupted" form, each Okazaki fragment is actually shorter than the stretch unwound in the replication fork. The RNA primer for DNA synthesis is constructed by the primase enzyme, followed by a small stretch of DNA synthesised by polymerase α (Pol α). Both enzymatic activities remain inside a single primase-Pol α protein complex. Pol δ or Pol ε (bound to the sliding clamp PCNA, loaded by RFC), and after that take over from Pol α. In lagging strand synthesis, when the replicative polymerase arrives at the completion of a preceding Okazaki fragment, it moves this fragment by continuing DNA synthesis, and a flap structure is produced. The flap structure-specific endonuclease-1 (FEN-1, Rad27 in *S. cerevisiae*) cuts out this structure and the cleaved site is sealed by DNA ligase1 (Cdc9 in *S. cerevisiae*). As the regularity between

the FEN1 and Pol δ is more efficient than regularity between the FEN1 and Pol ε, the Pol δ is thought to function on the lagging strand (Moldovan, Pfander & Jentsch 2007).

In the replication fork, the catenation and positive super coiling ahead are negated by topoisomerases I and II. Cyclin-dependent kinase (Cdk) activity remains high following S phase and during mitosis and to avoid the regeneration of the pre-replicative complex (Lambert, Carr 2005). Termination of Cdk activity allows for the gathering of pre-RC for the following cell cycle (Johnson, O'Donnell 2005).

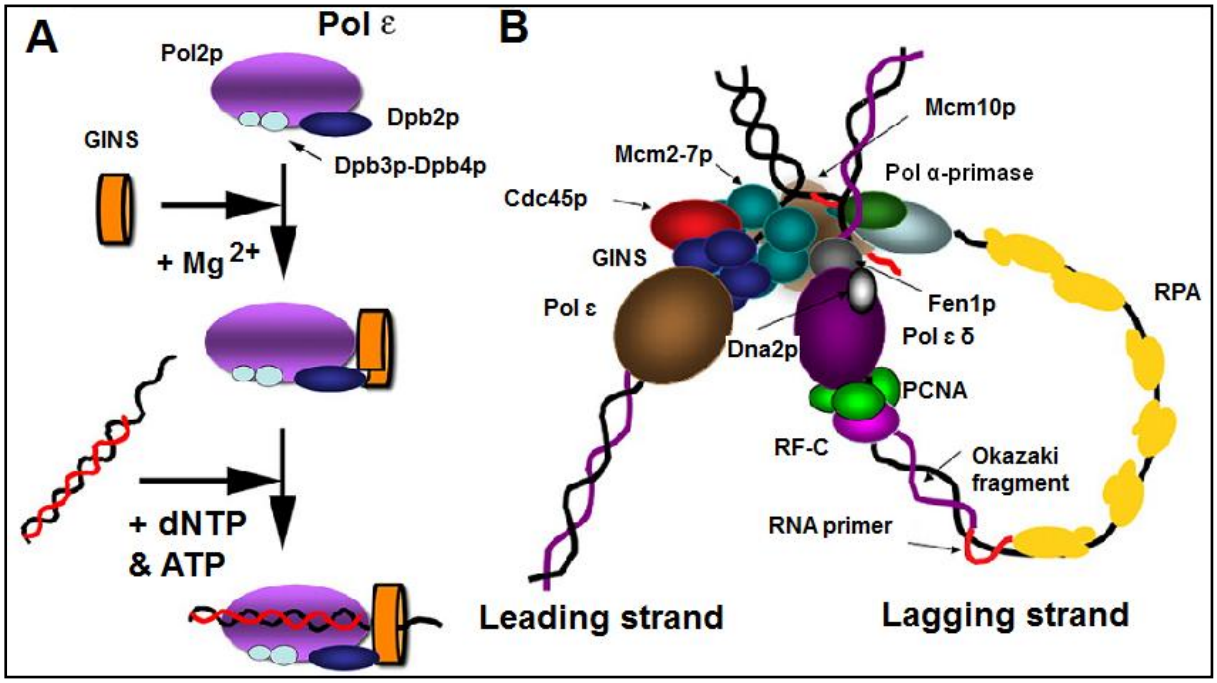



**Figure1.3 suggested structure illustrates the molecular activity at a eurokaryote replication fork.** A, elements of *S. cervisiae* Pol ε holoenzyme, which consists of Pol2, Dpb2, Dpb3, and Dpb4, reacts with a ring-shaped accessory element, GINS, and creates " an open complex" that is then used at Pol ε, which has a potential of both dsDNA- and ssDNA binding (Maki et al. 1998, Tsubota et al. 2003), as a rope to the DNA template, even though Pol ε complex itself may provide direct intraction to primer template DNA (Asturias et al. 2005). The red line shows a primer strand. B, in this structure, hexameric Mcm2-7 surrounded leading strand DNA and Pol ε is on the leading strand together with heterotetrameric GINS and Cdc45. Pol δ is on the leading strand with PCNA, RF-C, Fen1, and Dna2, along with Pol α-primase and RPA bound to the looping single-stranded DNA. Earlier researchs show that Dpb11 and Sld2 are detected at the replication origin, however

they do not travel with the replication fork (Masumoto et al. 2002). The purple curvy line shows newly synthesized DNA, and the red line shows the RNA primer synthesized by Pol α-primase.

### 1.15 Replication fork progression and transcription

Studies, both *in vivo* and *in vitro*, show that RNA polymerase complexes can actually hold back the process of replication forks and that cell viability is endangered as an outcome. The organisation of bacterial genomes showed that there is a bias to co-directional alignment of transcription units with replication, indicating selection against head-on collisions (Blattner et al. 1997). Studies in *E. coli* by (Mirkin, Mirkin 2007b) indicated that collisions in replication and transcription are preventable when both the processes continue in the co-directional orientation. By contrast, head on collisions cause critical inhibitions of the replication fork progression. This is basically collision between the DNA polymerase and RNA polymerase.

Replication fork stalling that requires a new fork to restart and/or activation of checkpoint processes due to the lack of any extrinsic DNA damage is a shared event in both bacteria (Cox et al. 2000) and eukaryotes (Cha, Kleckner 2002a). Replication forks could recommence from endogenous DNA damage such as oxidation or replication barriers. Modification of damaged replication forks is an increasingly important factor for effective chromosomal duplication and the prevention of genetic instability (Calzada et al. 2005a).

### 1.16 Regulation of the repliosome at a paused eukaryotic DNA replication fork.

Replication barriers can cause the repliosome to pause or stall. The S-phase checkpoint responds to replication fork (RF) stalling and to intra-S-phase damage (mainly ssDNA gaps and DNA DSBs), preventing the firing of late replication origins and entry into mitosis. In this function, the checkpoint participates in the preservation of running forks by preventing their collapse. Various elements function at the RF to prevent stalling or fork collapse. In *S. cerevisiae*, these involve the Rrm3 helicase (Ivessa et al. 2003), which is needed for RF stability at natural obstructions. Mrc1, which creates a complex with Tof1 and Csm3 and works in RF preservation jointly with the Sgs1 helicase (Katou et al. 2003a, Calzada et al. 2005b, Aguilera, Gómez-González 2008). The mammalian transducer kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3 related (ATR) are the central factors in starting the S-phase checkpoint response. ATR is stimulated in response to

stalled RFs and distinct kinds of damage that cause the generation of ssDNA, like UV-induced damage or resected DSBs, while ATM responds immediately to the DSBs to which it is enlisted during MRN complex processing (MRE11–RAD50–NBS1). ATR is enlisted by its cofactor ATRIP, which identifies RPA-coated ssDNA. However, it also needs activation at the RAD9–RAD1–HUS1 (9–1–1) replication processivity clamp (PCNA)-like complex, which is stopped by stalled forks by the RAD17 'RFC-like' complex. ATR and ATM kinases phosphorylate, the effector kinases CHK1 and CHK2 stimulating a checkpoint response. In this sequence of events, MCM is phosphorylated, which adds to its role in activating forks; in *S. cerevisiae*, the ATR orthologue Mec1 phosphorylates Sgs1 and Mrc1 obstruct replisome dissociation and collapse (Katou et al. 2003b) (See Figure 1.4).

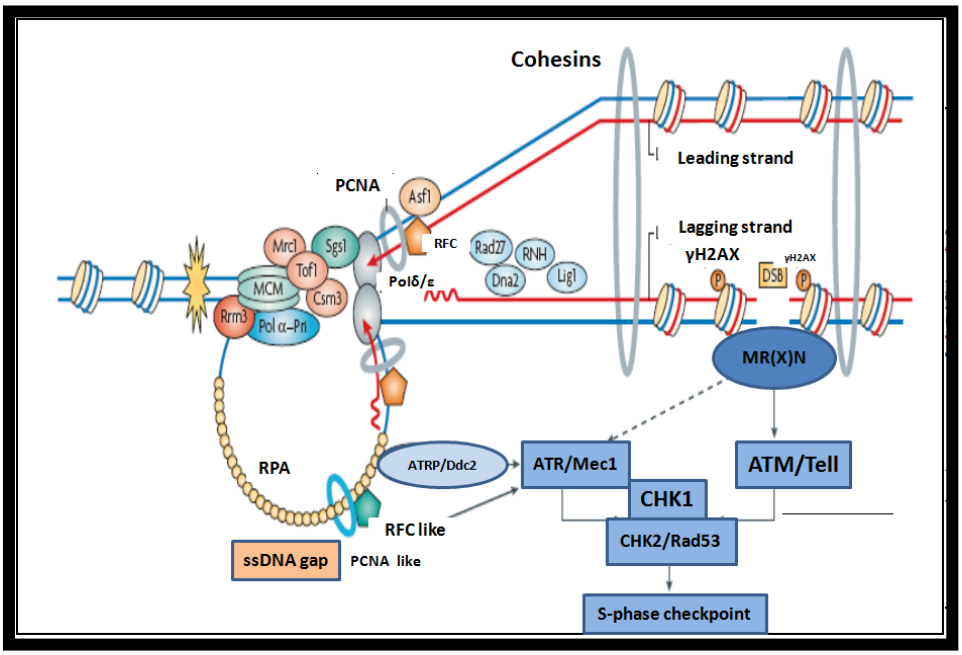



**Figure1.4 Replication fork progression and stalling.** Apart from protein complexes/factors required for DNA replication additional factors are recruited in the RF complex encountering an obstacle. Encountering an obstacle can cause RF stalling, leading to ssDNA gaps and double-stranded breaks (DSBs). Several factors associate with the RF prevent its collapse, including the *S. cerevisiae* Rrm3 helicase, the Mrc1 checkpoint mediator in association with Tof1 and Csm3, or the nucleosome assembly factor Asf1. ssDNA gaps and DSBs are sensed by the S-phase checkpoint which is activated through Tel1 (ATM in humans) and Mec1 (ATR in humans). In the case of a DSB, the checkpoint signaling spreads around the DSB site by histone H2AX phosphorylation (γH2AX) in humans (H2A in yeast). ATRIP/Ddc2, ATR/Mec1 interacting protein; CHK1/Chk1 and CHK2/Rad53, serine/threonine-protein

kinases; MCM, replicative helicase; MR(X)N, a nuclease complex; RPA, replication protein A; Sgs1, ATP-dependant helicase. (see text) Adapted from (Aguilera and Gomez-Gonzales, 2008).

Re-engaging of the RF is mediated by the S-phase checkpoint to avoid unscheduled recombination (Trenz et al. 2006). This was derived from the study of budding yeast S-phase checkpoint mutations (such to RAD53 and SGS1 respectively which result in a fork collapse in the entity of replication inhibitors, and the assembly of Holliday junctions (Cobb et al. 2005, Sogo, Lopes & Foiani 2002, Lambert et al. 2005).

If DSBs are produced at a fork, histone H2AX is phosphorylated (γH2AX) at its C-terminal tail as one of the earlier events at the break site. γH2AX spreads near the break, amplifying the primary damage signal and resulting in large, megabase-long chromatin domains that are suitable for the stable assembly of damage-response and cohesion elements that favour repair by sister-chromatid exchange (Strom et al. 2004). If this whole mechanism is interrupted by replication stress or S-phase checkpoint inactivation, breaks are created that could stimulate genomic instability.

### 1.17 Replication fork pauses / barriers and recombination

Replication arrests are related to genome rearrangements, which are thought to be caused by homologous or non-homologous recombination. Some proteins included in homologous recombination are also capable of changing an arrested replication fork into a recombination intermediate, which promotes replication restart and thus seemingly obstructs genome rearrangements (Michel 2000). Eukaryotic cells control the progression and integrity of DNA replication forks to preserve genomic stability and couple DNA synthesis to other mechanisms.

In eukaryotes, sequence-specific termination appears to be the exception rather than the rule. However, replication fork barriers (RFBs) and replication fork pauses (RFPs) have been located at different genomic sites. RFP sites are described as transiently arresting replication fork movement and RFBs are explained as elements that result in irreversible ‘blocks’ during replication of genomes (Hyrien 2000).

Natural RFBs occur within the rDNA and centromeric areas of different organisms and at other genomic loci like *tRNA* genes (Deshpande, Newlon 1996) and the *RTS1* (Replication

Termination Sequence 1) at the *mat1* locus in *S. pombe* (Vengrova, Codlin & Dalgaard 2002). An RFB has been detected in the 3' end of the rRNA genes in *S. cerevisiae*. This RFB needs the FOB1 gene (for fork blocking) for its activity. The biological roles of the RFB are believed to stop collisions at the replication and transcription machineries (Kobayashi 2003).

**1.18 Replication restarts from pauses.**

Chromosome replication is not a regular and constant process. The repliosome association with replication barriers in various stages relies on the nature of the block (McGlynn 2004, Michel et al. 2001). Initially, the repliosome might remain connected to the fork and simply pause, and then return to the start once the block has been removed (Figure 1.5a). Alternatively, the block might cause the collapse of the RF resulting in the fork being exposed to different processing molecules, like recombinases. The block might be dealt with following repliosome collapse and then the repliosome is re-recognised at the RF and replication proceeds (Figure 1.5i). It is possible that the fork might break, possibly because of a nick in one of the template strands, resulting in the creation of a DSB (Figure 1.5 b). This would need a recombination mechanism to re-establish the replication fork (Figure 1.5 c and d). Another option is that there is replication fork regression following detachment of the repliosome; this will give rise to a structure known as the 'chicken foot' (fork reversal) which had a similar appearance to a Holliday junction (Figure 1.5 e). Such a structure could be dealt with in a number of directions, involving resolution to a DSB (Figure 1.5 g), which would need subsequent recombination to reconstruct the replication fork, or reversion immediately to a running replication fork once the obstruction is detached (Figure 1.5 f).

Another possibility is that the replication structure can simply bypass the block, dependent on the nature of the block and the strand on which it is detected (Figure 1.6). Studies in *S. cerevisiae* showed that elimination of MEC1 (ATR orthologue) causes fork stalling and chromosome breakage by creating a genetically encoded replication slow zone (RSZ) (Cha, Kleckner 2002b), similar to that of mammalian fragile sites, which are precipitated by postponed progression during normally late-replicating areas (Laird et al. 1993, Letessier et al. 2011). These correspond with fragile sites in humans, which come to be more fragile in the absence of ATR. These areas in the chromosomes work to create secondary structures that could supply barriers for the replication fork, causing raised chromosomal rearrangements (Mirkin, Mirkin 2007a).

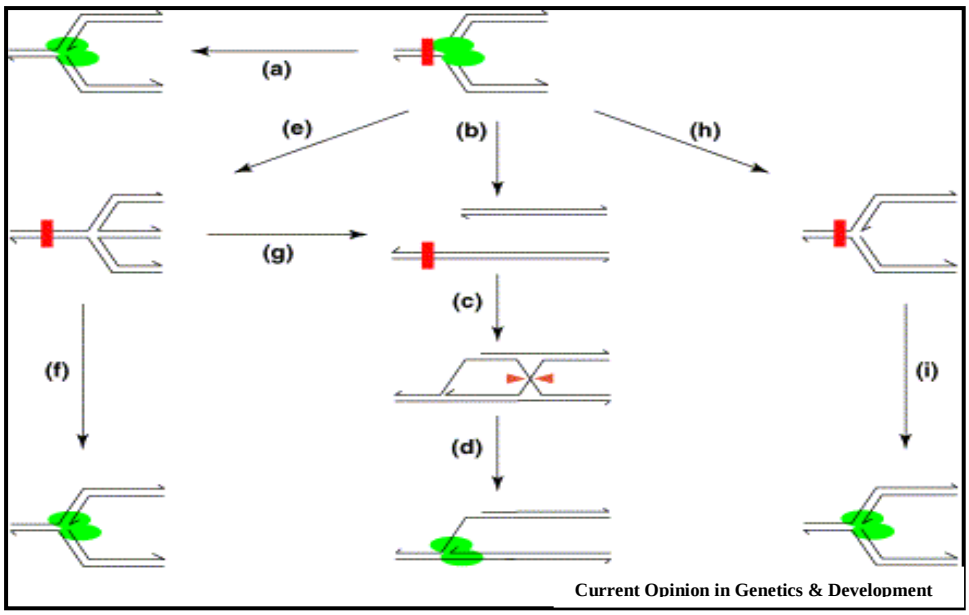



**Figure 1.5 Possible Pathways for dealing with replication blockage (McGlynn 2004).**

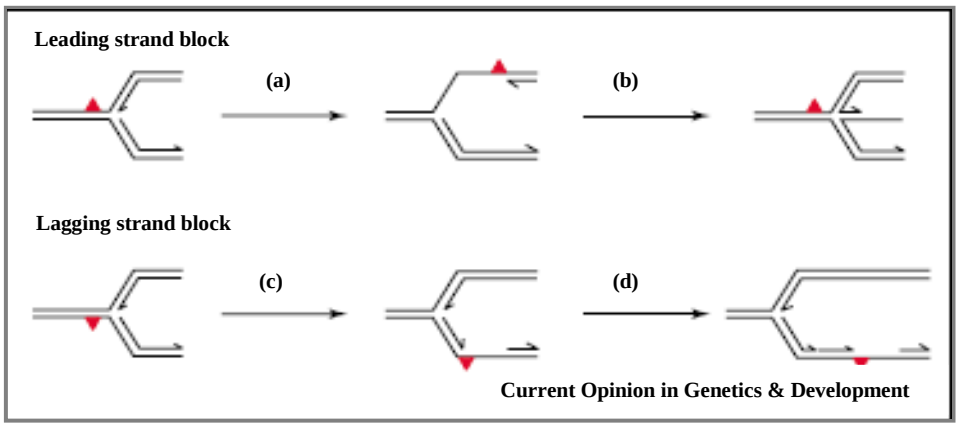



**Figure1.6 The way in which the replisome deals with a block can be dependent upon whether or not there is strand specificity to the block**. (a & b) leading strand block: this mar result in fork regression and the generation of a recombinogenic structure. (c & d) leagging strand block: this might result in block or lesion by pass, resulting in a gap which is repaired post replication.

## 1.19 Cancer stem cells

### 1.19.1 A definition of cancer stem cells

In 1937, Furth and Kahn provided the first determination of the frequency of malignant cells in leukaemia cell lines that could maintain hematopoietic tumours in mice, and the results

suggested that not all of these cells might initiate a tumour. Hence, cancer stem cells (CSC) were first proposed. Many studies in the 1960s and 1970s showed the functional heterogeneity in tumours in that just a tiny subset of tumour cells can re-initiate tumour growth *in vivo*. These studies formally initiated the CSC concept (Bruce, Van Der Gaag 1963, Becker, McCulloch & Till 1963, Buick, Till & McCulloch 1977, Goldberg 2005). Research into leukaemia has provided strong evidence for the existence of a supposed CSC subpopulation or a hierarchical model of cancer (Lapidot et al. 1994a). At the same time, CSCs have been found in various solid tumours including breast cancer, colon cancer, medulloblastoma, liver cancer, glioblastoma, pancreatic cancer, ovarian cancer and melanoma. The observation that leukemic stem cells (SCs) show a similar cell surface phenotype to that of normal hematopoietic SCs (HSCs) suggests that CSCs may initiate from their normal counterpart (Clarke et al. 2006a). In addition, an increasing amount of data indicate that CSCs may also derive from committed progenitors and even differentiated cells (Joseph et al. 2008, Zheng et al. 2008, Zheng et al. 2008). Jointly, CSCs may be obtained from stem cells that have acquired a tumourigenic capability, differentiated cells from committed progenitor cells that have the ability of self-renewal as well as tumourigenic properties. A CSC is a cell within a tumour that acquires the capability to self-renew to give rise to the heterogeneous lineages of cancer cells that constitute the tumour. After this, the reproduced tumours can be serially xenotransplanted (Clarke et al. 2006b, Clarke et al. 2006b).

### 1.19.2 Are stem cells implicated in cancer?

The above definition of CSCs does not indicate the origin of these cells, and these tumour-forming cells could assumedly result from stem, progenitor or differentiated cells (Rapp, Ceteci & Schreck 2008). The CSC hypothesis proposes that the malignancies accompanying the cancer result from a tiny population of stem-like, tumour-initiating cells. Even though cancer studies first isolated CSCs in 1994 (Lapidot et al. 1994b), this idea dates back to the mid-19th century. In 1855, German pathologist Rudolf Virchow suggested that cancers originate from the activation of dormant, embryonic-like cells that exist in mature tissue (Huntly, Gilliland 2005). Moreover, Virchow suggested that cancer does not develop naturally; rather, cancerous cells, like their non-cancerous counterparts, must result from different living cells. Solid support for the CSC hypothesis has come as a result of using cell-surface protein markers to recognise a proportionately rare population of stem-like cells in acute myeloid leukaemia (AML) (Lapidot et al. 1994c). Furthermore, other studies have

demonstrated that leukaemia-initiating cells from various AML subtypes were relatively immature in terms of differentiation (Bonnet, Dick 1997c). The cells were "stem-like" – i.e. more closely linked to primitive blood-forming (hematopoietic) stem cells than to mature, committed blood cells. The identification of leukaemia-inducing cells has led to an attempt to isolate and distinguish CSCs in solid tumours and stem cell-like populations have since been identified by utilising cell-surface protein markers in tumours of the colon (O'Brien et al. 2006a), brain (Singh et al. 2004, Li et al. 2007, Hermann et al. 2007), breast (Al-Hajj et al. 2003b), pancreas and prostate (Collins et al. 2005, Patrawala et al. 2006). However, recognising markers that clearly characterise a population of CSCs remains challenging, even when there is proof that the assumed CSCs can be found in a given solid tumour type. For instance, in hepatocellular carcinoma, cellular analysis has revealed the existence of stem-like cells (Sell, Leffert 2008). Nethertheless, standard markers still need to be recognised to distinguish these assumed CSCs, even though many possible candidates have been suggested (Yang et al. 2008a, Yang et al. 2008b). In some cancers, the CSCs still need to be identified and work is ongoing to associate stem-cell markers with malignant cancer cells. For example, the proteins Nanog, Nucleostemin and Musashi1, which are highly expressed in embryonic stem cells and are necessary for the maintenance of the pluripotency of those cells, are also highly expressed in malignant cervical epithelial cells (Ye et al. 2008). While this result does not demonstrate the presence of cervical cancer CSCs, it does suggest that these proteins may have essential functions in cervical carcinogenesis and progression.

### 1.19.3 Do CSCs originate from stem cells?

Regarding the analogies between stem cells and tumour-initiating cells, researchers have attempted to determine if CSCs arise from progenitor cells, stem cells, or differentiated cells existing in adult human tissue. The issue is currently under discussion (Croker, Allan 2008, Clarke et al. 2006c) (see Fig. 1.7).

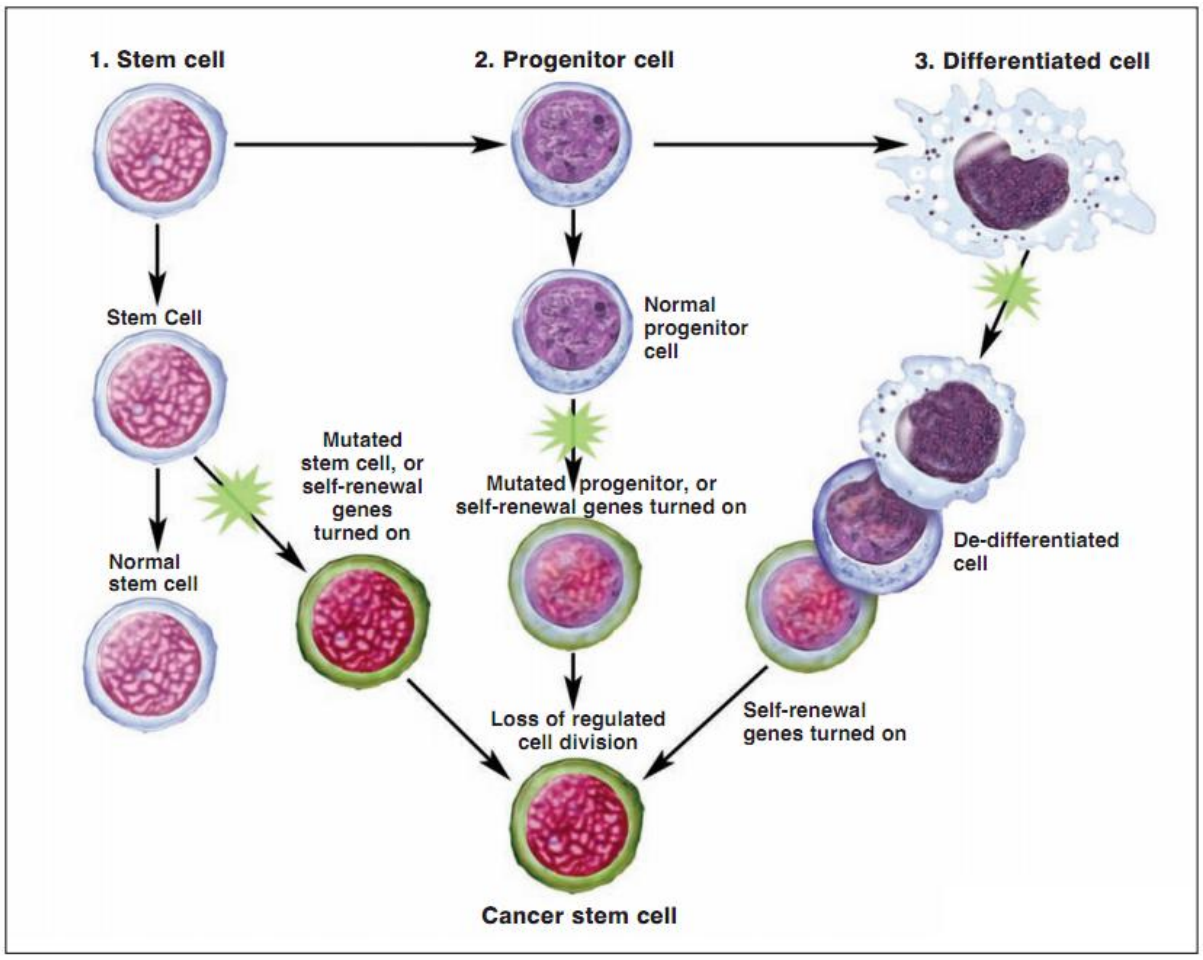



**Figure 1.7 How cancer stem cells may arise.** The processes that maintain "stem-ness" in stem cells are also active in several cancers. This correspondence has guided scientists to suggest that cancers may originate when an event generates a mutation in a stem cell, plundering it of the potential to regulate cell division. This figure illustrates three theories of how a cancer stem cell may appear: (1) a stem cell acquires mutations, (2) a progenitor cell acquires mutations, or (3) a wholly differentiated cell acquires many mutations that transfer it back to a stem-like state. In all three, the nascent or consequent cancer stem cell has lost the ability to control its own cell division.

### 1.19.3.1 Theory #1: Cancer cells originate from stem cells.

Stem cells can be distinguished from other cells by two features: (1) they can divide to generate duplicates of themselves, or self-renew, under a suitable environment, and (2) they are pluripotent, or capable of differentiating into the majority, if not all, mature cell kinds. If the CSCs originate from normal stem cells existing in the adult tissue, de-differentiation would not be essential for tumour formation. In this way, cancer cells could simply use the existing stem-cell regulatory pathways to promote their self-renewal. The capability to self-

renew provides stem cells with a long lifespan comparative to that of mature, differentiated cells (Allan et al. 2007). It has thus been theorised that the restricted lifespan of a mature cell reduces the likelihood that it will live long enough to undergo the many mutations required for tumour creation and metastasis. In support of this theory, research has revealed that CSCs associated with AML have been shown to contain distinct, hierarchically-arranged classes (like those observed in hematopoietic stem cells) that dictate obvious destinies (Hope, Jin & Dick 2004b).

#### 1.19.3.2 Theory #2: Cancer cells originate from progenitor cells.

The differentiation process from a stem cell to a differentiated cell ordinarily includes one or more intermediate cell types. These intermediate cells, which are more plentiful in adult tissue than are stem cells, are called precursor or progenitor cells. They are slightly differentiated cells that exist in foetal and adult tissues and normally divide to generate mature cells. Nonetheless, they maintain a partial ability for self-renewal. This characteristic, when considered along with their plentifulness, in comparison to stem cells in adult tissue, has led some researchers to suppose that progenitor cells could be an origin of CSCs (Li et al. 2006, Kucia, Ratajczak 2006).

#### 1.19.3.3 Theory #3: Cancer cells originate from differentiated cells.

Some studies have proposed that cancer cells could originate from mature, differentiated cells that by some means de-differentiate to become more like stem cells. In this way, the essential oncogenic (cancer causing) genetic or epigenetic changes would be required to lead to the de-differentiation pathway with the subsequent self-renewal of the proliferating cells. This opens up the probability that a relatively large population of cells in the tissue could have tumourigenic potential; however, only a tiny subset of these would really originate the tumour. Specific processes involved in which cells would de-differentiate have not been suggested. However, if a tissue includes an adequate population of differentiated cells, the rules of possibility show that a small proportion of them could, in principle, lead to the series of events necessary for de-differentiation. Furthermore, this series may involve several stages and recent studies have demonstrated that human adult somatic cells can be genetically "re-programmed" into pluripotent human stem cells by applying just four stem-cell factors (Yu et al. 2007a, Takahashi et al. 2007a).

### 1.19.4 Biological characteristics of CSCs

The characteristics of CSCs are: (1) sequent passage, (2) the potential for multilinage differentiation (such that they can recapitulate the multiple tumour cell types found in the parent tumour), (3) the tumourigenic capability or self-renewal, and (4) expression of a singular collection of surface markers that permit their recognition and purification (Clarke et al. 2006d). The CSC hypothesis postulates that in a given tumour, only an obvious phenotypic subset of cells has tumourigenic ability. At present, serial passages in xenotransplantation models offer the best standard assay to illustrate the CSC division (Frank, Schatton & Frank 2010). Normal tissue SCs firmly regulate the balance between self-renewal, proliferation, differentiation and quiescence (Boman, Wicha 2008, Reya et al. 2001c). Furthermore, the number of stem cells, in the context of the stem cell niche, is accurately maintained through the symmetric and asymmetric cell divisions and dysregulation of the self-renewal process occurs as an outcome in an excessive CSC population (Ricci-Vitiani et al. 2006, O'Brien et al. 2006b). This may result from an increase in symmetric divisions of the CSCs and may offer a possible drug target (Boman et al. 2007, Pece et al. 2010). As has been described in the hematopoietic process, the cells in solid organs develop to demonstrate a hierarchy in which stem cells lead to committed progenitor cells that result in rapidly proliferating cells. When ultimately become terminally differentiated cells. As SCs mature from self-renewaing stem cells to terminally differentiated cells, they gradually lose their ability for self-renewal and pluripotency; however, they show mitotic activity.

### 1.19.5 OCT4

OCT4 is a POU family transcription factor. It is known for its role in maintaining the pluripotency and self-renewal of embryonic stem cells (ESCs) and in producing induced pluripotent stem cells (iPSCs) as a fundamental reprogramming factor to date (Raymond, Ayala & Knuutila 2002). The expression and role of OCT4 in human tumours have been examined. OCT4 is putatively expressed in and correlated with germ cell tumours with pluripotent potential (Longhi et al. 2006a, Wittig et al. 2002a, Kubista et al. 2011, Mohseny et al. 2009). Many studies have also demonstrated OCT4 expression in adult stem cells and somatic cancers (Tang et al. 2008, Hogendoorn et al. 2003, Cleton-Jansen et al. 2009, Fuchs, Pritchard 2002, Sadikovic et al. 2010, Won, Kim & Park 2010, Ta et al. 2009, Ek, Dass & Choong 2006). OCT4-positive cells recognised in cancers may distinctly act as cancer stem cells (CSCs) (Clarke et al. 2006e, Ischenko et al. 2008, Koch, Krause & Baumann 2010), and

OCT4 expression is essential for maintaining the survival and self-renewal characteristic of cancer stem-like cells (Lapidot et al. 1994d, Bonnet, Dick 1997d). However, there is extensive evidence indicating that OCT4 is not expressed in tumour cell lines and somatic tumours (Longhi et al. 2006b, Wittig et al. 2002b, Tang, Ang & Pervaiz 2007, Iwasaki, Suda 2009, Bae et al. 2010). Expression of Oct4 in embryonic stem cells (ES)cells has been found to be associated with a poor prognosis (Chen et al. 2008, Zhang et al. 2010b, Karoubi et al. 2009). Further, deletion or knock down of Oct4 might cause apoptosis of a CSC-like population of lung cancer cells (Hu et al. 2008). Oct-4 has been identified to play an important role in cell viability, functioning as a stem cell survival factor, and causes induction of pluripotency in somatic cells (Ben-Porath et al. 2008). It also has a major function in maintaining self-renewal and the CSC-like, radio, and chemo-resistant characteristics of CD133+ NSCLC cells (Jeter et al. 2009). In squamous-cell carcinoma of the esophagus, Oct-4 expression is high in side population (SP) in contrast to non-SP cells (Klarmann et al. 2009). Oct4 is considered a hallmark of CSCs. A recent study has proven that cervical cancers comprise a sub-population of stem-like cancer cells containing Oct4 protein, indicating that Oct4 may be correlated with the initiation of cervical carcinogenesis (Feng et al. 2009). The expression of the Oct4 gene in different types of human cancer (Monk, Holding 2001a, Jin et al. 1999), a study illustrated function for Oct4 in adult stem cells (Tai et al. 2005), and the expansion of epithelial progenitor cells (Tai et al. 2005) sustain the hypothesis that cancer is a disease of stem cells. This hypothesis supposes that cancers appear in stem cells or early committed progenitors (Sell, Pierce 1994a) because of their incapability to differentiate in a controlled pattern. Oct4 clearly controls the transcription of genes, such as Trp53, Brca1, Parp1, and Bmi1, which serve a primary function in cells' tendency to sustain transformation, apoptosis, senescence, and differentiation.

ESCs are characterised by two features: pluripotency and self-renewal capability. Lately, the ectopic expression of the of transcription factors Oct4, Nanog, Sox2,c-Myc, Esrrb, and Klf4 has been induced to re-program human and mouse fibroblasts into a pluripotent stage. (Kaji et al. 2009, Okita, Ichisaka & Yamanaka 2007, Takahashi et al. 2007b, Takahashi, Yamanaka 2006, Woltjen et al. 2009, Yu et al. 2007b). Induced pluripotent stem cells (iPSCs) are very analogous to ESCs, and they keep the capacity for self-renew and differentiate into all three germ layers. Therefore, iPscs have great therapeutic potential in regenerative medicine (Amabile, Meissner 2009, Maherali et al. 2007, Wernig et al. 2007). The Wnt pathway is

implicated in stem cell maintenance (Anton, Kestler & Kühl 2007, Sato et al. 2003), perhaps by regulating the levels of pluripotency factors Nanog, Oct4, and Sox2 (Kalmar et al. 2009).

### 1.19.6 The role of niches in normal and cancer stem cells

Recent studies have generated an obvious notion of the tumour progression scheme for cancer growth, which is established on the cancer stem cell postulate (Fig. 1.8, (Dick 2008).Cancer tissues display the following characteristics: (1) self-sufficiency for growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) tissue invasion and metastasis, (5) sustained angiogenesis, and (6) limitless capability to duplicate (Hanahan et al., 2000). Since the majority of these characteristics involve normal stem cell features, it is thought that tumour tissue, and some normal tissue, are constantly repopulated from pools of self-renewing stem-like cells, called CSCs in the case of tumours (Table 1). CSCs are proposed exit as a small population at the top of the hierarchy in tumours, and they retain stem-like properties such as the ability for self-renewal and expression of stem cell-related genes/markers. Moreover, they are involved in tumour initiation.

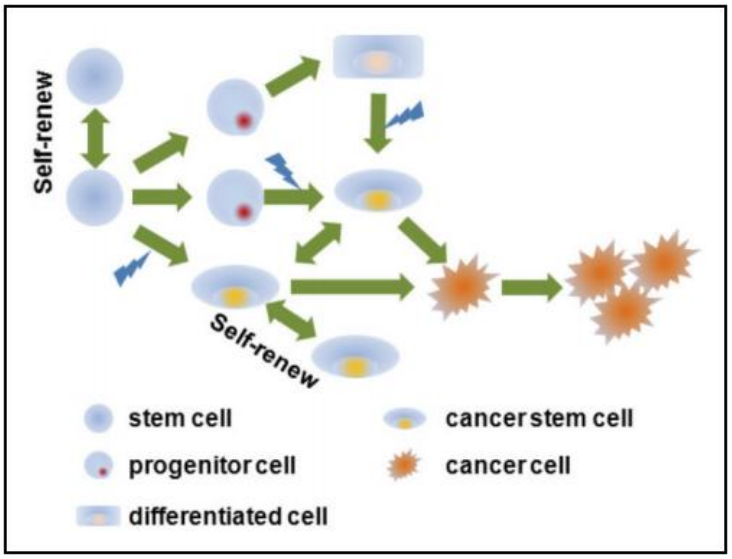



**Figure1.8 Hypothesis for the progression of cancer stem cells**. Stem cell and/or progenitor cells with the assembled genetic change indicate a sustained or resumed self-renewal ability, and at the definite stage, these cells, known as 'cancer stem cells' lead to more differentiation. However, not fully developed cancer cells with aggressive proliferating

**Table 1 Similarities and differences between cancers stem cell and normal somatic cells**

| **Cancer stem cell** | **Somatic stem cell** |
|---|---|
| Source of cellular lineage | Source of cellular lineage |
| Cancer initiation<br>metastasis and relapse | Organ generation<br>tissue regeneration |
| Self-renew<br>(Pluripotency) | Self-renew<br>(Pluripotency) |
| Tumour creation | Tissue reconstruction |

In normal adult tissues, stem cells rely on the combination of both cell-intrinsic and cell-extrinsic elements for appropriate, homeostatic tissue maintenance. It is likely that there is a functional microenvironment that assists cancer stem cells, a counterpart of normal stem cells and their niches. The participation of various elements has been demonstrated in the fundamental interaction between cancer stem cells and their microenvironment. Furthermore, studies have also shown that CD44 is essential for the homing and engraftment of the cancer stem cells to the niche in acute myeloid leukaemia and chronic myeloid leukaemia (Jin et al. 2006, Krause et al. 2006). Surprisingly, the molecular processes of leukaemia cell homing to the niche are similar to those of the interaction between normal hematopoietic stem cells and their vascular niches. CD133-positive brain tumour cells, that include CSCs, selectively attach to the endothelial cells that may form a vascular niche (Calabrese et al. 2007a). CD133-positive cells have been widely studied in pancreatic, colon and prostate cancers, and future research will show the fundamental molecular processes in the correlation between CD133-positve cancer stem cells and their niches.

Several cellular components have also been indicated as the CSC niche and have specific signal transduction pathways. For instance, endothelial cells in the vasculature of the brain maintain neural stem cell properties, in part through Notch signalling (Shen et al. 2004), and this is the same for brain cancer stem cells and their vascular niche (Hovinga et al. 2010). Another extensively studied example is the mesenchymal stromal cells in the cancer stem cell niche in the intestine. In the normal intestine, stem cells reside in a stem cell niche composed of epithelial cells and mesenchymal cells of the myofibroblast lineage that line the crypt. Currently, the Wnt signalling cascade is considered to be a prominent force in controlling cell

proliferation, differentiation and apoptosis to maintain the stem cell fate at this region (Clevers 2006). As the counterpart of the normal intestinal stem cell niche, tumour-associated myofibroblasts and mesenchymal stem cells are indicated as the main factors in colon cancer stroma (Elliott, Blobe 2005). Colon cancer stem cells have exhibited high Wnt activity, which is orchestrated by myofibroblasts remaining in the tumour stroma during processes in the hepatocellular growth element (Vermeulen et al. 2010).

These data suppose a strong connection between CSCs and their microenvironment and between normal stem cells and their niches, thus indicating the existence of a CSCs niche. Even so, a unlike normal stem cell niche, the conduct of CSCs might be regulated by the niche at various levels (Vermeulen et al. 2010). Under a normal environment, the stem cell niche usually represses stem cells from both differentiation and proliferation, and a transient proliferating signal is needed to activate tissue reproduction. On the contrary, in tumours, CSCs may be self-sufficient enough to undergo uncontrolled proliferation because of their internal mutations and/or alterations in the niche signals. This supports the hypothesis that the cancer stem cell niche is an environment that gives dominant signals in tumour cell proliferation and growth as well as more support compared to the normal stem cell niche.

### 1.20 The origin of niches for cancer stem cells and cancer cells

In normal cells the microenvironment or niche where the cells remain is significant for maintaining the stemness, for differentiation and for the regulation of proliferation. The analogies between CSCs and normal stem cells (as indicated in Table I) could help develop the interesting theory of the stem cell niche to the presence of CSCs niche. Various studies have demonstrated that specialised CSCs niches may be involved in tumour progression (Calabrese et al. 2007b, Gilbertson, Rich 2007), and they are to have a significant function in practically every aspect of the tumourigenic cascade, including the metastatic mechanism and drug resistance. Generally, metastasis takes place in an organ-selective manner as illustrated by the ‘seed and soil’ hypothesis, which shows that the localised microenvironment of particular organs appears to be more responsive to specific tumour cells than different organs. Thus, distributed tumour cells need to encounter an appropriate microenvironment in order to initiate and maintain a secondary tumour. The metastatic mechanism is very complex and inefficient and involves multi-steps such as circulation, intravasation, arrest, angiogenesis, extravasation and migration (Pawelek, Chakraborty 2008). Furthermore, tumour stromal cells

comprised of MSCs might be implicated in various phases of the metastatic process accompanied by the interaction of CSCs (Figure 1.9).

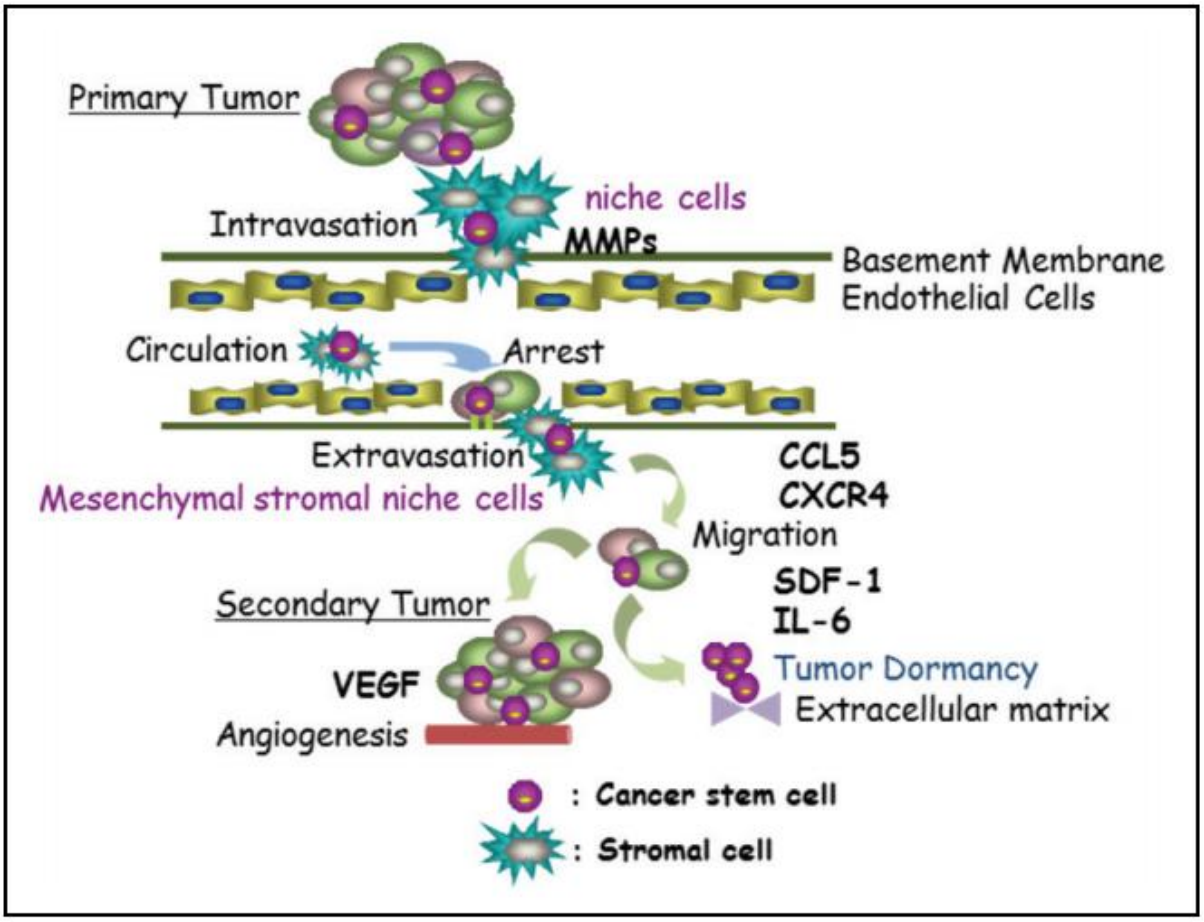



**Figure 1.9 Potential implications of tumour stromal cells involving MSCs/TAFs in a clustering metastatic model.** Metastatic tumour cells may comprise cancer stem cells, completing a very complex metastatic mechanism that collaborates with tumour stromal cells. Soluble factors such as MMPs, CXCR4, CCL5, IL-6, SDF-1 and VEGF secreted from MSCs/TAFs may be involved in the metastatic mechanism (Honoki, Fujii & Tsujiuchi).

Mesenchyaml stem cells have the ability to increase the growth and metastasis of particular tumours such as colon cancer (Shinagawa et al. 2010), and have been suggested to assist the progress of tumour-associated fibroblasts as well as promote tumour progression. The metastatic potential of osteosarcoma cells and breast cancer cells has also been indicated to be strongly increased when co-injected with MSCs during the paracrine signalling processes (Shinagawa et al. 2010). In this context, MSC-derived CCL5 seems to be a fundamental factor in increasing the growth and invasiveness of tumour cells. In addition, IL-6 from

MSCs is implicated in the growth promotion effect for osteosarcoma cells (Bian et al. 2010). MSCs also generate chemoattractant proteins such as MCP-1 and SDF-1 that attract spreading tumour cells such as B leukaemia cells and breast cancer cells (Burger, Kipps 2002, Burger, Kipps 2002).

The conversion of MSCs to tumor-associated-fibroblast (TAFs) (has been indicated to participate in tumour growth during fibrovascular network expansion and the generation of tumour-stimulating paracrine elements (Burger, Kipps 2002). Moreover, stimulated fibroblasts have also been found in liver metastasis, where they promote tumour outgrowth (Olaso et al. 1997). Fibroblast activation is apparently implicated in the priming of the premetastatic niche with fibronectin deposits (Kaplan et al. 2005), which pull tumour cells to sites for metastasis. Furthermore, the infiltration of activated fibroblasts or myofibroblasts precedes the recruitment of vascular endothelial cells in the hypoxic avascular metastatic environment, and they generate VEGF to promote transition and angiogenesis in a vascular step (Olaso et al. 2003). Furthermore, in paracrine signalling, MSCs preserve tumour cells against apoptosis and promote original tumour cell proliferation principally at direct cell – cell contact interactions (Roorda et al. 2010). All of this suggests that MSCs are implicated in the metastatic mechanism as the origin of the niche for metastatic tumour cells. In addition to the effect on tumour progression, tumour stromal cells may also contribute to drug resistance through complex mechanisms such as the direct cell contact, the interaction of extracellular matrices (ECM) and soluble factors (Nefedova, Landowski & Dalton 2003). Soluble factors that mediate drug resistance are produced by a dynamic interaction between tumour cells and stromal cells.

### 1.21 Similarities between CSCs and embryonic stem cells

Cancer cells share some features with embryonic stem cells (ESCs) such as self-renewal, uncertain proliferation, differentiation and migration (Pathak and Multani 2006). Together, CSCs and ESCs have capacity to “undergo rapid clonal proliferation”. Moreover, CSCs and ESCs contribute seven out of the nine signalling pathways associated with embryonic development and cancer. These are: the NOTCH signalling pathway, JAK/STAT pathway, the PI3K/AKT pathway, the MAP-Kinase/ERK pathway, the NFkB pathway, the TGFβ pathways and the Wnt pathway (Dreesen and Brivanlou 2007).

In addition, hESCs express a set of epitopes such as stage-specific embryonic antigens (SSEA-3 and SSEA-4 but not SSEA-1 unlike mESCs) and alkaline phosphatase (Adewumi et al. 2007; Stephenson et al. 2010; Takahashi et al. 2007). On inspection of 40 tumour forms with their normal tissue counterparts, Schoenhals and colleagues found that about half of the tumour types showed over expression of pluripotency factor(s) compared to the normal tissues. This elevated expression was linked to tumour progression and poor prognosis (Schoenhals et al. 2009).

Understanding the processes behind self-renewal and resistance of CSCs to therapeutics would result in a better comprehension of tumours, thereby increasing the possibility of creating efficient anti-cancer medications by targeting these CSCs (Ebben et al. 2010; T. Lin et al. 2009). The pathways implicated in self-renewal contain: Wnt, Notch, Hedgehog (Hh), transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), phosphatase and tensin homolog (PTEN) and BMI-1, a polycomb group member. Although these processes are used by both normal stem cells and CSCs, some compounds have been shown to target CSCs without affecting normal stem cells (e.g., parthenolide and rapamycin). This indicates that these processes may occur differently in CSCs and normal stem cells.

### 1.21.1 Classes of stem cell type

To date, four classes of stem cells have been identified: ESCs, induced pluripotent stem cells (iPSCs) (both pluripotent with the ability to produce all types of human cells), adult stem cells and chord blood/placental stem cells (both multipotent). (Dreesen and Brivanlou 2007; Takahashi and Yamanaka 2006; Takahashi et al. 2007). ESCs exhibit a distinct cellular morphology; that is, they are typically small cells with a high nuclear to cytoplasmic ratio and they grow in colonies (Thomson et al. 1998).

### 1.21.2 DNA repair in ESCs

The DNA inside a cell is continuously susceptible to damage. The cell manages this situation by employing different processes to repair the damage. This is essential as unrepaired DNA will instigate cell cycle arrest or apoptosis, and in cells with abnormal repair mechanisms, mutations will appear, go unchecked and may finally lead to cancers.

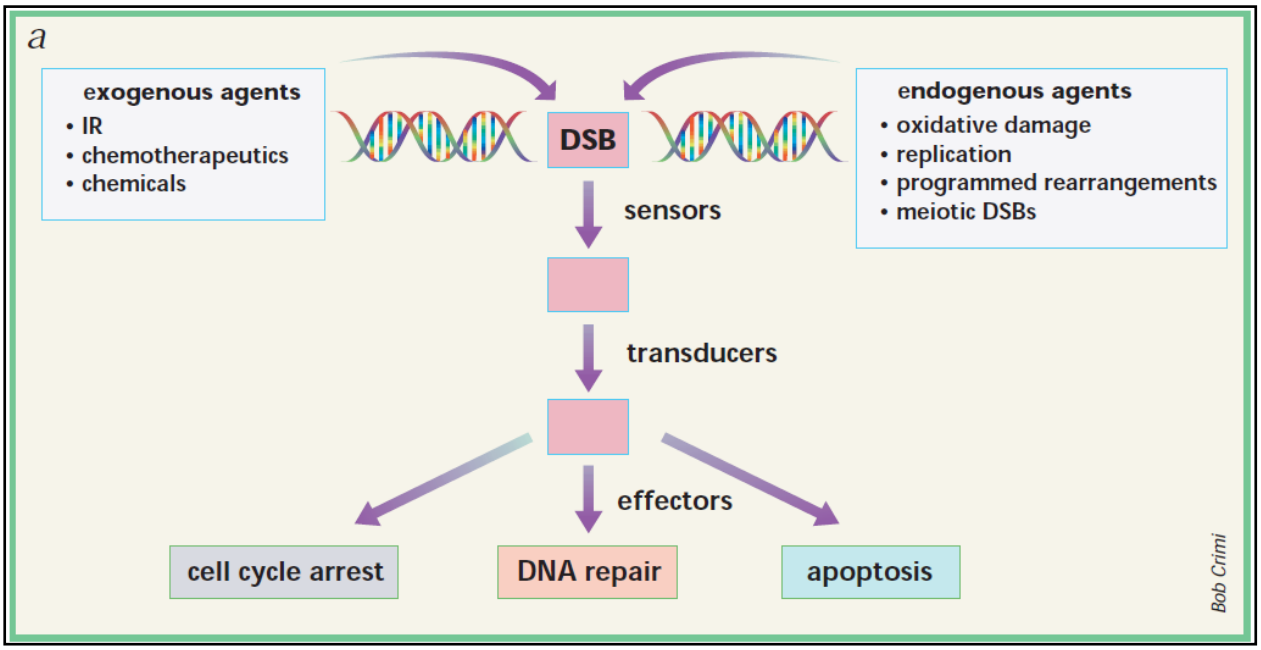



**Figure 1.10 Signalling in double strand breaks.** Organization of the DNA-damage response pathway. The presence of DSBs is recognised by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade, to activate signalling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable"(Khanna and Jackson 2001).

The outcomes of mutations in ESCs could be destructive to the body; therefore, it is necessary to have an efficient repair system to guarantee the maintenance of genomic integrity. While mutations in differentiated cells can give rise to a number of somatic diseases, mutations in ESCs can be disastrous, affecting many cell types, also passing them to progeny. The mutation frequencies measured in embryonic stem cells are indicated to be considerably lower than that of somatic cells. Except for X linked genes, the majority of the mutations in ESCs cause loss of heterozygosity (LOH). However, ESCs can adopt some processes to maintain genomic integrity such as suppression of mutagenesis, apoptosis or differentiation. In addition, genes associated with DNA repair, such as Msh2 and Xrcc1, have

been found to be highexpressed in ESCs (Park and Gerson 2005; Tichy and Stambrook 2008).

MESCs are found to have a p53-independent programme, i.e. they do not experience cell cycle arrest in response to DNA damage, in spite of the high expression of p53 (Savatier et al. 2002). However, the levels of apoptosis frequency among mESCs are much higher than that of differentiated cells. This guarantees that no DNA-damaged cells are supplied to the developing organism (Aladjem et al. 1998; Cervantes et al. 2002; Frosina 2010; Hong et al. 2007; Park and Gerson 2005; Serrano et al. 2010).

The most harmful DNA lesion to the cell is a double strand-break (DSB) (Tichy and Stambrook 2008). DSBs can result from free radicals that appear during metabolism, ionizing radiation, stalled replication forks and also through meiosis (Park and Gerson 2005). In ESCs, replicating chromatin is especially susceptible to strand breaks (Banath et al. 2009).

To date, the principal DNA repair mechanism recognised in stem cells comprises nucleotide excision repair (NER), mismatch repair (MMR) (BER), base excision repair, homologous recombination (HRR) and non-homologous end joining (NHEJ). In nucleotide excision repair, oligonucleotide pieces following irregular bases are removed and inappropriate or damaged bases are removed by base excision repair. In addition, the mismatch repair system recognises particular single mismatches or misaligned nucleotides (Park and Gerson 2005).

Homologous recombination mediated repair (HRR) and NHEJ are the chief mechanisms involved in DSB repair (Lieber et al. 2003). In HRR, the RAD52 protein family has a major role and this repair pathway is error free, taking place during the late S to G2 phases, when sister chromatids are present to function as a template. Furthermore, NHEJ is active in the G1 and early S phases when sister chromatids are missing (Morrison et al. 2000; Takata et al. 1998). Conflicting reports are available concerning the percentage of each pathway that is used by cells for DNA DSBs repair. However, most studies report a major role for the HRR pathway in ESCs ranging from 75-81%, and some studies have claimed that 92% of DNA DSBs were repaired by NEHJ in ESCs (Francis and Richardson 2007; Pierce et al. 1999). Since ESCs spend about 75% of their cell cycle in the S phase, this may indicate that these cells would favour HRR rather than NHEJ for DSB repair (Savatier et al. 2002). In addition, studies on the RAD51 protein, which plays a major role in HRR, have shown that its levels in ESCs is 20-fold higher than that in mouse embryonic fibroblasts (MEFs), where NHEJ is the

preferred mechanism for repair. Indeed, NHEJ deficiency in mice causes an increased susceptibility to IR exposure. However, ESCs are capable of repairing DSBs, caused by IR exposure, much faster than MEFs (Tichy and Stambrook 2008).

HESCs are more efficient in repairing DNA than human primary fibroblasts (Maynard et al. 2008), and through a series of hESC differentiation, the frequencies of DNA damage have been shown to increase and expression of DNA repair proteins to decline (Saretzki et al. 2008). Although homologous recombination is used to repair DSBs, it can also generate deletions and rearrangements of chromosomes. Furthermore, defects in proteins implicated in HRR may cause acute radiation sensitivity and cancer (Park and Gerson 2005).

### 1.22 Differentiation Therapy

The enhanced understanding of the molecular, genetic, and cellular characteristics of carcinogenesis over the last few years has resulted in various recent targets for intervention. One novel suggestion or method is differentiation therapy. Differentiation requires planned changes in gene expression directed at limiting the expression of a different repertory of genes in pluripotential cells to those needed to achieve the specialised phenotype in unipotent cells in a precise tissue. Cellular differentiation often results in the formation of non-dividing cells (terminally differentiated cells). This is the outcome of the modulation of genes implicated in the regulation of senescence, cell proliferation, and programmed cell death (apoptosis). The treatment of cancers may be possible by inducing the differentiation of stem cells, that is, via differentiation therapy (Pierce 1983) if the malignant cells of cancers are CSCs (Sell, Pierce 1994b, Reya et al. 2001d, Pardal, Clarke & Morrison 2003b, Bonnet, Dick 1997e, Singh et al. 2003b). The tumour stem cells of teratocarcinomas can be influenced by the environment of the maturing embryo to differentiate into normal adult tissues. If tumour cells can be compelled to differentiate and to stop proliferation, then their malignant potential will be regulated and controlled. Normal terminal differentiation always results in non-proliferating cells that eventually undergo apoptosis as they complete their normal lifespan. Therefore, an improvement in planning to activate the normal mechanisms of differentiation in pre-malignant and malignant cells is reasonable by utilising physiological or pharmacological agents that can avoid the epigenetic and genetic abnormalities repealing differentiation. This method, called differentiation therapy, can be used to stop, suppress, or reverse the malignant phenotype by inducing differentiation with the connected growth arrest,

apoptosis, and senescence (Figures 1.11a, b and c). Differentiation therapy has limitations, but it can alter malignant tumours into benign tumours. Here, data on chosen differentiation-inducing agents and their mechanisms of action are examined. A class of compounds, both natural and synthetic, is used to induce differentiation *in vitro* in different cell lines, including CSCs or stem cell-like termed NTERA2 cells, colorectal cells HCT116, and mouse embryonic cells. Some of these agents are illustrated as follows: (see page 38).

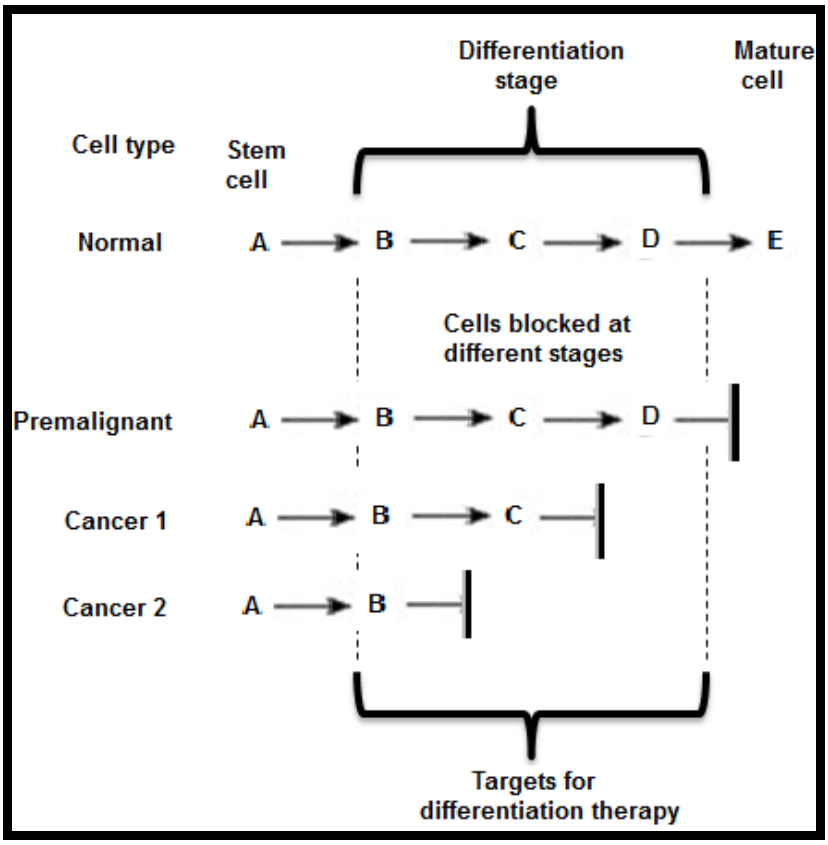



**Figure 1.11 a. Targets for differentiation therapy.** In a normal differentiation mechanism, a supposed stem cell indicated as A is needed to sustain the steps of progressive alterations in gene expression and the consequent phenotypic changes, symbolised by letters B, C, and D, before proceeding to the terminally differentiated cell stage, indicated as E. Cancer maturation is correlated with aberrant differentiation. This process is represented by a barrier in differentiation that can be formed before the development of premalignant cells. The design displays three possible blockages in late steps of the differentiation mechanisms (e.g., in stage D) and in early steps of the mechanism (e.g., at step B) (Lotan et al. 1990).

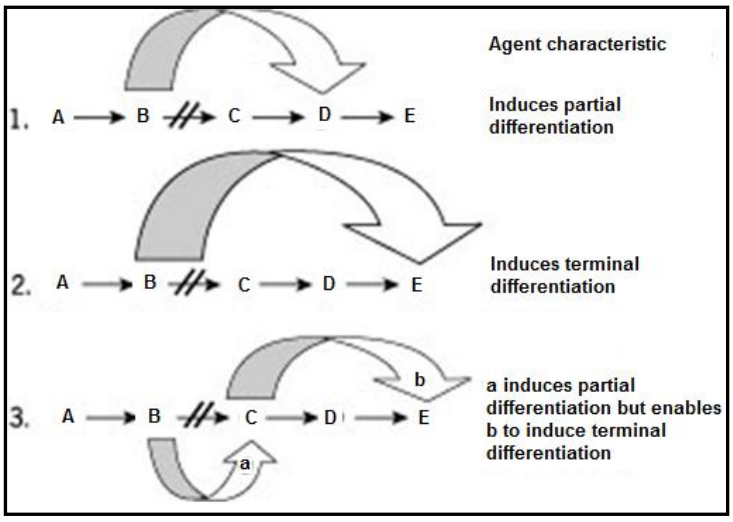



**Figure 1.11 b. Features of agents used to induce differentiation in malignant cells**. In Scheme 1, an agent can induce partial differentiation of cells obstructed in Step B by inducing them to sustain alterations to Step D. In Scheme 2, the agent can induce the cells obstructed in Step B to sustain full differentiation to the developed cell E. In Scheme 3, agent a can induce partial differentiation (from B to C), but at the same time, it makes the resultant cell C capable of responding to agent b which can complete the differentiation of cell C to the developed cell E. The latter example can serve as one of the rationales for the combination of agents for differentiation therapy. The designation of letters A–E is the same as in Figure 1 (Lotan et al. 1990).

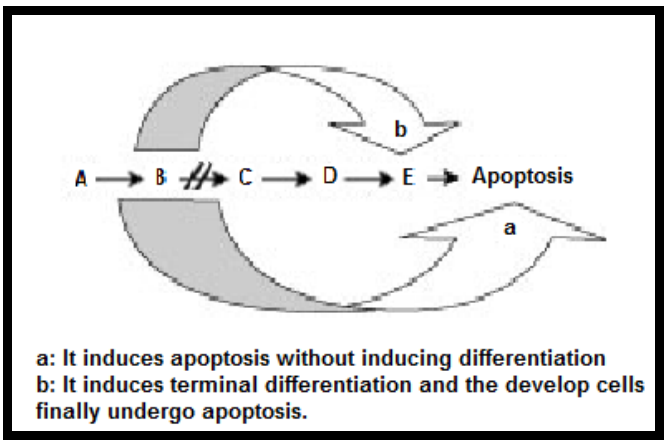



**Figure 1.11 c. Inter-relations between differentiation and apoptosis**. Several cell types sustain apoptosis as a normal result of terminal differentiation, so the treatment of cancer cells in Step B with agent b induces the cells in Step B to sustain differentiation to the

developed cell E and finally, to apoptosis. This scheme is contrary to that for an agent like a, which can induce apoptosis without inducing differentiation (Lotan et al. 1990).

### 1.23 N, N-Hexamethylenebisacetamide (HMBA)

HMBA is a hybrid polar compound that can induce the differentiation of both murine erythroleukaemia cells and several cell lines *in vitro* and in patients (Marks et al. 1995). Detailed studies using murine erythroleukaemia cells have described the early and late effects of HMBA (reviewed in Marks et al. 1995). The induction of differentiation by HMBA entails treatment at concentrations of 3–10 mmol L−1 for 3–15 days. Various studies have been performed with human cancer cells. HMBA induces the differentiation of human teratocarcinoma, glioma cells, and bladder carcinoma. Further, HMBA suppresses cell growth and reduces the tumourigenicity of the cells. The effects of HMBA were correlated with the expression and suppression of growth factors, such as transforming growth factor alpha, autocrine factor teratocarcinoma-derived growth factor-1, and a keratinocyte growth factor associated with the epidermal growth factor. HMBA induction, which results in a termination of proliferation, is partly mediated by the increased expression of cyclin-dependent kinase inhibitor p27, enhanced association of p27 with cyclin E/cyclin-dependent kinase 2 (CDK2 complex), and suppression of kinase activity associated with cyclin E/CDK2. The formation of E2F complexes with pRB and the protein p130 has been associated with in growth inhibition. Other effects of HMBA include enhancement of gap-junctional intercellular communication and induction of cell death, both of which can lead to the repression of growth.

### 1.24 Retinoic Acid (RA)

The all-trans RA (ATRA) is a metabolic compound derived from vitamin A and is extensively associated with neurogenesis (Lotan et al. 1990). Through embryogenesis, RA participates in the patterning of the neural plate and neural tube (del Corral, Storey 2004, Maden et al. 1996, Wilson et al. 2004). Retinoic acid plays a role in conservation of motor neurons (Novitch et al. 2003), and mammalian nerve regeneration (Zhelyaznik et al. 2003). RA is actually the most commonly used morphogen to generate *in vitro* neural progenitor cells and neurons from stem cells (Kim et al. 2009, Soprano, Teets & Soprano 2007, Martins et al. 2005, Hirami et al. 2009). It is one of the most important differentiation inducers. It can induce the differentiation and apoptosis of a number of tumour cells, including glioma cells (Haque, Banik & Ray 2007). ATRA represses growth and induces differentiation, so it has

therapeutic potential in cancer medication, particularly in combination with other therapeutic agents (Witcher et al. 2003). The combination of ATRA and paclitaxel-induced differentiation and apoptosis in human glioblastoma U87MG xenografts has been indicated (Karmakar, Banik & Ray 2008). However, the molecular processes of glioma cell differentiation induced by ATRA have not been completely elucidated. Retinoids, like RA, have been clinically used to treat many forms of cancer, but only a very small number of patients respond to them (for reviews, (Mongan, Gudas 2007, Freemantle, Spinella & Dmitrovsky 2003). This limitation is attributed to the effect of retinoid resistance, which refers to the deficiency in tumour cell response to the same pharmacological dosages of retinoids; normal cells respond by proliferation arrest or differentiation. Therefore, the problem of retinoid resistance remains to be overcome in cancer treatment.

New and favourable results can be found in experimental animals (Zhang et al. 2010a) and in human clinical trials utilising retinoids in combination with other medicines. For instance, vitamin A and TRAIL jointly generate apoptosis just in intestinal polyps and not in the normal intestinal mucosa in the ApcMin mouse model of intestinal carcinogenesis (Zhang et al. 2010a). Therefore, the description of the molecular processes by which retinoids act provides a significant precedent. Further, there is a need to learn more about how transcriptional mechanisms in undifferentiated versus differentiated cells are regulated by bioactive retinoids like RA in order to understand complex mechanisms, such as pattern creation in progression, cell differentiation, progression of cells to malignancy, and the steps of CSCs in driving tumourigenesis.

### 1.25 Mechanism of Activity of CPT: Poisoning of Topoisomesase I

Topoisomerases are ubiquitous enzymes implicated in topological troubles generated by many nuclear activities, such as DNA replication, chromatin assembly, transcription, repair, recombination, and chromosome segregation (Lorence, Nessler 2004). These enzymes exist in all living cells, including yeast, viruses, archebacteria, plants, flies, and humans (Wang 1996). Topoisomerases control DNA supercoiling and accomplish DNA decatenation using a complex exchange of DNA cleavage, rejoining, and manipulation reactions (Keck, Berger 1999). There are two major classes of topoisomerases: topoisomerase I (Topo 1) and topoisomerase II (Topo 2). Each topoisomerase type can be further divided into two sub-forms, A and B, which are unconnected in sequence and in construction (Keck, Berger 1999).

Topo 1 stimulates alterations in the linking number of DNA (i.e., the number of times one strand of DNA crosses the other) by one per cycle of the process through breakage, resealing of the phosphodiester bonds strands of DNA, and alteration in the linking number of DNA by two (Wang 1996). In all topoisomerase forms, a conserved tyrosine in the catalytic side acts to cleave DNA, forming a transient, covalent phosphotyrosyl intermediate (Keck, Berger 1999). Topo 1-mediated reaction can be classified into four stages (Lorence, Nessler 2004, Rothenberg 1997a, Rasheed, Rubin 2003, Rasheed, Rubin 2003, Pommier 2006) as depicted in Figure 1.12. Camptothecin increases DNA damage by regulating a normally transient covalent complex between DNA and Topo 1 (Hsiang et al. 1985). Camptothecin links very feebly only to normal B-DNA under a physiological environment and it does not independently link to Topo 1 (Lorence, Nessler 2004). Cross-linking studies have proposed that CPT acts with the Topo 1-DNA complex, thereby creating a ternary complex that stabilises the trans-esterification intermediate (Hertzberg et al. 1990, Pommier et al. 1995). Therefore, by regulation of the cleavable complex, CPT converts the normally useful enzyme Topo 1 into an intracellular, cytotoxic poison. As a result, CPT has structural similarities with topoisomerase inhibitors or topoisomerase poisons (Lorence, Nessler 2004).

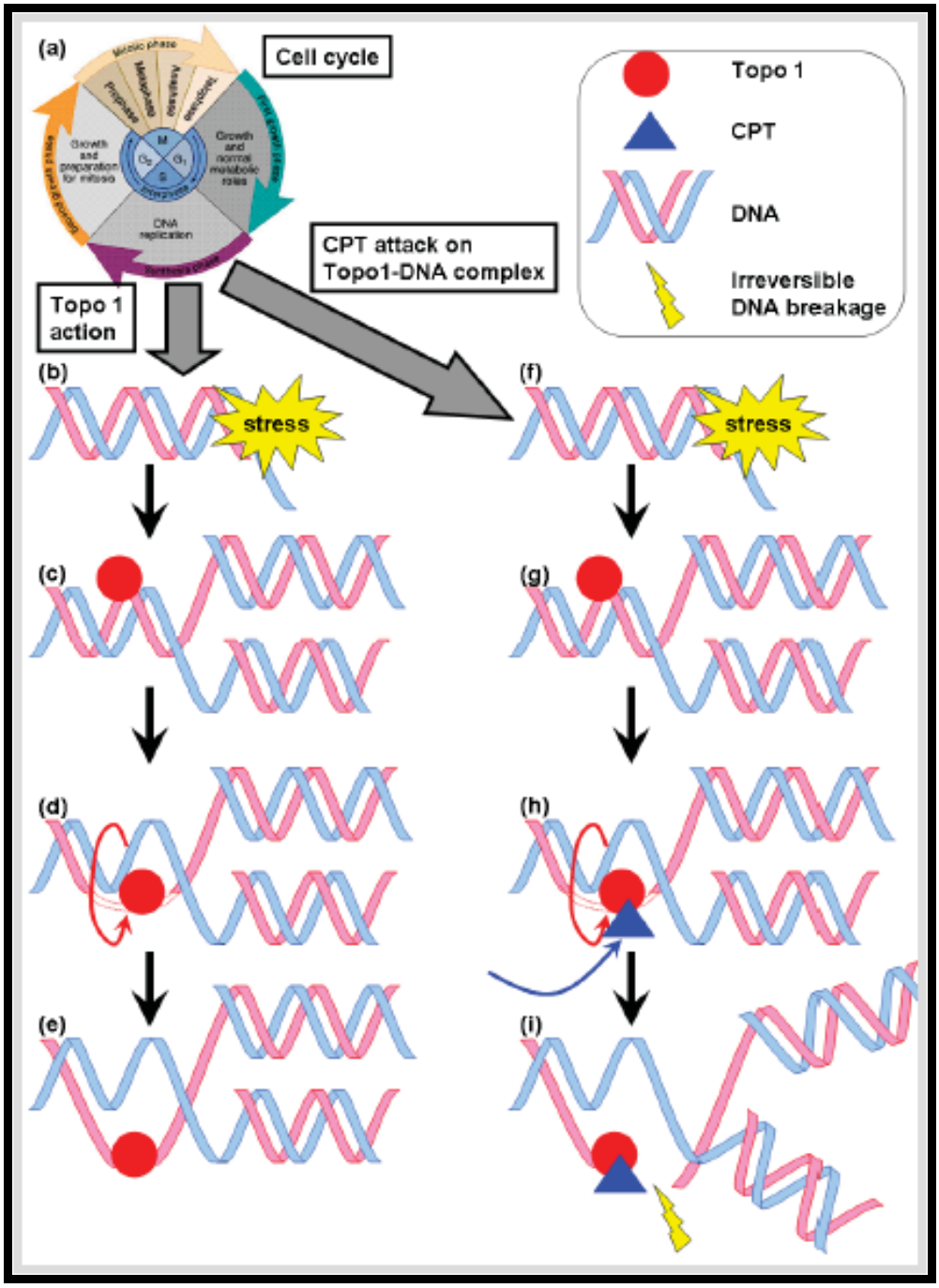



**Figure 1.12 Pathway of activity of Topo 1 and pathway of CPT attack on the Topo 1-DNA complex**. (a) The cell cycle; all conditions illustrated in parts (b-i) occur in the S-phase. (b,f) Increase in tension and supercoiling of DNA. (c,g) Topo 1 links to one DNA strand and incises it (cleavage reaction). (d) The undamaged DNA passes in the nick, resulting in the relaxation of the torsional strain. (e) Topo 1 reseals the cleaved DNA strand (re-ligation step). (h) Interaction of CPT with the Topo 1-DNA complex, creating a ternary complex that regulates the trans-esterification intermediate. (i) Irreversible breakage of DNA (Kusari 2010).

### 1.26 Aphidicolin

Aphidicolin, a tetracyclic diterpenoid acquired from *Cephalosporium aphidicola* (Brundret et al. 1972), represses the growth of cultured human cells (Bucknall et al. 1973a, Pedrali-Noy, Spadari 1979) and also growth herpes vaccinia and simplex viruses (Bucknall et al. 1973b). DNA replication stress (stalled DNA replication forks) frequently causes DNA damage. Aphidicolin (Aph), is an inhibitor of a-like DNA polymerases, has also been shown to increase DNA damage (Brox, Hunting & Belch 1984), most likely via degenerating replication forks. Aph-induced DNA damage may result in the formation of DSBs (Liu, Kuo & Melendy 2003). Aph is commonly used to synchronise cells in the cell cycle (Tobey, Oishi & Crissman 1990, Gong, Traganos & Darzynkiewicz 1995). H2AX phosphorylation in cells subjected to replication stress by hydroxyurea or aphidicolin appears to be mediated not by ATM (Kurose et al. 2006a) but by ATR (Ward, Minn & Chen 2004). Aphidicolin induces further delay in the progress of replication, which leads to the unreplicated in DNA (Hellman et al. 2002, Wang et al. 1999, Hellman et al. 2000). AT-rich dinucleotide flexibility islands represent one of the presumed inherent features of common aphidicolin-inducible fragile sites. These sites might contribute to replication upset either because of their high DNA flexibility (Chen, Rau & Charney 1985) or of their possible capability to form DNA secondary structures that can disturb the progression of the replication fork (Zlotorynski et al. 2003, LaDuca et al. 1983).

### 1.27 Hydroxyurea

Hydroxyurea leads to the exhaustion of deoxynucleotide triphosphates (dNTPs) in cells (Bianchi et al. 1986; Koc et al. 2004; Matsumoto et al. 1990). It is an inhibitor of ribonucleotide reductase, which is required for de-oxyribonucleotide synthesis, dNTPs are required by DNA polymereases as the building materials for DNA synthesis. With reduced levels of dNTPs, the DNA polymerase constituent of replication fork pauses which can result in fork collapse (Feng et al. 2006, Mirkin, Mirkin 2007c).

To arrest replication fork stalling, the cell may adapt to or overcome HU inhibition. It may also be released from stalling when HU is removed from the environment, and the dNTP supply is replenished in the cell (Kurose et al. 2006b, Lopes et al. 2001, Mulder, Winkler & Timmers 2005). Regardless of how long the replication fork is captured to make the necessary repairs, RFs must be capable of resuming so that the cell cycle can terminate. As a

further complication, the cells through the stalled RFs have an increased danger for DNA damage, resulting in alteration or cell death (Bernstein et al. 2009, Bryant et al. 2009, Froget et al. 2008, Kai et al. 2005, Mao, Kojic & Holloman 2009, Noguchi et al. 2003, Petermann et al. 2010c). Significantly, a short-duration (2–4 h) use with HU has been shown to cause global replication stalling. However, once the drug is removed, the stalled forks are reactivated in an XRCC3/RAD51-dependent manner (Petermann, Helleday 2010). By contrast, a lengthy treatment with the same drug results in the collapse of the stalled replication forks. As a result, homologous recombination repair is required to restore the integrity of the genome, whereas de novo activations of fresh replication origins are implicated in the re-commencement of replication. Therefore, prolonged treatment with hyrdoxyurea leads to increased events of cell death (Petermann et al. 2010a). The lengthy HU treatment results in the collapse of the activated replication forks, and replication is re-started with the activation of new origins (Petermann et al. 2010b).

**1.28 Phleomycin**

Phleomycin is a copper-containing protein, acquired from the culture medium of *Streptomyces verticillus* (Maeda et al. 1956a). In bacteria, it is a specific inhibitor of DNA synthesis (Falaschi, Kornberg 1964) and plays the role of an anti-tumour agent (Bradner, Pindell 1962a). These mechanisms supposedly take place via a direct influence on DNA, although the exact pathway has not been confirmed. Phleomycin has been recognised to link to DNA and to lead to the *in vitro* repression of DNA polymerase I of *Escherichia coli* (Falaschi and Kornberg 1964). Further, chromosome breaks have been discovered in human lymphocytes cultured in the existing drug (Jacobs, Neu & Gardner 1969). The biological properties of phleomycin are common. Phleomycin inhibits the growth of bean rust (Smale, Montgillion & Pridham 1961) and a diverse number of animal tumours (Shooter 1963, Bradner, Pindell 1962b). It also causes phage growth in lysogenic bacteria (Lein, Heinemann & Gourevitch 1962)and in Gram-positive and Gram-negative bacteria ( Maeda et al. 1956b, Tanaka, Yamaguchi & Umezawa 1963).

In *T. brucei,* RAD51 can be discovered in distinct foci in the parasite cell nucleus after phleomycin treatment (Proudfoot, McCulloch 2005, Hartley, McCulloch 2008) or is implicated in the production of a site-specific DSB by the I-*Sce*I meganuclease (Glover, McCulloch & Horn 2008). The creation or stabilisation of RAD51 foci additionally relies on

a number of elements in the eukaryotes, of which BRCA2, RAD51-3, and RAD51-5 have been shown to play a role in *T. brucei* following phleomycin treatment (Proudfoot, McCulloch 2005, Hartley, McCulloch 2008). These finding suggest phleomycin generates chromosomal strand breaks which require HR for their repair.

## Chapter 2 Materials and Methods

### 2.1 *S. pombe* strains, media and plasmids

*S. pombe* strains were grown and stored as described by Moreno et al. (1991). Media used is listed below. The *S. pombe* strains employed in this study are listed in Table 2.1. Plasmids employed in this study are pSRS5 (Pryce et al., 2009) and p*ade6-469* (Szankasi et al.,1988). General molecular biology techniques used are as described by Maniatis et al. (1982).

### 2.2 *S. pombe* Meiotic crosses protocol

Cultures of appropritate *S. pombe* strains were grown in yeast extract liquid (YE; see 2.8) supplemented with adenine (200 mg/l) to a density of approximately $2x10^7$ cells/ml. Equal volumes of each culture were mixed in microfuge tubes, pulse centrifuged and aspirated. Cell pellets were washed with 1 ml of $dH_2O$ and finally resuspended in 20 µl $dH_2O$.

Suspensions were spotted onto fully supplemented synthetic sporulation medium (SPA; see 2.8) and incubated at the required temperature for 3-4 days (4-5 days for room temperature crosses). After incubation, sporulating cells were scraped into a microfuge tube containing 1 ml of a 0.6% β-glucuronidase® (Sigma) in $dH_2O$ and incubated for 16 hrs at 25°C. After incubation, spores were harvested and resuspended in 30% ethanol and incubated at room temperature for not longer than 5 minutes. Spore suspensions were then centrifuged, aspirated dry and spore pellets were resuspended in 1 ml $dH_2O$.

### 2.3 Fluctuation analysis for calculation of mitotic recombination frequency

Cells were inoculated into appropriate liquid medium. The inoculated culture was incubated overnight at 30°C in a rotary incubator. A serial dilution of the culture was made and plated onto appropriate agar plates (selection was maintained for all cultures where strains contained plasmid). Plates were incubated for a period of no more than two days at 30°C until microcolonies could be observed. Single colonies were picked from the agar plate using a sterile Pasteur pipette and inoculated into 5 mls of appropriate liquid medium (selection was maintained when necessary) containing supplementary adenine (200 µg/ml) to avoid selection of recombinants. Cultures were incubated at 30°C in a rotary incubator until the culture was saturated (approximately 3 days). Serial dilutions were made and aliquots plated out onto selective NBA (no adenine) to quantify recombinants and NBA with supplementary

adenine (200 µg/ml; to count viable cells). For each strain a minimum of seven cultures per experiment were used and the median recombination frequency (adenine prototrophs / viable cell) was calculated and mean values of three independent median values were subjected to Student's *t*- test.

### 2.4 Construction of plasmid pSRS5 for plasmid by chromosome recombination assay (fluctuation analysis)

The plasmid based recombination assay, was carried out employing the plasmid pSRS5 which contains a mutated *ade6* gene. The *ade6* gene was mutated by deletion of one nucleotide 'G' at 1483 bp of the *ade6* gene (taking the A of the ATG start codon as position 1) (Pryce et al., 2009).

### 2.5 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was acquired employing the method as described in the GenEludeTM HP Plasmid Miniprep Kit. *E. coli* DH5∝ from the -70°C freezer stock was streaked on Luria Bertani (LB) media (see 2.8) with the appropriate antibiotic and grown overnight at 37°C. A single colony from this culture was inoculated into a 5 ml LB broth with the appropriate antibiotic and was incubated overnight at 37°C in an orbital incubator. The cells were collected by centrifugation at 3,000 g for 1 minute and resuspended in 200 µl of Resuspension Solution containing RNase A. Cells were then lysed with 200 µl of the lysis buffer. The cell remains were precipitated with 350 µl of Neutralization / Binding Buffer and parted by centrifugation at 3,000 g for 1 minute. The cleaning buffer was supplemented to the supernatant and centrifuged. Ultimately, the plasmid DNA was eluded out with 50 µl Elute solution utilizing the column supplied with the kit.

### 2.6 Preparation of *S. pombe* chromosomal DNA

5 ml cultures were grown to saturation in YEL+ adenine (200 µg/ml) then harvested by centrifugation at 5000 r.p.m. for 1 minute in a bench top Microfuge. Cell pellets were then transferred to screw-cap microcentrifuge tubes and washed with 1 ml of $ddH_2O$, re-centrifuged, and aspirated to an approximately 0.5 ml of $ddH_2O$. To each sample 0.2 ml of lysis buffere (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM

EDTA) was added and followed 0.2 ml of phenol:chloroform:isoamyl alcohol (25 : 24 : 1) and 0.3 g of acid-washed glass beads. Tubes were then vortex for 3 - 4 minutes and then spun in a bench top Microfuge for 5 minutes at 15,000 r.p.m.. The aqueous layer was then transferred to fresh 1.5 ml microfuge tubes and 1 ml of 100% ethanol was added. Tubes were then mixed by inversion, and spun for 2 mins at 15,000 r.p.m.. Pellets were then aspirated and resuspended in 0.05 ml of TE (pH 7.6-8.0) and stored at 4°C until use.

### 2.7 *S. pombe* transformation via electroporation

200 ml cell cultures of *S. pombe* cells were grown to a density of 1 x $10^7$ $ml^{-1}$ ($OD_{595}$ = 0.5) in supplemented nitrogen base (NB) medium. 50 ml samples where then chilled on ice for 20 mins before being harvested by centrifugation at 3000 *r.p.m.*. for 5 mins at room temperature. Each sample was then washed three times in ice-cold 1 M sorbitol before being resuspended in ice-cold 1 M sorbitol to a density of 1 x $10^9$ cells/ml. Samples were then mixed in pre-chilled Eppendorf tubes containing 1 µg DNA in 3 µl of TE buffer (pH 7.6) then immediately electroporated in pre-chilled Equibio ECU-102® cuvettes using settings of 2.25 kV, 201 Ohm, 25 µF. Immediately after pulsing 1 ml of ice-cold 1 M sorbitol was added to the cuvette, and the sample transferred to a pre-chilled Eppendorf tube. Samples where then washed and resuspended in 1 ml of ice-cold 1M sorbitol and 50 µl aliquots were spread onto EMM2 plates with or without required supplements and incubated for 6 days at 30°C.

### 2.8 Media, chemical, reagents

Reagents for yeast and bacterial media and supplements were obtained from Difco (Becton Dickinson) and Sigma. All other shared laboratory reagents were acquired from Sigma or BDH unless determined otherwise stated. Enzymes and buffers were from New England Biolabs (NEB), unless otherwise stated. When minimal media was used appropriate amino acid supplements were added to a final concentration of 200 mg/l. For liquid media the agar was omitted.

| **SPA** | **/500 ml** |
|---|---|
| Glucose | 5 g |
| $KH_2PO_4$ | 0.5 g |
| Agar | 15 g |
| Vitamins (x1000) | 0.5 ml |

1 ml 1000 x Vitamins in every 500 ml, Added after autoclaving media

| **NITROGEN BASE NB** | **/1 litre** |
|---|---|
| NB | 1.7 g |
| $(NH_4)2SO_4$ | 5 g |
| Glucose | 5 g |
| Agar | 10 g |

| **YE** | **/1 litre** |
|---|---|
| Yeast extract | 5 g |
| Glucose | 30 g |
| Agar | 14 g |

| **EMM2** | **/1 litre** |
|---|---|
| potassium hydrogen phtalate | 3 g |
| $Na_2HPO_4$ | 2.2 g |
| $NH_4Cl$ | 5 g |
| Glucose | 20 g |
| Vitamins ( x1000) | 1 ml |
| Minerals (x10,000) | 0.1 ml |
| Salts (x50) | 20 ml |
| Agar | 14 g |

| **SALTS x50** | **/500ml** |
|---|---|
| $MgCl_2.6H_2O$ | 26.25 g |
| $CaCl_2.2H_2O$ | 0.3675 g |
| KCl | 25 g |
| $Na_2SO_4$ | 1 g |

| **MINERALS x10,000** | **/500ml** |
|---|---|
| Boric acid | 2.5 g |
| $MnSO_4$ | 2 g |
| $ZnSO_4.7H_2O$ | 2 g |
| $FeCl_2.6H_2O$ | 1 g |
| KI | 0.5 g |
| Molybdic acid | 0.2 g |
| $CuSO_4$ | 0.2 g |
| Citric acid | 5 g |

| **VITAMINS x1000** | **/1 litre** |
|---|---|
| NB | 1.7 g |
| $(NH_4)_2SO_4$ | 5 g |
| Glucose | 5 g |
| Agar | 10 g |

After autoclaving add a few drops of

1:1:2chlorobenzene/dichlorethane/chlorobutane

**Table 2.1 The *S. pombe* strains used in this project**

| Strain Number | Genotype | Source |
|---|---|---|
| BP1 | *h- 972* (Wild type) | McFarlane collection |
| BP8 | *h+ 972* (Wild type) | McFarlane collection |
| BP88 | *h- leu1-32 ura-D18* | McFarlane collection |
| BP89 | *h+ leu1-32 ura4-D18* | McFarlane collection |
| BP90 | *h- ade6-M26 ura4-D18 leu1-32* | McFarlane Collection |
| BP1004 | *ade6-210 ura4-D18 swi1Δ::ura4*$^{+}$ | This study |
| BP1518 | *h- ade6-M26 ura4-D18 pade-6(469)* | This study |
| BP1519 | *h- ade6-M375 ura4- D18 pade-6(469)* | This study |
| BP1757 | *h- ura4-D18 leu1-32 swi1Δ::ura4*$^{+}$ | This study |
| BP1758 | *h+ ura-D18 leu1-32 swi1Δ::ura4*$^{+}$ | This study |
| BP1836 | *h+ swi1Δ**::**ura4*$^{+}$ *ade6-M26 leu1-32 ura4-D18* | This study |
| BP1837 | *h- swi1Δ**::**ura4*$^{+}$ *ade6-M26 leu1-3 ura4-D18* | This study |
| BP1905 | *h- swi1Δ **::** ura4*$^{+}$ *ade6-M26 leu1-32 ura4 -D18* | This study |
| BP1906 | *h- ade6-M26 ura4-D18 leu1-32* (pSRS5) | This study |
| BP1956 | *h- ade6-M375 ura4-D18 leu1-32 swi1Δ**::**ura4*$^{+}$ (pSRS5) | This study |
| BP58 | *h- ade6-M26 pat1-114* | This study |

### 2.9 Meiotic induction of *pat1-114*

Strain was streaked onto fresh YEA plate and incubated at 25°C for 4-5 days. Single colonies were taken to inoculate 5 ml YEL, incubated shaking AT 25°C to a concentration of 5-6 $\times 10^6$ cells/ml. The cultured was washed extensively in equal volume of MM-N (1% glucose; 40 mg/l leucine), and then resuspended in MM-N (1% glucose; 40 mg/l leucine) to a density of $4\times10^6$ cells/ml. The cell density is expected to be approximately $8\times10^6$ cells/ml after 6 hrs and 30 mins, with most cells expected to have arrested at G1. Equal volume of fresh MM-N (1% glucose; 40 mg/l leucine) pre-warmed to 34°C, and culture was transferred to shake at 34°C to induce meiosis. This was recorded as time-point 0 hrs.

### 2.10 Interpretation of 2D gel images

The results from the prepared 2D gel image are read. The interpretation of the image is as illustrated in Figure 2.2.

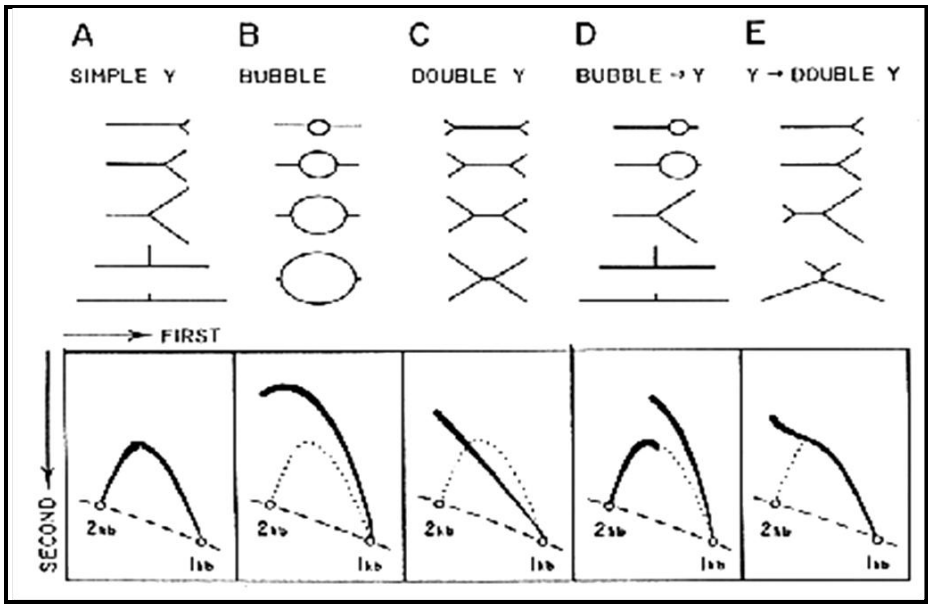



**Figure 2.1 Interpretation of two dimensional agarose gel electrophoresis (2D gel) analysis**. First dimension separation (arrow from left to right) of the replication intermediates is by size and second dimension separation (arrow from top to bottom on the left side of the image) by shape. The pre-digested DNA with restriction endonuleases incisions the replication bubble, causing replication intermediates (RIs) of various forms. These look as simple 'Y' (A), bubble (B), Double 'Y' (C), bubble or 'Y' (D) and 'Y' to double 'Y'.

A spot would be caused on the Y arc when a similar size replication intermediate assembles at particular part of the replication fork; this shows a replication fork is being blocked from progression.

### 2.10.1 Two- dimensional gel electrophoretic analysis of DNA replication intermediates

*S. pombe* cells were grown to a cell count of 1 x $10^7$ cells / ml in 400 ml of yeast extract liquid, then decanted into pre-chilled centrifuge tubes. Sodium azide was added to a final concentration of 0.1% to stop metabolic growth. Ice flakes were added and the culture was chilled on ice. The cells were harvested at 3,000 *g* for 10 minutes at 4ºC. The supernatant was removed and the cells were washed once with ice cold $dH_2O$ and finally harvested at 3,000 *g* for 10 minutes. The cell pellet was resuspended in 2 ml NIB buffer [nuclear isolation buffer; 50 mM MOPS (pH 7.2), 150 mM KAc, 2 mM $MgCl_2$] and transferred to a cold Falcon tube containing 2-3 ml of acid washed glass beads. Cells were then vortexed for 30 seconds followed by incubation on ice for 30 seconds; this was repeated 15 times. The lysate was removed and transferred to ice cold Oakridge tubes. The beads were washed with three volumes of ice cold NIB buffer and the wash was pooled with the lysate. The lysate was centrifuged at 13,000 r.p.m. for 30 minutes at 4ºC. The supernatant was removed and the pellet was resuspended in 4 ml TEN buffer (5 mM TRIS, 50 mM EDTA, 100 mM NaCL: pH 8.0) containing 1.5% sodium sarksyl and 0.5 mg/ml proteinase K. Samples were then gently mixed and incubated at 42ºC for a minimum of 2 hours. Following incubation the samples were centrifuged at 5,000 r.p.m. for 5 minutes at 4ºC and the supernatant was transferred to a 15 ml Falcon tube containing 4.2 g cesium chloride. Samples were then loaded into Quick-Seal centrifuge tubes (Beckman) and Hoescht dye (5 µl of a 5 mg/ml stock) was added. The sample was then centrifuged at 50,000 *g* for 18 hours at 20ºC. DNA was visualized with a long wave ultra violet light trans illuminator and the middle band was collected using a syringe and washed five times with equal volumes 5:1 isopropanol:$dH_2O$. DNA was precipitated using two volumes of ice cold 70% ethanol, spooled out and washed three times with 3 ml 70% ice cold ethanol. The DNA was air dried and resuspended in 400 µl TE buffer (pH 8.0) at 4ºC. DNA was digested over night with appropriate restriction enzymes followed by purification with phenol/chloroform and ethanol precipitation. The DNA was finally resuspended in 200 µl NET buffer (10 mM TRIS, 1 mM EDTA, 1 M NaCl, pH 8.0). To isolate replicative intermediates the DNA was enriched for structures containing single-stranded DNA using BND cellulose. 2 ml BND cellulose solution was added to a 3 ml syringe plugged with glass wool. The liquid was allowed to drip through and the column was washed with 2 ml NET buffer. The DNA solution was loaded and fractions were collected and the column was washed twice with 1 ml NET buffer. The DNA was eluted with two 750 µl volumes of 1.8% caffeine in NET buffer, collected and centrifuged at 10,000 r.p.m. for 10 minutes to remove residual BND. The supernatant was transferred to a fresh tube and precipitated with ethanol and finally resuspended in 15 µl TBE. Samples were run in the first dimension in a TBE gel of 0.5% agarose for 16 hours at 20 V. Slices of this gel containing the DNA were then run in the second dimension in a TBE gel of 1.2% agarose containing 0.5 µg/ml ethidium bromide at 200 V. DNA was blotted onto a Gene Screen™ membrane (NEN Life Science Products) as described in the

manufacturer's guide. Membranes were probed with an appropriate probe labeled with $P^{32}$-γATP using the Mega Prime Labeling System™ (Amersham Biosciences).

## 2.11 Origin of cell lines

**Table 2.2 Origin of cell lines**

| Name of cell line | Type of cell line | Source |
|---|---|---|
| NTERA2 (NT2) | Testicular germ cell tumor cancer | P. W. Andrew (University of Sheffield) |
| HCT116 | Colon Cancer cells | European Collection of Cell Cultures (ECACC) |
| E14 | Mouse Embryonic stem cells | European Collection of Cell Cultures (ECACC) |

### 2.11.1 NTERA2 cell culture

The NTERA2 cells were a kind gift from Dr. Peter W. Andrews (University of Sheffield, UK.). They were seeded ($5 \times 10^6$ $cm^2$ flask) in high glucose Dulbecco's modified Eagle's medium 1X (DMEM + GlutaMAX™-I) (Invitrogen, GIBCO 61965) supplemented with 10% foetal bovine serum (FBS) (Invitrogen, GIBCO 10270-098) and maintained in a humidified atmosphere of 10% $CO_2$/ 90% air at 37°C. Cells were passaged on reaching confluency by means of mechanical separation every three days by splitting a confluent flask of cells at ratios 1:2, 1:3, 1:4.

### 2.11.2 HCT116

Cells were cultured in 1× McCoy's 5A medium + GlutaMAX$^{TM}$I, (Invitrogen, GIBCO 36600) supplemented with 10% FBS.

## 2.12 Freezing cells

Cells were cultured in T75 flasks, washed with PBS and trypsinized media was added after the cells started to detach, and after a centrifugation of 5 minutes at 2,000 *r.p.m.*. Cells from each flask were resuspended in 1ml freezing media (1% DMSO, 90% FBS). Tubes were then moved to a ice box including isopropanol and incubated at -80°C for overnight. Next day the frozen vials of cells were transferred to a liquid nitrogen container for longer term storage.

### 2.13 Thawing cells

The cell suspension was rapidly thawed in a water bath at 37°C then the cell suspension was diluted in 5 ml of growth media and centrifuge at 2,000 *r.p.m.,* for 5 minutes. The cells were re-suspended in 14 ml growth media and transfer into T75 flasks. The cells were then incubated in 37°C incubator at 10% $CO_2$.

### 2.14 Cell counting by Haemocytometer

Cells were cultured as desired, media aspirated, washed with PBS and trypsinized. The trypsin (Sigma, T3924) was deactivated by adding media to it. 10 µl of a trypain/ media mixture) was added to the two chambers of the coverslips on a Hemocytometer. Under a microscope, the number of cells was counted on both the grids of the Haemocytometer. For each grid, cells were counted on 25 squares. The mean number of cells per grid was taken for both the grids (two chambers), divided by 2 (since we counted 25 squares). The total number of the cells was multiplied by $10^4$ to get the number of cells per ml. The number of cells per flask can now be calculated dependent on the volume of media in the flask.

### 2.15 Western blot

Cells were grown as required, washed twice with 1X PBS and trypsinize to detach the cells from the flask surface by adding 0.5 ml in 6-well plate or 1 ml in T75 flask to detach the cells from the flask surface/ 6 well plate surfaces. Cells were washed once with medium containing serum (to inactivate the trypsin), followed by two washes with ice cold 1x PBS. Cells were counted during one of these steps by using haemocytometer. cells were then centrifuged at 2,000g for 5 minutes and resuspended in lysis buffer {50 mM Tris-HCl [pH7.4], 200mM sodium chloride, 0.5% Triton X-100, 1mM AEBSF (4-(2- aminoethyl)-benzenesulfonyl fluoride) and one complete, mini, EDTA-free protease inhibitor cocktail tablet (Roche- cat#11836170001) per 10 ml of lysis buffer, each tablet contains 0.02 mg/ml Pancreas-extract, 0.0005 mg/ml Thermolysin, 0.002 mg/ml Chymotrypsin, 0.02 mg/ml Trypsin, 0.33 mg/ml Papain}. An equal volume of 2X (loading) Laemmli buffer [(20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 4% (w/v) SDS (sodium dodecyl sulphate), 200 mM DTT (Dithiothreitol), 100 mM Tris-HCl (pH6.8)] was added and the lysates were boiled for 5-10 minutes until the cells were disrupted. The lysates were then loaded on 1 mm thick 4-12% TRis glycine precast gels (Invitrogencat# EC60352BOX) or 0.75 mm thick 7.5% SDS-PAGE (sodium dodecyl sulphate –polyacrylamide gel electrophoresis). Precision Plus Protein

Dual colour standards (BioRad- cat#161-0374) were also run as markers to detect the protein sizes. Gels were run at 125 volts for 2-3 hours. Proteins were transferred to methanol wet Immobilon-P PVDF membrane (Millipore- cat#IPVH00010) at 500 mA for 15-18 hours in 2x Towbin buffer (380 mM Glycine, 50m M Tris). After transfer, the membrane was blocked in 10% milk in PBS overnight at 4°C or 1 hour at room temperature. Immunodetection was performed in 10% non-fat dry milk + 0.1% Tween 20 (Sigma- cat#D-1379) for monoclonal primary antibodies and 0.5% Tween 20 for polyclonal primary antibodies. Primary antibody solutions were incubated at room temperature for one hour or 4°C overnight, after which a 15-minute cleaning is done in 10% non-fat dry milk + 0.1% Tween 20 at room temperature. A secondary antibody was incubated again at room temperature for one hour or 4°C overnight. The details of antibody can be seen in tables 2.3 and 2.4. Following incubation with secondary antibody membranes were washed for 10 minutes in 10% non-fat dry milk, followed by two washes for 10 minute in PBS/0.1% Tween 20 (for monoclonal primaries) or PBS/0.5% Tween 20 (for polyclonal primaries). Bands were visualised on Kodak X-OMAT AR film with Super signal west pico chemiluminesent ECL substrate (Pierce- cat#34080).

**Table 2.3 Primary Antibody.**

| **Antibody** | **Clone no. / Cat no.** | **Lot no.** | **Source** | **Host** | **Clonality** | **Sto-ck conc.** | **IF** | **Western** |
|---|---|---|---|---|---|---|---|---|
| Anti-Ki-67 | PP-67 | - | Abcam | Rabbit | polyclonal | 2 µl/ml | | |
| Anti-Lamin A+C | 636/ sc-7292 | H2007 | Santa-Cruz | mouse | monoclonal | 200 µg/ ml | - | 1/750 |
| Anti-Oct4 | Ab19857 | - | abcam | rabbit | polyclonal | 0.1 mg/ml | 1 µg/ml | 1 µg/ml |
| Anti-SSEA-1 | MAB4301 | - | Millipore | mouse | monoclonal | 100 µg/ml | 1µg/ml | - |
| Anti-Tubulin | T6074 | - | Sigma | mouse | monoclonal | 2 mg/ml | - | 1: 5000 |

**Table 2.4 Secondary Antibody.**

| **Antibody** | **Conjugate** | **Stock conc.** | **Require of con.** | **Source** |
|---|---|---|---|---|
| Goat anti-mouse | AlexaFlour 488 | 2 mg/ml | IF (ML) 1/400 | Molecular Probes |
| Goat anti-mouse | AlexaFlour 568 | 2 mg/ml | IF (ML) 1/400 | Molecular Probes |
| Goat anti-rabbit | AlexaFlour 488 | 2 mg/ml | IF (ML) 1/200 | Molecular Probes |
| Goat anti-rabbit | AlexaFlour 568 | 2 mg/ml | IF (ML) 1/200 | Molecular Probes |
| Donkey antirabbit | HRP | 0.8 mg/ml | W 1/25000 | Jackson Immunoresearch |
| Donkey antimouse | HRP | 0.8 mg/ml | W 1/25000 | Jackson Immunoresearch |

### 2.16 Immunostaning

Cells were seeded in 24- well plates. Sterile coverslips were plated under the cells prior to cell loading, and the cells were incubated according to the condition of an experiment. Cells were then washed twice in 1X PBS (phosphate-buffered saline) then fixed by adding 4% paraformaldehyde and incubated for between 15-20 minutes at room temperature. The cells were then washed gently twice with 1X PBS then blocking by adding 1X PBS + 5% FBS for one hour at room temperature or leave them overnight at 4°C. Then the cells were incubated with primary antibodies (see Table 2.3) diluted in 5%FBS for 30 minutes at 37°C or overnight at 4°C. The cells were then washed three times with PBS for 10 minutes or five times for 5 minutes. The secondary antibody immunostaning suspention was incubated at 37°C for 30 minutes or overnight at 4°C and then washed three times in 1X PBS for 10 minutes or five times for five minutes . Cells were then washed with 1X PBS and on the second washing the propidium iodide staning (1 µg/ml) was added to PBS after secondary antibody incubation. The cells were then imaged by using a Zesis LSM 510 confocal microscope.

### 2.17 Chemically induced differentiation

Cells were differentiated by two inducer agents, hexamethylene bisaetamide (HMBA) and retinoic acid (RA). Cells were treated with four poisons [camptothecin (CPT), aphidicolin

(APH), hydroxyurea (HU), phleomycin (PHL)] see table and four types of poisons were added to all the cells (NTERA2, HCT116, E14) as follows:

**Table 2.5 Poisons and inducers.**

| **Type of agent** | **Origin** | **Cat. No** | **Concentration** |
|---|---|---|---|
| hexamethylene bisaetamide (HMBA) | Sigma | | 3 mM |
| Retinoic acid (RA) | Sigma | R2625 | $10^{-2}$M (3 mg/ml) |
| camptothecin (CPT) | Sigma | C9911 | 1 µM, 2 µM, 0.5 µM, 0.05 µM, 0.1 µM, 0.25 µM and 0.025 µM |
| aphidicolin (APH) | Sigma | A0781 | 1 µM and 2 µM |
| hydroxyurea (HU) | Sigma | H8627 | 1 µM |
| phleomycin (PHL) | Sigma | P9564 | 100 mg/ml and 200 mg/ml |

**2.18 Mouse Embryonic stem cell method**

**Table 2.6 Mouse Embryonic stems cell method**

| **Item** | **Volume** | **Company + Cat.No** |
|---|---|---|
| DMEM Glutamax, 4.5 g/l glucose | 410 ml (Store in Fridge) | GIBCO (32430-027) |
| 15 % Serum (EmbryoMax FETAL CALF SERUM) | 75 ml (Store in Freezer – 20) | MILLIPORE (ES-009-B) |
| Amino acids non-essential 1X | 5 ml (Fridge) | GIBCO (11140-035) |
| Pen/strep. | 5 ml (Freezer – 20) | GIBCO (15140-122) |
| Sodium Pyrovate (NaPyr) | 5 ml (Fridge) | GIBCO (11360-039) |
| B- mercapto ethanol | 1 ml (Fridge) | GIBCO (31350-010) |
| (ESGRO) LIF ($10^7$)(1000 u/ml) | 50 µl (Freezer -20) | MILLIPORE (ESG1107) |

| | | |
|---|---|---|
| Can store in 50 µl aliquots ready for use in 500 ml) | | |
| UltraPure water with 0.1% Gelatin | | Millipore (ES-006-B) |
| Trypsin: EDTA (0.05% trypsin 0.53 mM EDTA in Hanks salt | | Millipore (SM-2002-C) |
| 2x Freezing media w/DMSO for ES cells | | Millipore (ES-002-F) |

### 2.18.1 Plating Mouse Embryonic Fibroblasts (MEFs)

The optimal MEF density per dish was determined by Millipore protocol. MEFs were thawed and plated at density of 1 x $10^6$ cells per dish. Mouse ES (mES) cells were passaged onto MEFs and maintained for 2 days.

### 2.18.2 Plating MEFs

A 10 cm tissue culture plate was coated with 0.1% gelatin and incubated for a minimum of 30 minutes in the tissue culture hood at room temprature. One vial of MEFs was thawed by swirling in a 37°C water bath and 10 ml of warmed DMEM medium was mixed in gently. The cells were centrifuged at 1,500 *r.p.m.* for 3.5 minutes. Medium was aspirated and the pellet was resuspended in 2 ml of DMEM medium. Gelatin were aspirated and replaced with 9 ml/dish of DMEM medium. 1 ml of resuspended MEFs was transferred to each plate and incubated for 24 hours at 37°C at 5% $CO_2$.

### 2.18.3 Thawing Mouse ES cells

MEFs were plated on to a gelatin-coated plate at least one day prior to mES flating to permit adherence and flattening. Cells were taken from liquid nitrogen/dry ice and thawed quickly in a 37°C water bath. Cell suspensions were moved to sterile 15 ml tubes comprising 8 ml warmed growth medium, and cells were mixed. Conical tubes containing cells were centrifuged at 1,500 *r.p.m.* for 5 minutes at room temperature. The freezing medium was aspirated and cells were resuspended in 2 ml of the warmed ES growth medium. Cells were

plated in 10 cm dish ($10^6$ cells per dish) containing MEF feeder cells. Cells were maintained by daily medium exchange. Cells were a passage at 75% - 90% confluence.

### 2.18.4 Passaging of MouseES cells

Cells were passaged every 2-3 days depend upon the growth rate of the cells. The optimal condition was to maintain cells at roughly 80% confluent on day 2 to prevent spontaneous differentiation. Fractions range was spilt in a range from 1:4 to 1:10. The optimal cell numbers for seeding in dishs were $10^5$ –$10^6$ cells per 10 cm dish. Used medium was taken off from cultures and culture tissue washed with PBS. 1 ml/dish of trypsin was added and set in an incubator for 5 minutes or up to the time where the cells start to visibly dissociate from the plate. Trypsin solution was used 2-3 times to separate cells from the plate. DMEM media was added to the plate; the volume of the media added was depended on the splitting ratio. A Suitable amount of cell suspension was transferred to new gelatine-coated plates. Plates were rocked slowly to achieve a uniform cell distribution.

### 2.18.5 Cyropreservation of murine ES cells

Medium was removed from cell cultures and cells were washed with PBS. 1 ml/dish was supplemented by trypsin and put in an incubator for 5 minutes or up to the time of cells start to separate from the plate. Trypsin solution was used for 2-3 times to separate cells from a plate. The detached cell aggregate was moved to a 15 ml conical tube including 5 ml mES growth medium. The dish was washed with an additional 1 ml of a growth medium to collect any remaining aggregates. The conical tube containing cells was centrifuged at 1,500 *r.p.m.*. for 5 minutes at room temperature. Supernatant was taken off and cells were resuspended in 0.5 ml media then transferred to a cryovial containing 0.5 ml of freezing medium. The cryovial was placed in an isopropanol freezing vessel and stored at -80°C overnight. The vial was moved to liquid nitrogen vapour for storage.

# Chapter 3 Analysis of a developmentally programmed recombination hotspot during mitotic proliferation

## 3.1 Introduction

In most organisms stem cells and the germ line are formed early during embryogenesis following the formation of the zygote by the fusion of two gamete cells. Gametes are generated during the sexual cycle when a specialist cell division, meiosis, reductionally segregates the chromosomes of a diploid cell into haploid gametes. In most organisms the homologous chromosomes of a diploid progenitor germ cell undergo programmed rearrangements during meiosis resulting in gametes with an unique genetic configuration. This process is mediated by meiotic recombination between homologous chromosomes and recombination intermediates serve to physically conjoin homologues during the first meiotic division to ensure homologues align and segregate correctly in a reductional configuration (Figure 3.1) (Phadnis, Hyppa & Smith 2011).

During mitotic cell division recombination is used as a mechanism for repairing random DNA strand breakages and these pathways have an intimate association with the DNA replication machinery, enabling them to respond to damage incurred during S-phase (McFarlane, Al-Zeer & Dalgaard 2011). However, during meiosis DNA double-strand breaks (DSBs) are specifically generated to initiate recombination and complex chromatin associated mechanisms direct the repair partner choice to ensure an increased bias towards inter-homologue events, which in mitotically dividing cells could be highly detrimental due to the potential to drive loss of heterozygocity events. Inter-homologue events in meiosis form physical recombination intermediates, either single (Cromie et al. 2006) or double Holliday junctions (Schwacha, Kleckner 1995) which ultimately result in chiasmata, the inter-homologue connections required to enable homologues to resist the pulling forces of the meiosis I spindle apparatus.

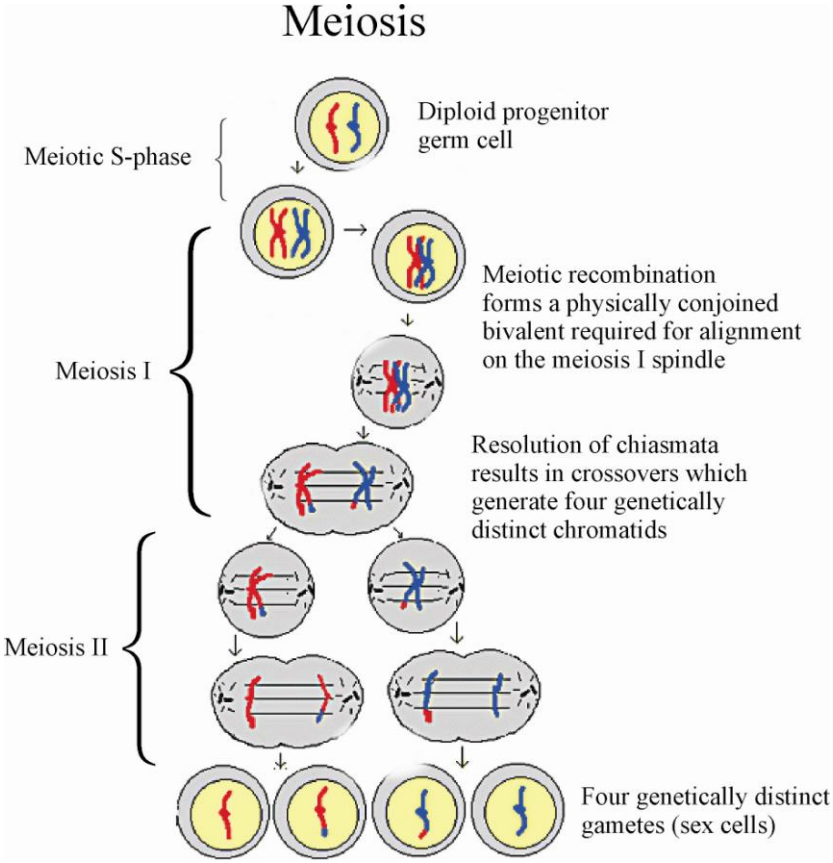



**Figure 3.1 Schematic representation of the basic overview of the meiotic cell division**. A diploid progenitor cell (top) under goes meiotic DNA replication to generate four chromatids (two pairs of homologous chromosomes). Homologues align and become conjoined via genetic recombination intermediates to form a bivalent which is required for alignment of the chromosomes on the metaphase I plate. Recombination which results in chiasmata (the sites of conjoining) occur at specific chromosomal loci termed hotspots. Resolution of chiasmata results in separation of homologues at meiosis I and the crossing over of chromosomal arms between participating chromatids; this results in the formation of four genetically distinct chromatids. Meiosis II then ensues where centromerically conjoined recombined sister chromatids separate generating four genetically distinct haploid gametes (sex cells).

DSBs are formed by a meiosis-specific topoisomerase II–like protein, Spo11 (Rec12 in *S. pombe*), and they can be processed down distinct pathways, only some of which culminate in chiasmata, which are associated with the formation of cross over events between participating chromatids; others will be dissolved/resolved without the formation of an associated crossover event (Whitby 2005). DSBs and recombination do not occur with a random distribution throughout genomes during meiosis; rather, there are genomic sites known as hotspots which are the preferential targets for DSB formation and there are genomic regions which are refractory to Spo11, which are termed cold spots, for example centromeric heterochromatin (Petes 2001, Wahls, Davidson 2010). What features of the genome constitute a hotspot has been the subject of much research and debate, although an overarching feature of hotspots is that they are regions in which the chromatin takes on a more open configuration during meiosis, the so called meiotic hotspot chromatin transition (Petes 2001). Hotspots tend to be in non-coding regions of the genome and some hotspots in both humans and yeast have been found to have a sequence specificity to them which indicates that they bind specific *trans* acting factors to mediate hotspot activation. Recent work in humans has shown a 17 base pair motif, which is the binding site for the zinc finger histone methyl transferase PRDM9 serves as a human meiotic recombination hotspot (Berg et al. 2010, Hinch et al. 2011). Intriguingly, the binding sites for PRDM9 have been associated with human genetic disease breakpoints and this suggests that the unscheduled activation of these sites in somatic, mitotically dividing cells might result in disease-inducing genetic rearrangements (De Raedt et al. 2006, Myers et al. 2008).

The study of sequence-specific meiotic recombination hotspots has largely been previously confined to the study of the *S. pombe M26* and *M26*-like family of hotspots (Pryce & McFarlane, 2009), as prior to the discovery of the PRDM9 motif, this was the only characterised family of sequence-specific meiotic recombination hotspots, although these are not the only hotspot-determining elements within the *S. pombe* genome (Pryce & McFarlane, 2009; Steiner et al., 2011). *M26* is a heptameric DNA sequence (5'-ATGACGT-3') which binds to the heterodimeric stress response transcription factor Atf1-Pcr1 (Mayr & Montminy, 2001). Other Atf1-Pcr1 binding sites, the so called cAMP response elements (CREs) also serve as meiotic recombination binding sites indicating that the binding of Atf1-Pcr1 is critical for hotspot activation. Indeed, this is the case and both Atf1-Pcr1 have been shown to be required for *M26*/CRE hotspot activity (Wahls & Smith, 1994; Kon et al., 1997). The binding of Atf1-Pcr1 to CREs is dependent upon the activation of these factors via a mitogen-

activated protein kinase (MAPK) pathway which is required both for Atf1-Pcr1 transcriptional activation in response to osmotic stress and meiotic hotspot activation (Kon et al., 1998; Gao et al., 2008). This activation is then linked to an array of poorly defined functions which mediate the chromatin transition at *M26*/CRE sites during meiotic entry although the mechanism by which Rec12 (Spo11) is then recruited to these sites remains unknown (Pryce & McFarlane, 2009).

DNA binding proteins, including RNA polymerase II are known to have the potential to generate barriers to the progression of the DNA replication fork (for example, see Prado & Aguilera, 2005). These DNA replication fork barriers (RFBs) can have recombinogenic potential, but not all barriers serve as mitotic recombination hotspots under normal conditions. A recent study from the McFarlane group demonstrated that a RFB generated by *tRNA* genes bound to RNA polymerase III did not serve as a recombination hotspots (Pryce et al., 2009). However, when they mutated a component of the replisome progression complex (RPC), the Timeless orthologue Swi1, they found that the *tRNA* gene-mediated RFB became a mitotic recombination hotspot (Pryce et al., 2009), a finding later corroborated for *tRNA* genes throughout the genome (Rozenzhak et al., 2010). The function of Swi1 (Timeless) and the RPC is proposed to be to monitor replicative barriers and ensure that they do not result in a DNA replication fork collapse which might generate recombinogenic lesions (McFarlane et al., 2010).

Previous work has demonstrated that the recombination hotspot activity of CRE sites is restricted to meiosis (Pryce & McFarlane, 2009). However, analysis of meiotic recombination potential has not been carried out under conditions which would trigger the MAPK pathways which activates Atf1-Pcr1 meiotic hotspot activation. Moreover, it is not known whether the binding of Atf1-Pcr1 to CRE sites within the genome under stress conditions generate a *de novo* barrier to the progression of the replisome which must be accommodated via the action of the RPC. It has been demonstrated that stresses which trigger failings in DNA replication are a major oncogenic factor (for example, see Bartkova et al., 2006) and this, in combination with the observation that a human meiotic recombination hotspot motif is associated with disease-related genomic rearrangements (Raedt et al., 2006; Myers et al., 2008; Berg et al., 2009), led us to hypothesis that under stressed conditions meiotic recombination hotspots form barriers to DNA replication progression which require the RPC to prevent them from driving unscheduled and potentially genome damaging, recombinogenic lesions. Here we test the specific hypothesis that the *M26*-containing meiotic recombination hotspot *ade6-M26*

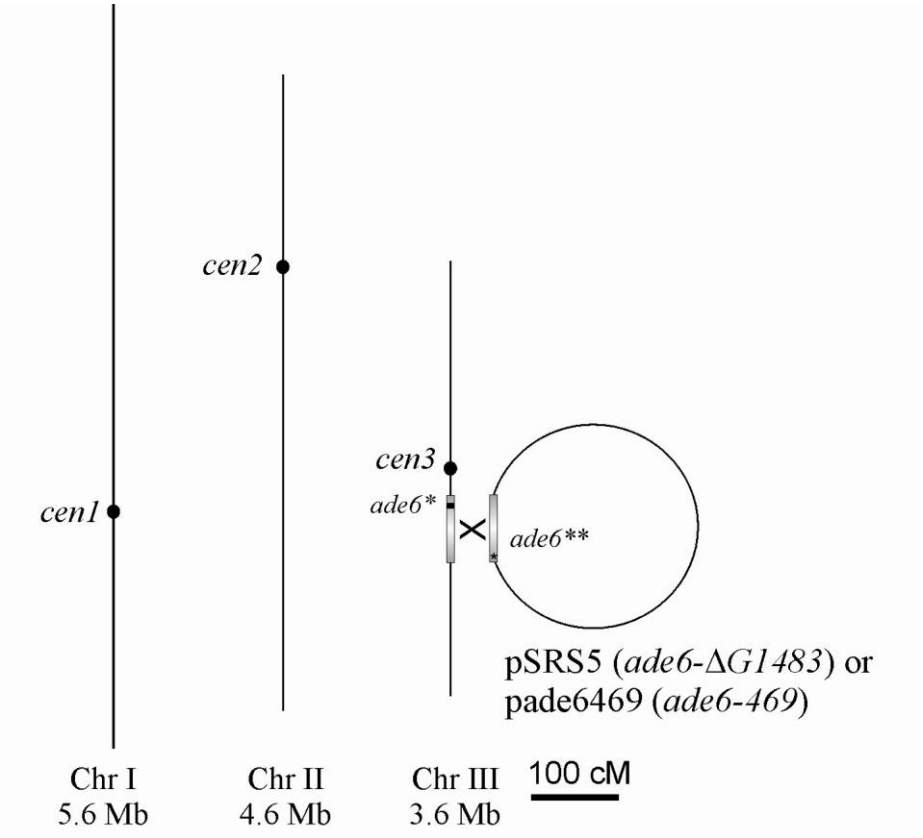
takes on mitotic recombinogenic potential in response to stresses which induce Atf1-Pcr1 binding at *M26*, and that Swi1 is required to suppress this recombinogenic potential.

## 3.2 Results

### 3.2.1. *ade6-M26* does not serve as an inter-molecular mitotic recombination hotspot under non-stressed conditions

During meiosis the *ade6-M26* allele serves as an inter-homologue recombination hotspot, stimulating recombination by approximately one order of magnitude relative to a non-hotspot (no Atf1-Pcr1 binding site) control allele, *ade6-M375*, which carries a single point mutation adjacent to the *M26* Atf1-Pcr1 DNA binding sequence (Pryce & McFarlane, 2009). Mutant alleles of *ade6* can be used to measure recombination frequency as one can measure the frequency of the occurrence of prototroph ($Ade^+$) recombinants within a defined population of cells or spores (for example, see Pryce et al., 2005). The McFarlane group has recently developed an inter-molecular recombination assay in which the frequency of mitotic recombination can be measured between a chromosomally bourn allele of *ade6* and a plasmid bourn allele *ade6* (Figure 3.2; Pryce et al., 2009). We set up this system so that the chromosomal allele of *ade6* was either *ade6-M26* (meiotic recombination hotspot) or the *ade6-M375* (non-hotspot control allele); the plasmid bourne allele was a marker mutant allele, *ade6-ΔG1483*, in cases where a $LEU2^+$ plasmid marker was required (when using *swi1::ura4*$^+$ strains) and pade6-469 when a $ura4^+$ marked plasmid could be used. We then carried out fluctuation analyses to determine the median recombination frequency for both hotspot (*ade6-M26*) and non-hotspot (*ade6-M375*) chromosomally encoded alleles.

Initially, we measured mitotic plasmid-by-chromosome recombination in strains carrying *ade6-M26* and *ade6-M375* chromosomally encoded alleles with no stress added to the media and a RPC proficient background ($swi1^+$). We observed no statistically meaningful difference between the hotspot (*ade6-M26*) and non-hotspot (*ade6-M375*) alleles (Figure 3.3). This confirms previous studies which demonstrate that *ade6-M26* has no measurable mitotic recombination hotspot activity (Pryce & McFarlane, 2009).




*ade6** - chromosomal allele of *ade6*[*ade6-M26* (hotspot) or *ade6-M375* (non-hotspot)]
*ade6*** - plasmid encoded allele of *ade6* (*ade6-ΔG1483* or *ade6-469*)

**Figure 3.2. A schematic of the mitotic inter-molecular (plasmid-by-chromosome) recombination system employed in this study**. *S. pombe* has three chromosomes, indicated here by the vertical lines [centromere (*cen*) positions are indicated by black circles]. The *ade6* gene is located on chromosome III (Chr III), the smallest chromosome. The alleles used in distinct strains in this study (*ade6**) were *ade6-M26* (hotspot encoding) and *ade6-M375* (non-hotspot). The large open circle represents the plasmids which bears the partner *ade6* allele for the inter-molecular recombination assay (this is not to scale). The two plasmids employed in this study were pSRS5 and pade6-469 which carried distinct *ade6* alleles (*ade6***), which were *ade6-ΔG1483* or *ade6-469* respectively. Mitotic recombination between the chromosomal and plasmid bourn alleles can result in an $ade6^{+}$ wild-type recombinant which generates a prototroph (Ade+) and the frequency of prototrophs within a population can be measured to give Ade+ cells per $10^{6}$ viable cells.

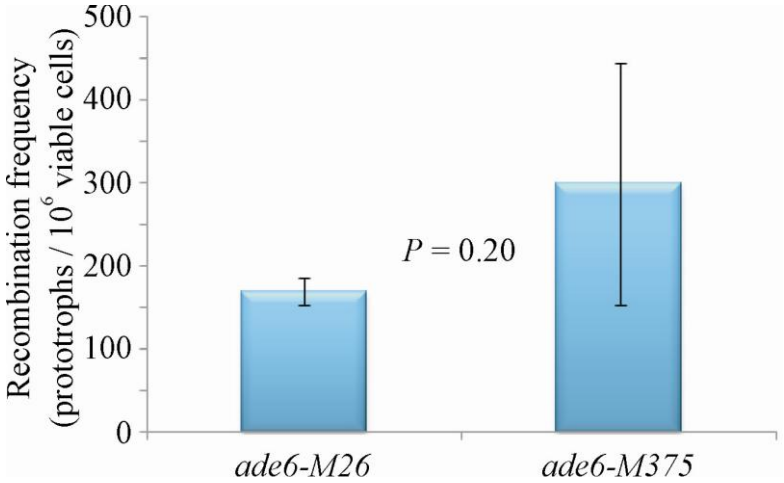



**Figure 3.3. Analysis of mitotic inter-molecular recombination for the *ade6-M26* meiotic recombination hotspot**. The plot shows the mean values for three (or more) median values derived from fluctuation analyses for plasmid-by-chromosome recombination frequencies for *ade6-M26* (left) and *ade6-M375* (right) alleles. The plasmid employed in these analyses was pade6-469. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N ≥ 3 in all cases.

### 3.2.2. *ade6-M26* is not activated for mitotic inter-molecular recombination hotspot activity in the presence of stress.

Whilst the mitotic recombination activity of *ade6-M26* has previously been demonstrated to be no different from the non-hotspot control allele, an observation confirmed using our system (Figure 3.3), it has not been tested under stress conditions. Atf1-Pcr1 bind to the CRE consensus sequence and serve as a transcriptional activator under stress conditions as they represent part of a stress response pathway. We postulated that under stressed conditions Atf1-Pcr1 bind to the *M26* site in the *ade6-M26* allele and trigger mitotic hotspot recombinogenic potential. This postulate has not been previously tested, to our knowledge. To test this, we employed our plasmid-by-chromosome inter-molecular recombination system to determine whether *ade6-M26* would serve as a mitotic recombination hotspot in stress conditions which would mediate Atf1-Pcr1 binding to the *M26* heptamer; we used both osmotic stress (1.2 M sorbitol) and salt (0.2 M KCl), both of which are known to trigger an Atf1-Pcr1-mediated transcriptional response. Fluctuation analyses were carried out as described above, but cells were cultured in the presence of osmotic (Figure 3.4) or salt (Figure 3.5) stress. As can be seen, neither stress condition activated the *ade6-M26* hotspot for inter-molecular mitotic recombination.

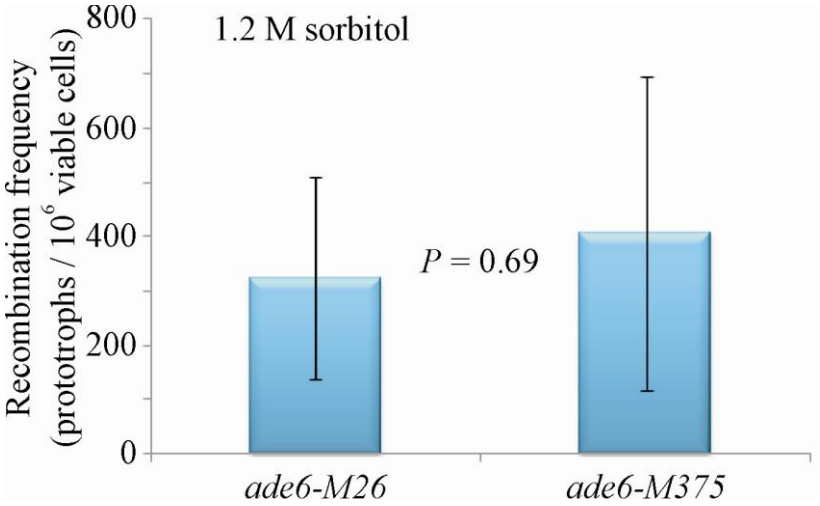



**Figure 3.4. Analysis of mitotic inter-molecular recombination for the *ade6-M26* meiotic recombination hot spot under osmotic stress (1.2 M sorbitol)**. The plot shows the mean values for three (or more) median values derived from fluctuation analyses for plasmid-by-chromosome recombination frequencies for *ade6-M26* (left) and *ade6-M375* (right) alleles. The plasmid employed in these analyses was pade6-469. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N ≥ 3 in all cases.

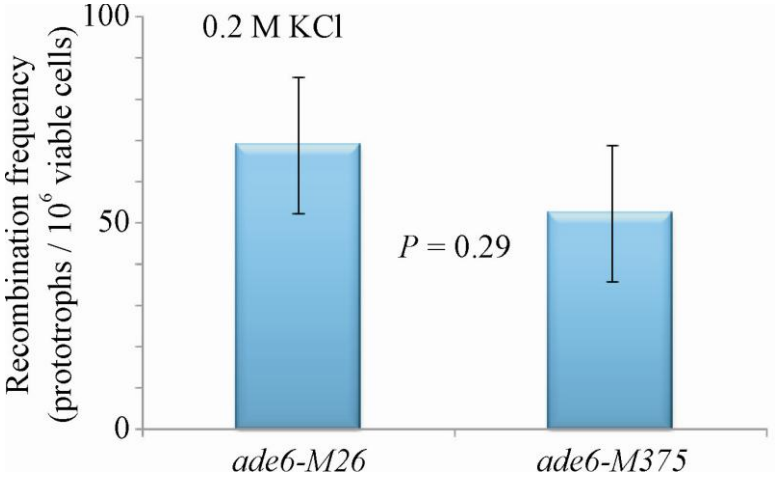



**Figure 3.5. Analysis of mitotic inter-molecular recombination for the *ade6-M26* meiotic recombination hot spot under salt stress (0.2 M KCl).** The plot shows the mean values for three (or more) median values derived from fluctuation analyses for plasmid-by-chromosome recombination frequencies for *ade6-M26* (left) and *ade6-M375* (right) alleles. The plasmid employed in these analyses was pade6-469. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N ≥ 3 in all cases.

### 3.2.3. Loss of Swi1 function elevates recombination under stress conditions, but does not induce hotspot activity for *ade6-M26*

Given the finding that stress conditions in which Atf1-Pcr1 should bind to the *M26* heptamer within the *ade6-M26* allele did not induce hotspot activity for *ade6-M26* we concluded that any Atf1-Pcr1 binding to this site could be tolerated during DNA replication and did not result in the formation of replication fork collapse-mediated recombinogenic lesions. This was not entirely surprising as the replication machinery must encounter many such transcription factors bound to the replication template during each S-phase and the fork collapse avoidance mechanisms must cope well with such events (Labib & Hodgson, 2007). Indeed, the McFarlane group recently demonstrated that *tRNA* genes, where RNA polymerase III is associated with the DNA duplex and can mediate a RFB, are not recombinogenic when the RPC is fully functional. Given this, we set out to address whether loss of RPC function, in this case loss of Swi1 function, would make the *ade6-M26* hotspot recombinogenic in our plasmid-by-chromosome inter-molecular mitotic recombination assay; the idea here is that in the absence of a function required to avoid replication fork collapse (Swi1) Atf1-Pcr1 bound to the replicative template will cause a recombinogenic lesion at the site of the *M26* heptamer (where Atf1-Pcr1 bind).

We firstly tested this theory in the absence of any stress (Figure 3.6). We compared the mitotic recombination frequencies of the hotspot (*ade6-M26*) and non-hotspot (*ade6-M375*) alleles in strains in which the *swi1* gene had been deleted (Figure 3.6A). As can be seen in Figure 3.6A there was no statistically meaningful difference between the hotspot and non-hotspot allele, indicating that in the absence of stress the loss of the Swi1 RPC component does not render *ade6-M26* recombintionally active.

In addition we compared *ade6-M26* mitotic recombination activity with and without mutation of *swi1* (Figure 3.6B). Again, no statistically meaningful difference was observed. This was slightly unexpected as previous studies have shown that the loss of Swi1 activity results in an elevation of all basal recombination during mitosis (Sommariva et al., 2005; Pryce et al., 2009), which does not appear to be the case in this system in the absence of stress.

Following this we explored the possibility that *ade6-M26* would only become recombinationally active as a meiotic hotspot when RPC function was diminished (*swi1* mutation) and Atf1-Pcr1 was induced to bind to the *M26* heptamer within the *ade6-M26*

allele by stress activation; both osmotic (Figure 3.7) and salt stress (Figure 3.8) conditions were tested.

A comparison of hotspot (*ade6-M26*) and non-hotspot (*ade6-M375*) alleles under osmotic stress (1.2 M sorbitol) conditions in a *swi1*Δ strain showed there was no hotspot activation of *ade6-M26* under these conditions (Figure 3.7A), although a comparison of *ade6-M26* with and without an active RPC (*swi1*$^{+}$ *vs. swi1*Δ) demonstrated that loss of Swi1 function did elevate the recombinogenic potential at *ade6* in a statistically meaningful fashion, although the meiotic hotspot allele (*ade6-M26*) was not activated further than the non-hotspot (*ade6-M375*) control (Figure 3.7A). Likewise, salt stress conditions resulted in no enhancement of recombination potential of the *ade6-M26* allele over the non-hotspot *ade6-M375* allele (Figure 3.8A), but again loss of Swi1 function did give a statistically meaningful elevation in recombination frequency at *ade6* (Figure 3.8B), but not one which is hotspot specific.

One minor point of some note is that using the plasmid pSRS5 gave a relatively low recombination activity with the *ade6-M26* chromosomal allele in the RPC competent cells (*swi1*$^{+}$) under salt and osmotic stress conditions (Figure 3.7B and 3.8B) compared to these same conditions used with the pade6-469. The reason for this is not clear, but we believe that this might simply reflect the relative stability of these two distinct plasmids under the stressed conditions.

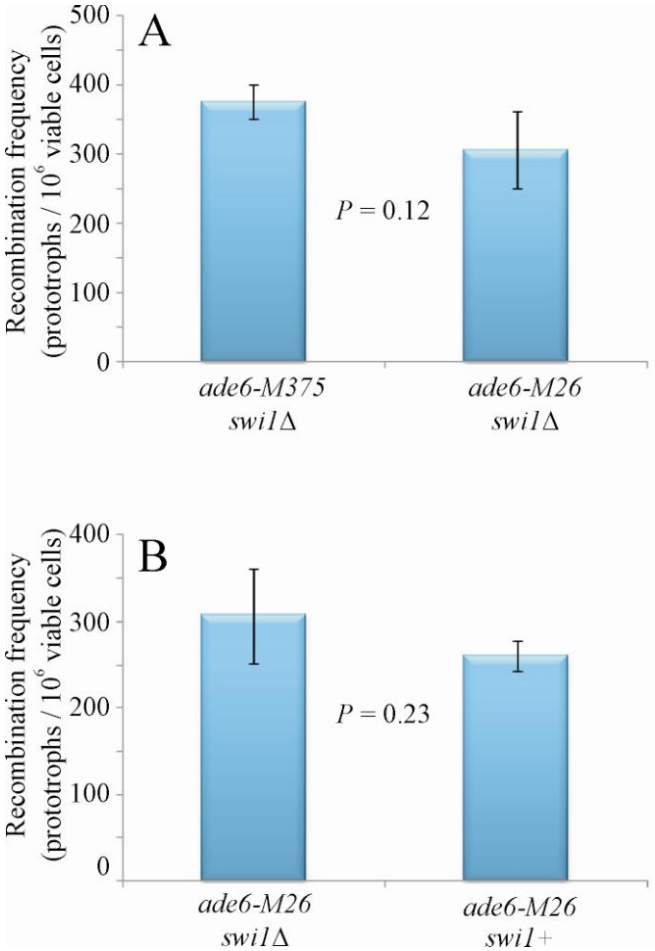



**Figure 3.6. Analysis of the inter-molecular recombinogenic potential of the *ade6-M26* meiotic recombination hot spot in cell defective for Swi1 function. A.** A comparison of the mitotic recombination activity of the *ade6-M26* meiotic recombination hot spot in a *swi1*Δ background compared to a non-hotspot control (*ade6-M375*). **B.** Comparison of *ade6-M26* hotspot activity in both wild-type and *swi1*Δ cells demonstrates that mutation of *swi1* does not increase the mitotic recombination potential of the hotspot alleles. The plasmid employed in these analyses is pSRS5. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N = 3 in all cases.

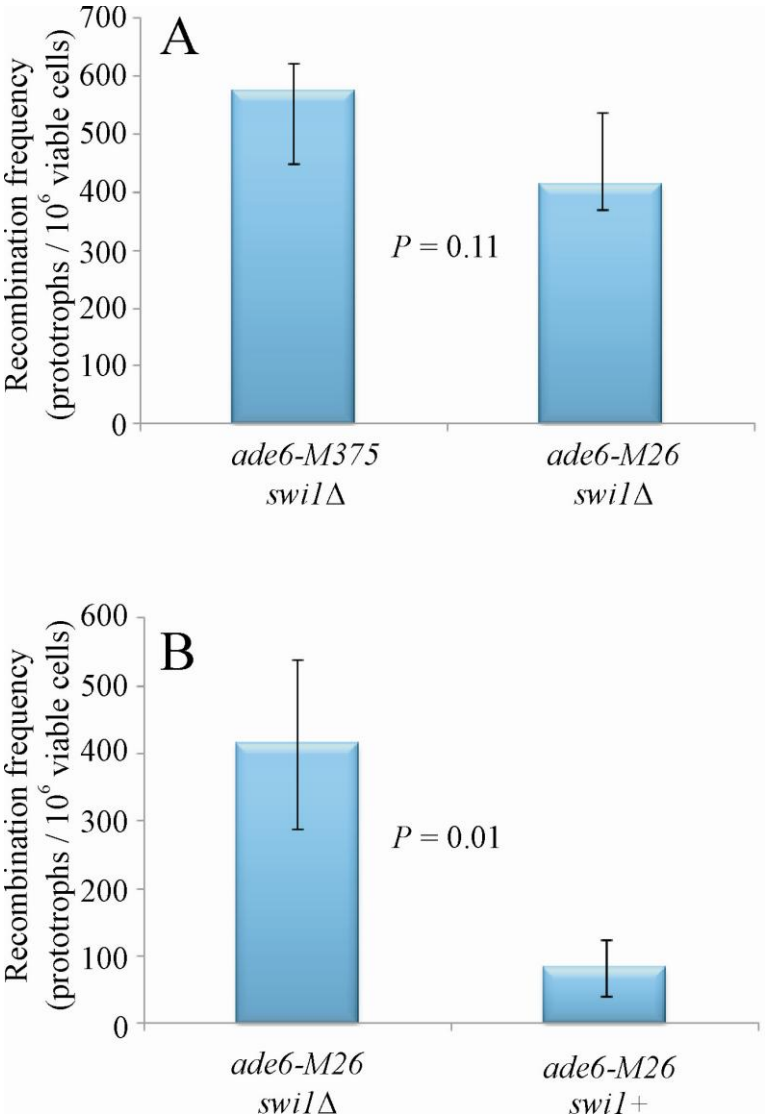



**Figure 3.7. Analysis of the inter-molecular recombinogenic potential of the *ade6-M26* meiotic recombination hot spot in cell defective for Swi1 function in the presence of osmotic stress (1.2 M sorbitol). A.** A comparison of the mitotic recombination activity of the *ade6-M26* meiotic recombination hot spot in a *swi1*Δ background compared to a non-hotspot control (*ade6-M375*). **B.** Comparison of *ade6-M26* hotspot activity in both wild-type and *swi1*Δ cells demonstrates that mutation of *swi1*$^+$ does not increase the mitotic recombination potential of the hotspot alleles. The plasmid employed in these analyses is pSRS5. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N = 3 in all cases.

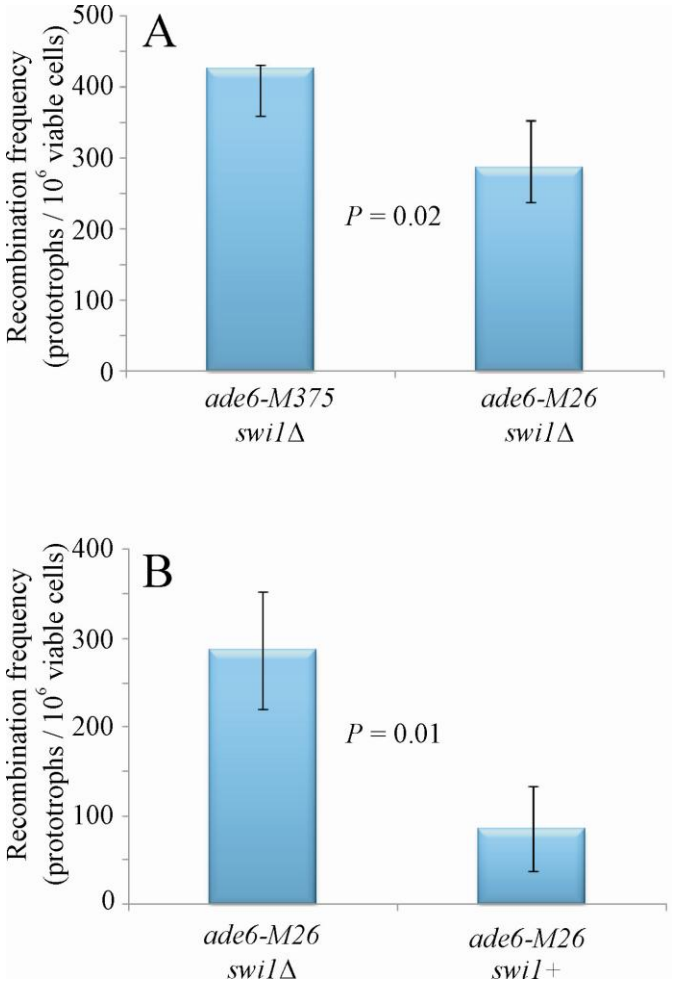



**Figure 3.8. Analysis of the inter-molecular recombinogenic potential of the *ade6-M26* meiotic recombination hot spot in cell defective for Swi1 function in the presence of salt stress (0.2 M KCl). A.** A comparison of the mitotic recombination activity of the *ade6-M26* meiotic recombination hot spot in a *swi1*Δ background compared to a non-hotspot control (*ade6-M375*). **B.** Comparison of *ade6-M26* hotspot activity in both wild-type and *swi1*Δ cells demonstrates that mutation of swi1 does not increase the mitotic recombination potential of the hotspot alleles. The plasmid employed in these analyses is pSRS5. Error bars represent one standard deviation and the *P* value is derived by Student's *t*-test. N = 3 in all cases.

### 3.2.4. Physical analysis of DNA replication fork progression through the *ade6-M26* allele.

The genetic analysis of inter-molecular mitotic recombination of the meiotic recombination hotspot *ade6-M26* (see above) reveals no evidence for the activation of the hotspot under the conditions we tested. To investigate this further we initiated a physical analysis of DNA replication intermediates at the *ade6* locus in strains carrying the *ade6-M26* allele. For this two dimensional gel electrophoresis and Southern blotting was used. Two dimensional gels separate DNA molecules based on both mass (first dimension) and structure (second dimension); this enables Y structures generated by the progression of a DNA replication fork through a specific region to be resolved on an agarose gel, which can then be subjected to Southern blotting to identify the replicative structures associated with a specific genomic region (Brewer & Fangman, 1988). Y structures for a given region of the genome, as defined by restriction digest sites, will vary in the lengths of the replicated and un-replicated section of the Y structure (Figure 3.9), which results in a distinctive arch structure on the two dimensional gel (Figure 3.9). The *ade6* locus is replicated in a unidirectional fashion (Ahn et al., 2005) which gives a replication Y arc of a uniform intensity, indicating that there are no normal significant impediments to DNA replication fork progression in this region. If DNA replication fork barriers are present in a specific region then Y structures will accumulate as the fork stalls at this particular position, resulting in a region of greater intensity on a replicative Y arc on a two dimensional gel.

We firstly analysed the *ade6* locus in a wild-type *ade6-M26* mitotically dividing haploid grown without stress. This gave an arc of uniform intensity indicating no measurable pausing of the DNA replication fork as it passed through the *ade6-M26* allele (Figure 3.9). We failed to obtain enough DNA for analysis from mitotically dividing cells under stress (salt or osmotic stress) and so we extracted DNA from haploid *ade6-M26* cells which had been induced to traverse meiosis using a temperature sensitive mutant, *pat1-114*, which initiates meiosis at the restrictive temperature and activates the *M26* hotspot in the *ade6-M26* allele. Here we could see no significant pausing on the Y arc associated with the position of the *M26* heptamer (the site of Atf1-Pcr1 binding). We did observe a small increased intensity in one region of the arch, but this was not reproducible (work carried out by Dr. D. Pryce) and we believe this to be a signal emanating from the RNA polymerase II binding site within the *ade6* promoter with no evidence for a direct causal link to *M26*. Analysis of *swi1* mutants

was initiated by others within the group independently and no *ade6-M26*-specific replicative pauses were observed (D. Pryce, personal communication).

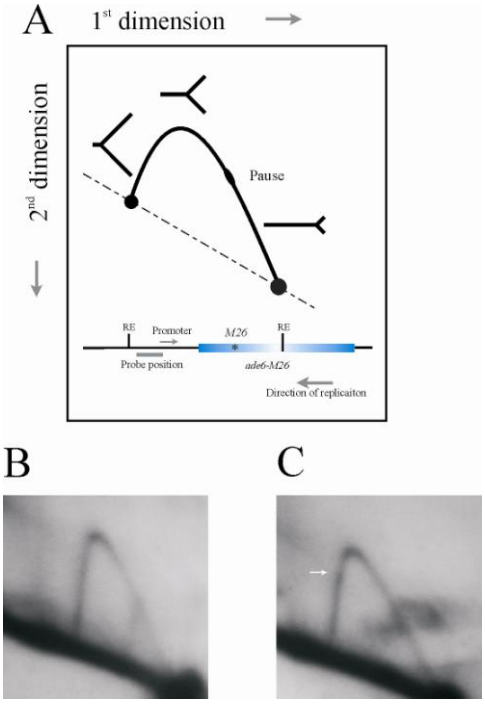



**Figure 3.9. Two dimensional gel analysis of the *ade6-M26* allele in mitosis and meiosis.** **A.** A schematic of a simple Y arc two dimensional gel. The arc represents the mobility path for a continuous DNA replication fork through a specific genomic region (as determined by a specific Southern blot probe). The inflection point of the arc represents the point at which sections of daughter duplexes are of an identical length to the remaining un-replicated section for a particular genome restriction fragment. A pause in DNA replication fork progression will be apparent by a build up of Y structures at a specific point on the arc (as illustrated). The 1st and 2nd dimensions are shown; the first dimension separates restriction digested fragments of the genome based on mass and the second dimension separates based on non-linear structure. Following running all restriction digested genomic DNA on the gel, the replication structures for specific genomic regions can be detected using Southern blotting with a region-specific probe (in this case an *ade6*-specific probe). The lower part of A shows the *ade6-M26* allele with schematic approximations of the positions of the restriction sites used (RE), the probe position, the *ade6* promoter, the direction of DNA replication and the approximate position of the *M26* heptamer. **B.** The two dimensional gel for the *ade6-M26* region in mitotically dividing wild-type cell grown in non-stress conditions. The uniform intensity of the Y arc demonstrates no discernable impediment to DNA replication progression. **C.** The two dimensional gel for the *ade6-M26* region going through meiotic S-phase from a haploid induced meiosis. The white arrow indicates the position of a possible weak DNA replication fork barrier which is believed to correspond to the approximate position of the *ade6* promoter and not the *M26* heptamer.

### 3.3. Discussion

Unscheduled genomic rearrangements and mutations are essential for the process of evolution of living systems; however, when these occur in human somatic or germ line tissues they can cause serious genetic disease, including cancers (for example, see Moynahan & Jasin, 2010). Despite the potential dangers of lesions which can initiate genomic rearrangements, homologue alignment and bivalent formation during meiosis I is dependent upon the programmed formation of one of the most genotoxic lesions, DSBs. The formation of DSBs is elevated at hotspot regions and they are processed to drive recombination events with an elevated inter-homologue bias (Schwacha & Kleckner, 1997). To reduce the potential for these lesions to be generated in non-meiotic cells where they could he highly deleterious, the molecular mechanisms controlling DSB formation and inter-homologue partner choice must be regulated in such a way as to ensure a very tight meiosis-only restriction. To date, little work has been done to determine whether deregulation of the meiotic DSB and partner choice programme in somatic tissue has any role to play in oncogenic progression. However, evidence is starting to emerge which might indicate that such deregulation may indeed have oncogenic potential. For example, the meiosis-specific protein NUT, which has no known function, has been shown to form an oncogenic fusion protein which mediates the epigenetic down regulation of p53 gene expression causing aggressive tumours in juveniles known as NUT midline carcinomas (Reynoird et al., 2010).

At the onset of this work it was becoming apparent that there was a link between the DNA sequences associated with meiotic recombination hotspots in humans and cancer-associated genomic rearrangement sites (Raedt et al., 2006; Myers et al., 2008; Berg et al., 2009). This led us to speculate that a primary oncogenic driver might be the activation of a meiotic recombination hotspot in somatic tissues. Indeed, the recent identification of the motif associated with the human meiotic hotspot regulator, PRDM9, provides further support to this argument as this motif has also been linked to genetic disease-associated break points (Raedt et al., 2006; Myers et al., 2008; Berg et al., 2009).

At first viewing this hypothesis appears flawed in that there seems to be a high number of meiosis-specific genes which would be required to generate DSBs in the same way that they are generated in meiosis. This would suggest that the deregulation of the meiotic DSB inducing programme would need to be on a significantly large enough scale to ensure that all minimal component parts for DSB formation were present in the somatic tissue undergoing oncogenic change. However, there is existing evidence which suggests that when one meiotic

gene is aberrantly expressed in somatic cells, then many others are also de-repressed, which might be indicative of a dysfunction in a master regulator (Fratta et al., 2011).

Not withstanding the possibility of a fully de-regulated and activated minimal DSB-inducing pathway we presented another hypothesis to provide a possible explanation for the activation of meiotic recombination hotspots in somatic tissues which is not dependent upon there being a need for the full activation of a minimal DSB pathway. We postulated that the activation of the primary step in hotspot activation, the binding of the primary *trans* activator, in this case the Atf1-Pcr1 heterodimer binding to *M26* (but it could also be PRDM9 binding to the 13 bp human motif) generates a novel and unscheduled block to the progression of the DNA replication machinery. This blockage thus increases the likelihood of the replication fork collapsing at this site resulting in a recombinogenic lesion which might drive a genomic instability event, particularly if the partner choice is aberrant. This hypothesis is also consistent with the finding that one of the major factors leading to instability is DNA replication stress. Previous work has demonstrated that pauses in DNA replication can result in gross chromosomal rearrangements (Ahn et al., 2005; Lambert et al., 2005) and that protein complexes which are associated with DNA, such as RNA polymerase II, can drive recombination when a collision with the replisome occurs (Prado & Aguilera, 2005). However, this is not true for all protein complexes associated with the replicative template and indeed the more normal case might be that the replication machinery can prevent replicative pauses from becoming recombinogenic (Pryce et al., 2009).

To test our hypothesis we took a stepwise approach in a simple genetically tractable model system, *S. pombe*, in which a well defined sequence-specific meiotic recombination hotspot had been characterised. Firstly, we explored whether or not the *ade6-M26* meiotic recombination hotspot served as a mitotic hotspot in the plasmid-by-chromosome system we have developed. We find that this is not the case and this is consistent with earlier work and demonstrates that under normal conditions in a wild-type cell that the hotspot activity of *M26* is confined to meiosis. However, this does not dismiss the possibility that *M26* does cause recombinogenic lesions during mitosis which are processed by inter-sister chromatid recombination which would not give a genetic readout in the inter-molecular plasmid-by-chromosome system we employed, although we believe that this is unlikely as we could find no evidence of replication pauses by two dimensional gel analysis of *M26* within *ade6-M26* in the wild-type (Figure 3.9).

Next, we asked whether stress, in this case salt and osmotic stress could activate *M26* in mitosis. The idea being that these stresses are known to induce Aft1-Pcr1 transcriptional activation and so this should elevate the levels of Atf1-Pcr1 bound to *M26* in mitotic cells, which, in turn would generate a replication pause inducing barrier at the position of *M26* which would drive the formation of recombinogenic lesions. Again, we found no evidence for stress-induced elevations in recombination. We did not directly test binding of Atf1-Pcr1, increased replication pausing at *M26* or lesion formation at *M26* under stressed conditions. Had we done this we could have established whether or not Atf1-Pcr1 binding was indeed generating replicative pause sites, and if so, were they forming recombinogenic lesions. Rather than executing these extensive physical assays we simply set out to now ask whether mutation of the machinery which is responsible for suppressing recombination at DNA replication fork barriers could induced mitotic hotspot activity at *M26* under stressed or non-stressed conditions. It is known that loss of Swi1 activity can convert a replicative barrier, which is normally encountered in the genome, to become a source of recombinogenic lesions and signals (Sommariva et al., 2005; Pryce et al., 2009; Rozenzhak et al., 2010) and so we believed that this genetic test was appropriate prior to embarking on extensive physical analyses. We found that whilst loss of Swi1 function gave an elevation of recombination under stressed conditions, it did not result in an *M26*-specific hotspot elevation, indicating that loss of Swi1 function does not result in the activation of *M26* under any of the conditions we tested. The basal elevation in recombination is to be expected due to the loss of RPC activity (Sommariva et al., 2005; Pryce et al., 2009; Rozenzhak et al., 2010).

These findings can be interpreted in a number of ways. Firstly, there may be no significant binding of Atf1-Pcr1 in our system, even under stress conditions. We believe this to be unlikely as *ade6-M26* has previously been demonstrated to be a target for binding (Gao et al., 2008). Secondly, Atf1-Pcr1 binding to *M26* may not cause a significant pause to DNA replication progression. This is partly supported by the finding that we do not observe a meiosis-specific measurable pause at the *M26* using two dimensional gels (Figure 3.9C). Moreover, it may be he case that the pausing generated by Atf1-Pcr1 binding, if any, is not of sufficient magnitude to require the function of Swi1 and the RPC to prevent recombinogenic lesions forming, or these lesions, if they are formed, are always driven down the inter-sister chromatid route and do not result in measurable inter-molecular recombination in the plasmid-by-chromosome assay we have established.

### 3.4. Final comment

These studies have not generated any evidence for the hypothesis we set out with, which postulates that a defined meiotic recombination hotspot can be activated in mitotically proliferating cells by generating a recombinogenic DNA replication fork perturbation. Whilst this preliminary study has its limitations (for example, limited physical analyse) we feel that there is not enough of a platform to justify taking the work further in this system at this stage. This brings back into focus the question of how meiotic hotspot sequences might be linked to break points in human genetic diseases and it raises the other possibility that meiotic hotspots do become activated by a switching on of a minimal DSB-inducing programme which is normally tightly restricted to meiosis. This possibility has now taken on more favour within the McFarlane research group. A recent finding from the group has demonstrated that many of the factors which are thought to be meiosis-specific, including Spo11, are indeed switched on in normal, non-cancerous somatic cells (R. McFarlane, personal communication of unpublished data). This remarkable and unexpected finding might indicate that there is not the need for a significant and wide-spread de-regulation of key DSB mediators as they are already present. Rather, there may only be the need for some specific factors to be combined with these factors to mediate DSB formation which has mechanistic similarities to meiotic DSB formation. In yeast cells Spo11 (and Rec12) are tightly regulated and are highly meiosis-specific; the situation in humans appears to be different and this might mean we must re-think some long held pre-conceptions. Interestingly, the human hotspot activator, PRDM9, unlike Spo11, is not present in normal somatic cells and gene expression is limited to the testis, but it is switched on in approximately half of the cancer tissues / cell lines studied to date (33 analysed to date), indicating that regulation of the production of this key regulator may be the trigger for activating the DSB machinery to function in a meiotic hotspot activating fashion. In this regard genes such as PRDM9 may be proven to be significant genotoxic oncogenes.

## Chapter 4 Effect of camptothecin on the embryonal cancer line NTERA2

### 4.1 Introduction

The NTERA-2 cL.D1 cell line is a pluripotent human testicular embryonal carcinoma cell line obtained by cloning the NTERA-2 cells isolated from a nude mouse xenograft of the TERA-2 which was initially obtained from a metastasis of a human testicular teratocarcinoma (Andrews et al. 1984a, Dewji, Singer 1997). NTERA2 xenografts include various cell forms, especially neural precursors. NTERA2 was isolated as single-cell clone, and NTERA2 clone D1 (NT2/D1) became the standard line that is at present used. NTERA2 cells express features shared with other human EC cells such as SSEA4 and SSEA3, also TRA-1-60 and a high ratio of the liver isozyme of alkaline phosphatase. Not only do NTERA2 EC cells form well-differentiated teratomas when produced as xenografts in nude mice, they also respond to retinoic acid and other treatments *in vitro* (Andrews 1984a). After exposure to $10^{-5}$ or $10^{-6}$ M retinoic acid, NTERA2 cells rapidly lose their EC phenotype, obtaining a considerably different growth pattern and cellular morphology. Cultures exposed to retinoic acid characteristically lack expression of EC markers like SSEA4, SSEA3, or TRA-1-60 over a one-two week time period. Simultaneously, different antigens, especially ganglioseries glycolipids, appear on the surface of the cells (Fenderson et al. 1987). Mostly, a 2- to 3-day exposure to retinoic acid is enough to cause nearly all the cells to differentiate, and in less than 2–3 weeks, EC cells cannot be identifing in the cultures.

Differentiation of NTERA2 EC cells is distinguished not just by variations in surface antigen expression, but as well as by variations in predisposition to infection with particular viruses, especially human immunodeficiency virus (HIV) and human cytomegalovirus (HCMV). For instance, NTERA2 EC cells are immune to infection with HCMV and HIV, while the differentiated cells are permissive for the replication of both viruses (Gonczol, Andrews & Plotkin 1984a, Hirka et al. 1991a). In the situation of HCMV, resistance effects from inactivity of the main immediate early promoter of the virus in the EC cells (Lafemina, Hayward 1988a, Nelson, Groudine 1986a).

Several genes have a marked regulation through NTERA2 differentiation. Oct4 for example, which is characteristically expressed by EC cells and ES cells, is down-regulated after retinoic acid induction of NTERA2 cells (Przyborski et al. 2000). Simultaneously, other

genes are induced. One of these is the Wnt family member, Wnt13, which is not expressed by NTERA2 or other human EC cells, however is induced substantially by retinoic acid induction (Wakeman, Walsh & Andrews 1998a). No other expression of different members of the Wnt family through NTERA2 differentiation while, for instance, Wnt1 has been notable for be induced through differentiation of the murine EC line P19 (Papkoff 1994a).

NTERA2 EC can be induced to differentiate with different agents, the most notably hexamethylene bisacetamide (HMBA) (Andrews et al. 1990) and proteins of the BMP family (Andrews et al. 1994). The differentiation potential of the NTERA-2 cL.D1 cell line has been compared to a recognized pluripotent human ES line, BG01 (Mitalipova et al. 2003). It is suggest that NTERA-2 cells can be a potential alternative to human ES cell source of help in the domain of embryonic stem cell biology perfecting alternative human ES cells.

Although, EC stem cells can supply a simple and robust experimental model, their differentiation potential is frequently restricted, dissimilar to ES cell differentiation, which is unlimited (Przyborski et al. 2004).

Undifferentiated NTERA2 EC cells, like human ES cells, express the connexin 43 protein and, consequently, possess functional gap junctions (Bani-Yaghoub, Bechberger & Naus 1997, Wong et al. 2004). The NT2/D1 cell line is a human EC which does not needs any feeder layer to prevent its undifferentiated potential, and it may produce embryoid body-like forms *in vitro*, a characteristic also noticed in other EC tumours (Parchment, Gramzinski & Pierce 1990, Soprano et al. 1988, Takeuchi, Watanabe & Uno 1983). The presence of some differentiating agents, such as as RA, in the culture medium, seems to activate the neuroectodermal differentiating program (Andrews 1984b), where the NT2/D1 cells cause neural cells but as well as nonneural cells.

### 4.2 Cancer therapy

Cancer or tumor cells at various stages of the cell cycle will deal with chemotherapy or radiotherapy very differently (Marx 1994). It has been proposed that there might be a small population of resting period cells residing in the quickly proliferating tumor populations, and a different subpopulation of cancer stem cells (CSCs) and SP (side population). It is proposed

that dormant cells were closely connected with tumor progression, disease return or drug resistance (Pang et al. 2010, Dalerba et al. 2007, O'Brien et al. 2006c).

Removal of tumor-initiating and tumor-maintaining cell populations is thus potential cancer treatment. To improve such treatments, the cellular processes of resistance leading to failure of demonstrated therapies must be avoided or removed.

Here we postulate that cancer stem cells can become more sensitised to a cancer therapeutic agents when they are progressing through the differentiation process. We believe that this will also apply to embryo stem cells and this work has implications for how we therapeutically eliminate cancer stem cells and for understanding how neoplastic transformations can be avoided in regenerative medicine approaches using stem cells.
The aim of this chapter was to investigate the different DNA damage responses in G0 phase and proliferating NTERA2 cells after camptothecin (CPT) exposure in different culture densities and treatment in different the periods.

## 4.3 Camptothecin and mechanism of resistance

### 4.3.1 Camptothecin inhibits topisomerase I

Topoisomerase l is an enzyme which non-covalently binds to torsionally strained, supercoiled, double-stranded DNA and produces a transient single-strand break in the DNA molecule. This enables for the passage of an entire complementary DNA strand through the break. This mechanism is active throughout transcription, replication, recombination and other DNA functions (Maxwell, Gellert 1986). The enzyme-bridged DNA breaks, and is then resealed by the topoisomerase I enzyme. Disconnection of the enzyme returns an entire, freshly relaxed DNA double helix. Camptothecin inhibits the action of topoisomerase I and has been employed as chemotherapeutic.

### 4.3.2 Camptothecin and mechanism of antitumor and resistance activity

Camptothecin binds to the cleavable complex between the free 3′-phosphate of the DNA and topoisomerase I. The resulting enzyme-linked DNA breaks cannot be religated during the poison is existing (Hertzberg et al. 1989a). S-phase-specific cytotoxicity arises when the

progress replication fork and bound complex collide, causing irreversible fork breakage (Fig 4) (Pommier et al. 1996).

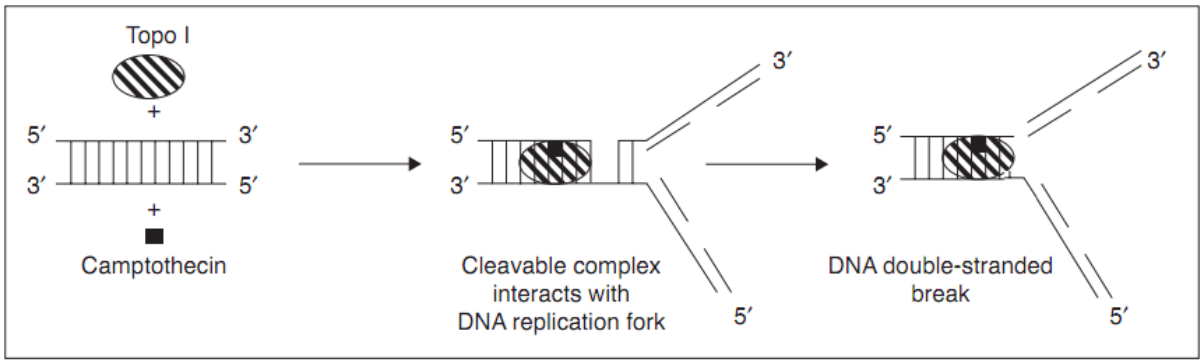



**Figure 4. Mechanism of function of the camptothecin**

However, whilst single-strand DNA breaks are downgraded quickly upon deletion of the topoisomerase I inhibitor, double-strand breaks persist for long times after drug elimination (Hertzberg et al. 1989b). Camptothecins are not typical enzyme inhibitors; they alter the activity of a normal endogenous protein to create a cellular toxin (Takimoto et al. 1999).

Resistance to camptothecin analogs *in vitro* is frequently connected with either lowered levels of cellular topoisomerase (McLeod, Keith 1996, Saleem 1997) or with gene alterations that downregulate or change the topoisomerase I enzyme (Gupta et al. 1988, Gupta, Gupta & Goldstein 1996, Benedetti et al. 1993, Fujimori et al. 1996). Decreased uptake of camptothecins because of overexpression of the multidrug resistance-associated protein (Jonsson et al. 1997, Chen et al. 1999, Chu et al. 1999) and to a smaller degree of P-glycoprotein (Chu et al. 1999, Hendricks et al. 1992) has also been demonstrated. Nevertheless, the degree of multidrug resistance to camptothecin is clearly less than that noticed with different multidrug resistance substrates.

We hypothesized that transiently exposing NTERA2 as cancer stem cell or stem cell-like to CPT would have damaging effects on DNA replication causing their death while simultaneously having little or no effect on replication after differentiation by two inducers such as HMBA and retinoic acid see (chapter 5 and 6). This hypothesis arises from work which demonstrated camptothecin was high toxic to undifferentiated mouse ES cells, but not

proliferating differentiated embryonic fibroblasts (MEF) (Wakeman & McFarlane, unpublished data).

## 4.4 Results

Previous work had exposed mouse ES cells to CPT and found that they were sensitive to chronic exposure (24 hours) at a CPT concentration of 2 μM. However, mouse embryonic fibroblasts (MEFs) which were subjected to the same treatment did not show any loss of viability following treatment (Wakeman & McFarlane, unpublished data). Unfortunately, the MEFs and the ES cells employed in that preliminary studies were not isogenic and were derived from distinct laboratory mouse strains. However, this work potentially indicates that ES cells have distinct sensitivity to CPT in comparison to proliferating differentiated cells in the mouse. Here we hypothesised that this would be true for human cells with stem-like characteristics and employed the well characterised EC cell line NETRA2, as these cells can be cultured and differentiated *in vitro*.

Prior to using NTERA2 we set out to establish the experimental conditions to be employed for this cell line. The first aim was to establish the appropriate cell density of the starting culture which would permit cell proliferation for the required experimental time period (which in later chapters is up to 8 days). The second aim was to determine whether NTERA2 at the chosen cell densities could continue to proliferate and show no loss of viability in response to DMSO, the solute required for CPT. Thirdly, the previous work had employed two CPT concentrations with both an acute (3 hour) treatment and a chronic (24 hour treatment); establishing which conditions, if any, gave significant loss of viability for NTERA2 was important.

### 4.4.1 NTERA2 can tolerate chronic and acute exposure to the solute DMSO

Because CPT is solubilised in DMSO we grew NTERA2 cells to different cell numbers in 2 ml of medium in six well plates. Cell numbers tested were 25,000, 50,000, 75,000 and 100,000. Cells were treated with 1 μl or 2 μls of DMSO for 3 hours (acute) and then the DMSO containing medium was washed off and replaced with fresh, pre-warmed medium. Cultures were followed for up to 5 days, with cell number being counted after 3 hours and every 24 hours thereafter. In all cases cells treated with DMSO exhibited no loss of viability relative to the untreated control; Figure 4.1 shows the data for 50,000 cells and a similar trend was observed for the other cell densities tested (data not shown). A similar study was carried out for cultures treated for 24 hours of exposure to 1 μl or 2 μl DMSO and again, no

significant loss of viability was observed when comparisons were made between treated and untreated cultures. Figure 4.2 shows the 100,000 cells as an example; a similar pattern was seen with other starting cell numbers (data not shown). These data confirm that DMSO is not cytotoxic to NTERA2 under the treatment regime to be used for treatment with CPT.

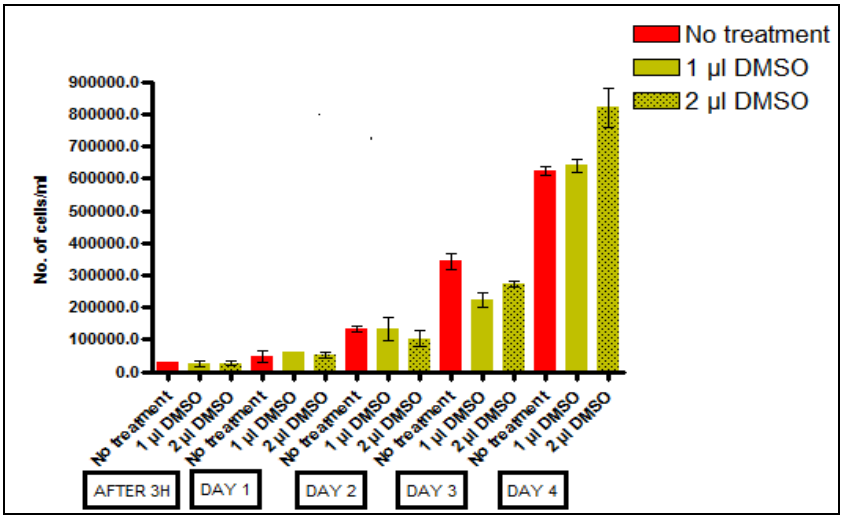



**Figure 4.1. Total number of NTERA2 cells following exposure to DMSO as control**. 50000 of NTERA2 cells were exposed to 1 µl and 2 µl of DMSO in 6 well plates, 2 ml/ well for 3h, the total number of cells remaining at each time point determined by cell counting using haemocytometer. Error bars represent Standard Error of Mean and the *p* value is derived by one way ANOVA in all cases.

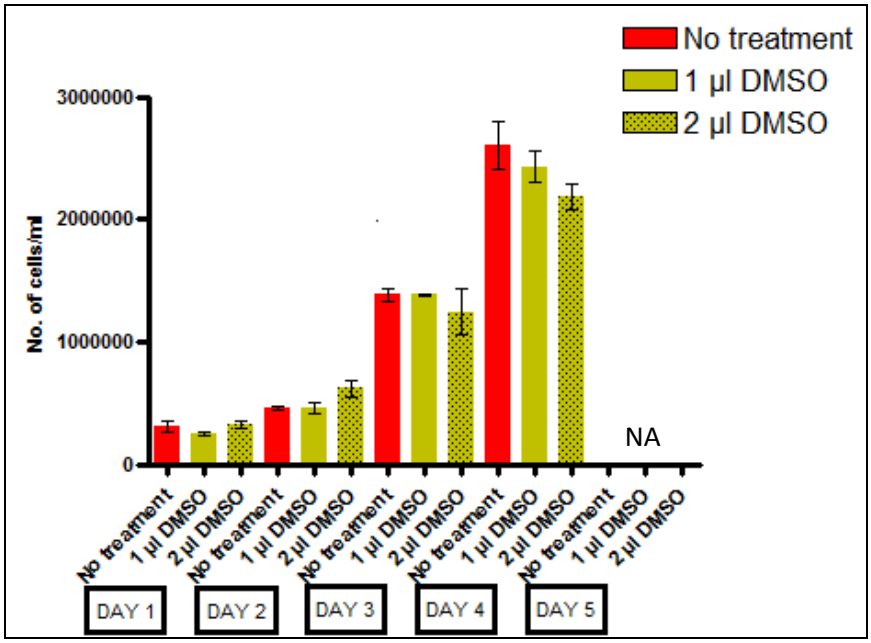



**Figure 4.2. 100,000 NTERA2 cells were exposed to 1 µl and 2 µl of DMSO** in 6 well plates, 2 ml/ well for 24h, the total number of cells remaining at each time point determined by cell counting using haemocytometer. Error bars represent Standard Error of Mean and the *p* value is derived by one way ANOVA in all cases.

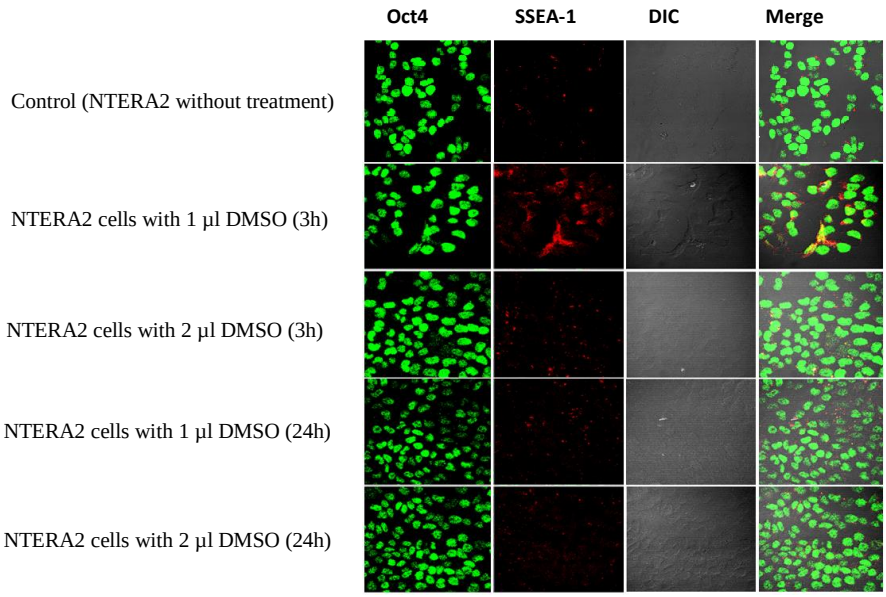



**Figure 4.3 Confocal image showing presence of Oct4 in NTERA2 undifferentiation cells (stem cell-like) which were treated with 1 µl and 2 µl DMSO to confirm that DMSO has no affect for on growing cells during 3h or 24h.** Dapi staining (blue) stains the nucleus of the cells. DIC image shows the cells without any staining and merged image shows the expression of oct4 (green) and down-regulated SSEA-1 (red) in the cells. Last image, NTERA2 cells without treatment but the (red) indicate to Pi (nucleus stain).

### 4.4.2 Treatment with DMSO does not alter the levels of the stem cell marker Oct4

Previous studies have demonstrated that prolonged exposure of NTERA2 cells to high concentrations of DMSO can result in cellular differentiation (Andrews 1984a). Whilst DMSO treatment does not result in loss of viability or reduced cell proliferation (see above), it is important to confirm that the NETRA2 cells retain their stem cell-like characteristics in response to the relatively low levels of DMSO employed here. To test this we carried out confocal microscopy to determine whether or not the stem cell marker Oct4 remains present in the cells after DMSO treatment. Moreover, as cells differentiate away from the pleuripotent state they turn on expression of the SSEA-1 marker. Figure 4.3 shows cells immunostained with antibodies against Oct4 and SSEA-1 following acute exposure to DMSO (3 hours; Figure 4.3. A) and chronic exposure (24 hours; 4.3. B). in both cases Oct4 is clearly present in the majority of the cells within the population and SSEA-1 is almost undetectable; this pattern is identical to that seen for untreated NTERA2. Moreover, cell morphology does not alter upon treatment with NTERA2.

### 4.4.3. Acute exposure to CPT results in a limited loss of cell viability

Having established that DMSO did not result in cell death, growth or proliferation arrest or differentiation of NTERA2 the response to an acute dose (3 hours) of CPT could be tested. A range of cell numbers were tested (cell numbers are given as the total number of cells per 2 ml culture medium in a six well plates; cell numbers tested were 25,000, 50,000, 75,000 and 1000,000 cells). Following addition of the CPT and 3 hours incubation the CPT-containing medium was removed and replaced with fresh pre-warmed medium which did not contain CPT. Figure 4.4. shows the data obtained for acute treatment of 75,000 cells with both 1 µM and 2 µM CPT, although a similar pattern was observed for all cell numbers tested (data not shown). As can be seen treatment with 2 µM resulted in an approximate 10-fold loss of viability during the exposure period, whereas as 1 µM did not generate this same viability loss. Following this, both sets of treated cells show a maximal reduction in cell counts of no greater than approximately 100-fold and appear to maintain a constant population of cells for up to 4 days post treatment. These data indicate that a significant population of NTERA2 cells survive acute exposure to CPT.

### 4.4.4. NTERA2 is exquisitely sensitive to chronic exposure to CPT

Having determined that a significant number of cells can survive acute exposure to CPT it was postulated that these survivors were not inherently resistant, but had not traversed an appropriate cell cycle period to take on significant CPT-dependent DNA damage. To test this NTERA2 cells were subjected to a more chronic exposure to CPT (24 hours). Cell cultures with different cell numbers (25,000, 50,000, 75,000 and 100,000 cells in 2 ml culture medium) were treated with 1 µM or 2 µM CPT for 24 hours, following which the medium was washed off and replaced with fresh pre-warmed medium. Figure 4.5 shows the data for the culture starting with 50,000 cells, although all cultures showed a similar pattern to chronic CPT exposure (data not shown). As can be seen chronic treatment (24 hours) results in a total loss of cell numbers and only cell debris could be observed following 24 hours exposure to CPT, where as the DMSO control shows cell numbers increasing, indicating the cells were continuing to proliferate in the absence of CPT (Figure 4.5.). Total loss of cell viability is seen immediately following (24 hours) of exposure and there was no measurable resistant population. An acute exposure (3 hours) was carried out alongside the chronic exposure (24 hours) and the data for 50,000 cells can also be seen in Figure 4.5., again showing only a limited loss of cell number.

Whilst mouse ES cells had been over 100 times more sensitive to CPT than the MEFs, this total loss of countable cells was not observed and there was a small population of survivors (Wakeman & McFarlane, unpublished data). It was noted that slightly higher numbers of cells had been used for the mouse ES population (approximately 140,000 cells in 2 ml medium). To determine whether this higher cell density could result in a reduced sensitivity (possibly due to fewer proliferating cells, although this was not directly tested), a cell number of 147,000 in 2 mls was tested for NTERA2 with both acute and chronic exposure to 1 µM and 2 µM CPT. As can be seen in Figure 4.6. a similar pattern was observed for this high cell number as had been observed for the lower cell numbers. This indicates that for NTERA2 higher cell density does not result in a CPT resistant population following chronic CPT treatment.

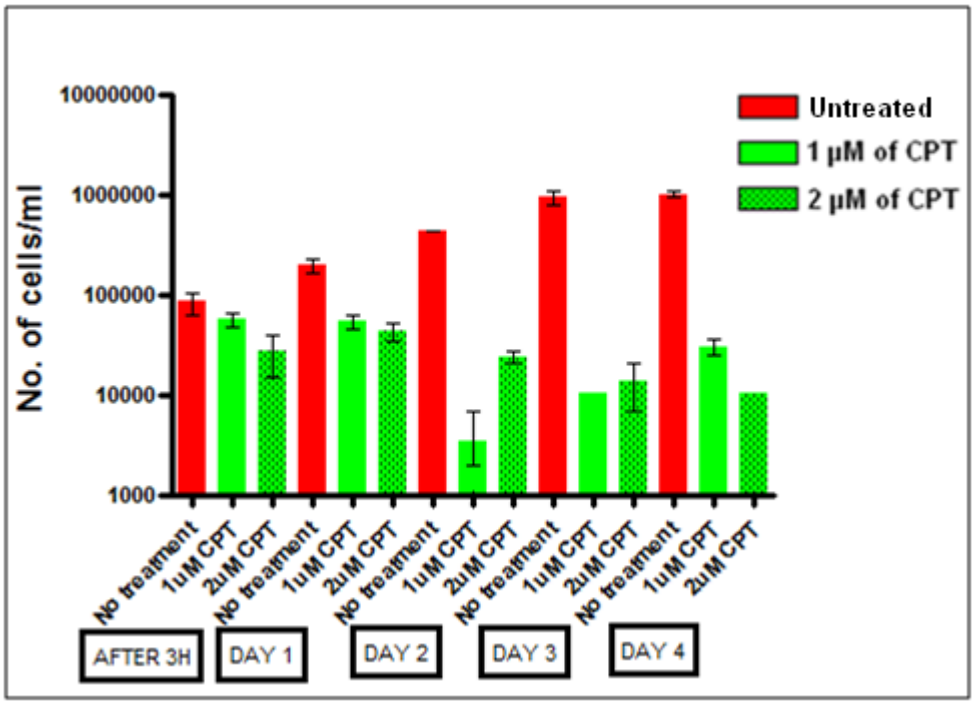



**Figure 4.4 Total number of NTERA2 cells following exposure to camptothecin**. 75000 of NTERA2 cells were exposed to 1 µM and 2 µM of camptothecin in 6 well plates, 2 ml/ well for 3h and 24h, the total number of cells remaining at each time point determined by cell counting using haemocytometer. Error bars represent Standard Error of Mean and the *p* value is derived by one way ANOVA in all cases.

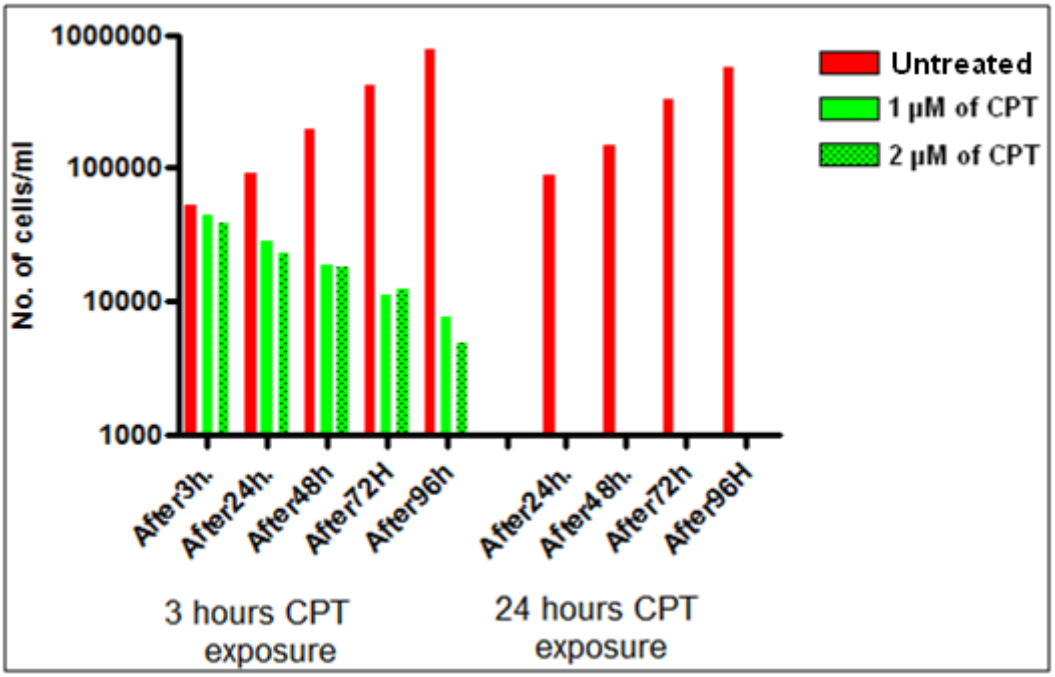



**Figure 4.5. Total number of NTERA2 cells following exposure to camptothecin**. 50,000 NTERA2 cells were exposed to 1 µM and 2 µM of camptothecin in 6 well plates, 2 ml/ well for 3h and 24h, the total number of cells remaining at each time point determined by cell counting using haemocytometer.

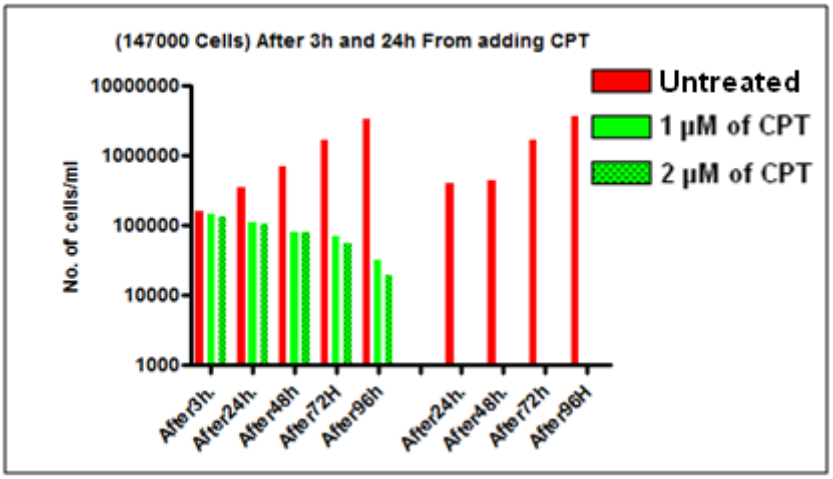



**Figure 4.6. Total number of NTERA2 cells following exposure to camptothecin**. 150,000 NTERA2 cells were exposed to 1 µM and 2 µM of camptothecin in 6 well plates, 2 ml/ well for 3h and 24h, the total number of cells remaining at each time point determined by cell counting using haemocytometer.

## 4.5 Discussion

### 4.5.1 NTERA2 cells treated with anticancer drugs such as camptothecin

Chemotherapy for cancer treatment is extremely toxic, relatively nonspecific, and frequently fails to achieve long-term patient survival. Mortality can occur due to tumour relapse as an effect of chemotherapy-resistant CSCs. Therefore, it is vital to develop new therapeutic strategies that specifically target CSCs. Selective differentiation therapy specifically affecting CSC differentiation and with fewer toxic side effects could be used to treat tumours. Camptothecin is an anti-tumour agent with robust inhibitory effects against DNA topoisomerase I. Topoisomerase l is an enzyme that affects the topology of DNA by transiently breaking and resealing the phosphodiester acid backbone, and its expression is altered in human cancer cells (van der Zee et al. 1994, Boonsong et al. 2002, Gouveris et al. 2007). Therefore, we selected CPT as the anti-cancer agent and used two different concentrations—l μM and 2 μM—as described previously in this chapter.

Many investigations have established that camptothecin induces apoptotic cell death (Nelson & Kastan 1994, Piret & Piette 1996, Shao et al. 1999, Alexandre et al. 2000a, Davis et al. 1998a, Morris & Geller 1996a). However, it is still unclear whether this is the primary anti-tumour mode of action of camptothecin. Apoptotic cell death induced by camptothecin has been shown to occur solely at high concentrations of camptothecin (1 μM and 2 μM) (Alexandre et al. 2000b, Davis et al. 1998b, Morris & Geller 1996b). At lower concentrations, camptothecin leads to primarily S-phase cells and does not result in cells exhibiting all the features of apoptotic cell death (e.g. cell shrinkage and nucleosomal DNA laddering) (Morris, Geller 1996b, Davis et al. 1998c, Morris & Geller 1996c). The relevance of apoptotic cell death at clinical doses of camptothecin is debatable. The TOP1-mediated double-strand breaks could be responsible for apoptotic cell death induced by high concentrations of camptothecin.

It has previously been suggested that increased proliferative kinetics, self-renewal ability, high tumorigenicity and invasiveness are the common features of CSCs (Jordan, Guzman & Noble 2006, Shackleton 2010, Fulda & Pervaiz 2010). We demonstrated that exposure of undifferentiated NTERA2 cells at different densities to different concentration of

camptothecin led to death of all the cells when exposed for long periods (24 hours), whereas some of the cells were not affected when they were exposed to camptothecin for shorter period (three hours). Our study on a small population of NTERA2 cells showed cell survival and cell resistance to camptothecin, and the NTERA2 cells were undifferentiated in most conditions but some cells underwent differentiation; therefore, we think that those cells are small populations of NTERA2 cells may have been resistant to camptothecin. Figure 4.8 shows the three different culture conditions: super-confluent, sub-confluent and confluent cells. A small number of differentiated cells were present in all the three culture conditions. Immunostaining revealed Oct4 expression in more than 95% of cells.

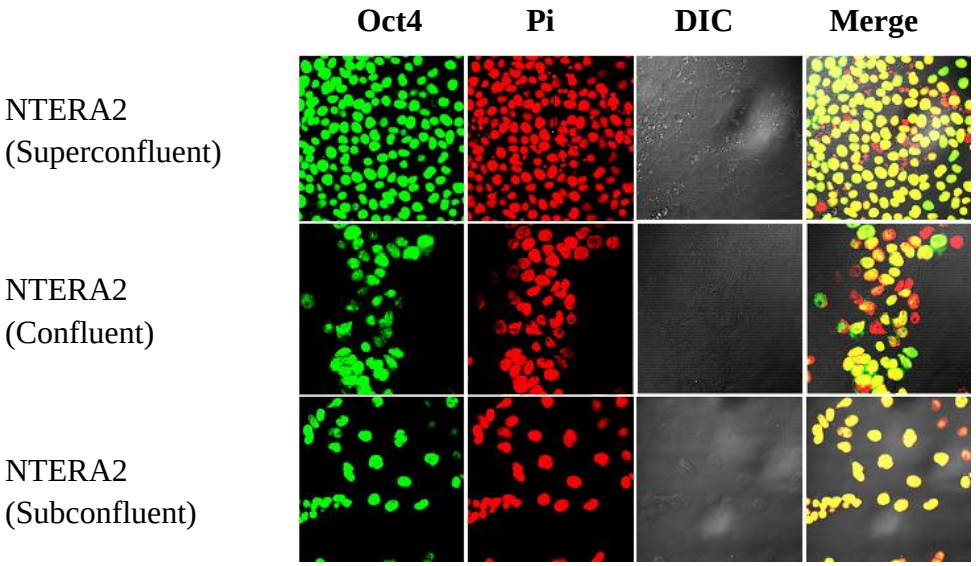



**Figure 4.7 shows Oct4 expression in three different cultures of NTERA2 cells.**

Finally, further studies are required to identify the pathways implicated in the behaviour of drug-resistant CSCs that survived exposure to camptothecin for three hours. A better understanding of the molecular pathways that control CSC differentiation and identification of new differentiation-inducing agents that specifically target these CSCs may offer novel treatment options for cancer. We will describe some of these pathways in detail in the subsequent three chapters in this thesis.

## Chapter 5 The effects of differentiation on DNA damage response pathways

### Introduction

Despite the recent advances in the diagnostic, protective and therapeutic modalities for cancer, it remains a serious health concern worldwide. Over the last few years, studies have to improved knowledge of the genetic, molecular and cellular mechanisms involved in carcinogenesis, resulting in the development of various intervention procedures. This chapter deals with one such novel intervention procedure, namely, differentiation therapy.

### 5.1 Differentiation the NTERA2 cells by hexamethylene bisacetamide

NTERA2 was obtained after passage of TERA2 in an athymic (nu/nu) (nude) mouse wherein a xenograft tumour with obvious teratoma characteristics was created (Andrews et al. 1984b). TERA2 and NTERA2 xenografts included several types of cells, primarily glandular structures and neural elements. Single-cell clones of NTERA2 were isolated, and the NTERA2 D1 clone (commonly known as NT2/D1) is a well-known cell line that is extensively used. NTERA2 cells exhibit features similar to human embryonic carcinoma (EC) cells such as 2102Ep, namely, the presence of the markers TRA-1-60, SSEA4 and SSEA3 and high levels of alkaline phosphatase (ALP). Interestingly, unlike many other human EC lines, TERA2-derived lines do not express any placental-like ALP activity and indicate no confirmation of trophoblastic differentiation when cultured at low cell density.

Differentiation of NTERA2 EC cells is identified not only by alterations in specific surface antigen but also by changes in predisposition to infection with certain viruses, especially human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV). While NTERA2 stem cells are immune to HIV and HCMV infections, the differentiated cells permit the replication of both these viruses (Gonczol, Andrews & Plotkin 1984b, Hirka et al. 1991b). In the case of HCMV, the resistance occurs due to inactivity of the major immediate-early promoter of the virus in the EC cells (Lafemina, Hayward 1988b, Nelson, Groudine 1986b). Further, several other genes exhibit marked regulation during NTERA2 differentiation. For instance, Oct4, which is expressed by EC and ES cells, is downregulated following exposure of NTERA2 cells to retinoic acid (Alexander Przyborski, Smith & Wood 2003)(S.A. Przyborski & P.W. Andrews, 2001). Simultaneously, the expression of some of the genes is induced following retinoic acid exposure. One such gene is a member of the Wnt family—Wnt13—which is not expressed by NTERA2 or different human EC cells, but is strongly

induced following retinoic acid exposure (Wakeman, Walsh & Andrews 1998b). Curiously, while Wnt1 is well known to be induced through differentiation of the mouse ECline P19 line (Papkoff 1994b), none of the other members of the Wnt family were expressed during NTERA2 differentiation. Members of the frizzled family of genes that encode putative receptors for Wnt are also expressed at different levels during NTERA2 differentiation, and a previous study hypothesised that Wnt signalling could play a potential role in regulating cells obtained through differentiation (Wakeman et al. 1998), which is a part of the Wnt signalling processes, is as well capable of inducing NTERA2 differentiation, and it has been hypothesised that this might show a possible route for EC cell differentiation to be modulated by Wnt signalling (Giesberts et al. 1999).

NTERA2 EC cells are susceptible to induction not just through retinoic acid but also via several agents, particularly hexamethylene bisacetamide (HMBA) (Andrews et al. 1990) and members of the bone morphogenetic protein (BMP) family (Andrews et al. 1994). NTERA2 cells induced with 3 mM HMBA can be distinguished from those induced with retinoic acid, as neural factors are usually not expressed, although NCAM is an obvious marker of differentiation in HMBA-induced cells. Some substances interfere in the nature of the cells induced by these agents. A previous study reported that smooth muscle actin induction occurs after exposure to BMP7 and, to a slightly lower extent, retinoic acid (Qualtrough 1998). Even if several of the markers typical of retinoic acid induction are not expressed shortly after HMBA treatment; eventually, the HMBA-induced cultures frequently express some of the markers typical of retinoic acid-treated cultures, and the presence of neurons is sometimes observed. Therefore, although the process of differentiation induced by these two agents (retinoic acid and HMBA) is dissimilar, these agents appear to interfere with one another and the pathways are not reciprocally exclusive. However, the nature of the non-neural cells observed in the differentiating NTERA2 cultures, induced by retinoic acid or HMBA, and has not been wholly distinguished.

### 5.1.1 Hexamethylene bisacetamide

HMBA is a clinically employed agent (Andreeff et al. 1992a) that was initially developed as an anticancer drug, which could be involved in the reactivation of the dormant reservoirs. HMBA leads to the release of pTEFb from HEXIM1 and precipitates Cdk9 enrolment to the HIV-1 5′ LTR via an unforeseen interaction with the transcription element Sp1 (Choudhary,

Archin & Margolis 2008). HMBA has been demonstrated to induce gene expression in latently-infected T-lymphoid and monocytic cell lines, and to cause downregulation of the receptor CD4 but not of the coreceptors CXCR4/CCR5 at the surface of peripheral blood mononuclear cells (PBMCs) (Klichko et al. 2006). Pilot human clinical trials have proposed that HMBA or other parallel compounds may therapeutically function in cells latently infected with HIV-1.

### 5.1.2 Action of HMBA

HMBA can induce apoptosis in isolated human myeloma cells. All the cell lines exhibited downregulation of Bcl-2, which is a recognised inhibitor of apoptosis. Further, this finding established that Bcl-2 overexpression rendered myeloma cells immune to HMBA-induced cell death, thereby reinforcing the relationship between HMBA and apoptosis, suggesting the possible benefit of HMBA and related compounds in the medication of multiple myeloma (Siegel et al. 1998a). Ouyang et al. 2004 studied human hepatocellular carcinoma cells and demonstrated that at low concentrations, HMBA can arrest cell growth and at higher concentrations it can induce apoptosis by lowering the Bcl-2/Bax ratio, thereby proposing another possible use of HMBA in the treatment of liver cancer (Ouyang et al. 2004). Further studies implied the potential application of HMBA in T acute lymphoblastic leukaemia (T-ALL), where it was shown to increase the predisposition of leukemic cells to apoptosis (Chiaramonte et al. 2008). The molecular mechanism of HMBA is yet to be elucidated; thus far, no studies have investigated the effects of HMBA in lung cancer. NFκB is a transcription factor that plays a central role in the control of inflammation, apoptosis and cell proliferation. The NFκB mechanism is constitutively switched on in most human cancers, and several studies have focussed on recognising the molecules that inhibit the NFκB mechanism (Gilmore, Herscovitch 2006, Greten et al. 2004, Aggarwal et al. 2006). The Akt pathway is important for cell survival and is an essential regulator of NFκB activation. (Gustin et al. 2004, Madrid et al. 2000, Mayo et al. 2002, Ozes et al. 1999, Coffer, Jin & Woodgett 1998). The ERK/MAPK pathway is another significant mechanism implicated in cell survival (Dent et al. 1999, Tran et al. 2001, Xia et al. 1995a) and has been indicated to induce NFκB responses by stimulation of p90 Rsk. (Richards et al. 1999, Panta et al. 2004, Ryan et al. 1993, Schouten et al. 1997). In an effort to identify the mechanism of HMBA, a study has investigated the influence of HMBA on the NFκB mechanism. It was found that HMBA-mediated sensitisation to cell death could be attributed partly to its capability to downregulate

the NFκB mechanism (Dey et al. 2008a). Studies have reported that HMBA simultaneously targets the MAPK and Akt mechanisms, both of which are critical for cell survival. Downregulation of both these mechanisms consequently affects the NFκB mechanism; this could be the mechanism by which HMBA represses NFκB activation (Dey et al. 2008a). The same study also suggested the possibility of application of this HMBA mechanism in multi-targeted therapy for controlling lung cancer and as therapy in combination with kinase inhibitors. They also supplied possible biomarkers to determine the tumour response to treatment with HMBA (Dey et al. 2008a).

HMBA is a hybrid polar compound that has been deliberately used to induce terminal differentiation of transformed cells. It has been shown to control cell proliferation many cancers such as acute myelogenous leukaemia (AML), solid tumours (liver) and myelomas (Li, Du & Huang 1996, Rifkind, Richon & Marks 1996, Andreeff et al. 1992b, Siegel et al. 1998b). Nevertheless, the mechanism of action of HMBA has not yet been clarified. Figure 5.1 illustrates a suggested mechanism of action of HMBA. Studies have demonstrated that HMBA increases cell cytotoxicity and represses *NF*κB gene expression in response to TNFα and IL1. The same study also shows that HMBA simultaneously downregulates the Akt and MAPK pathways, both of which are necessary for cell survival and growth and feed into the *NF*κB pathway (Ghosh et al. 2006). Therefore, HMBA may repress the TNFα-induced activation of *NF*κB via both these pathways (Fig. 5.1). Moreover, a previous study indicates that HMBA decreases the IKK kinase activity, *IκBα* phosphorylation and p65 phosphorylation at the essential Ser536 residue (Dey et al. 2008a). This study also suggested that some of the tumour response markers to HMBA in lung and breast cancer are downregulated in Akt and ERK phosphorylation. The Akt/PKB pathway is an essential survival pathway that plays a primary role in several cancers, including lung cancer (Vivanco, Sawyers 2002). The Akt/PKB pathway has been shown to be constitutively active in non-small cell lung cancer (NSCLC) cell lines, resulting in immunity to chemotherapy and radiation (Brognard et al. 2001). Consequently, improving the inducers that repress the Akt pathway would be of considerable benefit. Tissue microarray studies of tumour samples obtained from patients with NSCLC suggested p-Akt overexpression as an independent prognostic indicator, and patients with increased p-Akt had a significant survival disadvantage over patients with lower Akt phosphorylation, while p53 or Ki-67 expressions were not statistically significant prognostic indicators (David et al. 2004). A study by (Tang et al. 2006) compared immunohistochemical staining of normal and lung cancer samples and

reported that p-AKT overexpression and lack of PTEN expression in lung cancer cases is a poor prognostic indicator and is associated with poor differentiation and metastasis. Various agencies have illustrated how Akt activates the *NF*κB pathway. Sizemore et al. suggested that in response to IL1, Akt could activate *NF*κB by inducing p65 phosphorylation independent from its liberation from *IκBα* (Sizemore, Leung & Stark 1999). Madrid et al. suggested that Akt activates *NF*κB directly by utilising IKK or indirectly through IKK via p38 (Madrid et al. 2001). Another study reported Akt-dependent activation of IKK following the interaction of mTOR and IKK, thereby providing fresh insights into these processes and their clinical relevance (Dan et al. 2008). Established data propose that HMBA represses *NF*κB by downregulating Akt phosphorylation, followed by repression of an IKK kinase process and following decrease in p65 phosphorylation at the Ser536 residue, which is essential for chromatin remodelling. The extracellular signal-regulated (ERK) or mitogen-activated protein kinase (MAPK) cascade is another significant pathway in cell survival and proliferation (MacKeigan, Collins & Ting 2000, Xia et al. 1995b). Its activation is mediated by MEK1, a necessary intersection in the pathway with ERK1 and ERK2 being its identified targets. (Vicent et al. 2004) carried out immunohistochemical investigations of samples obtained from patients with NSCLC, and observed increased phosphorylation of ERK1/2 in the patient population with histologically proven NSCLC, suggesting that ERK1/2 was stimulated. This ERK1/2 activation was found to be connected with metastases and advanced and aggressive NSCLC tumours. (Brognard, Dennis 2002) demonstrated the role of ERK1/2 activation in promoting immuno to chemotherapy and apoptosis. HMBA represses TNFα-induced phosphorylation of ERK1/2 in a time-dependent manner. If studied in detail, the Akt and ERK/MAPK cascade have great potential as prognostic indicators in lung cancer and further studies on inducers that inhibit both these processes, such as HMBA, have considerable potential in therapy.

It is possible that HMBA affects a phosphatase or kinase linked to both the Akt and MAPK pathways, and efforts are ongoing aimed at further understanding its mechanism of action (Dey et al. 2008a). While inhibition of both these pathways are being extensively studied in the context of lung cancer, a recent study (Tang et al. 2006) indicated that p65 tissue nuclear expression was increased in both small cell and non-small cell lung cancers, suggesting the possible function of inflammation in the early pathogenesis of lung cancer. Therefore, inducers such as HMBA that repress the *NF*κB pathway and also inhibit TNFα-induced activation of *NF*κB target genes are implicated in anti-apoptosis (Bcl-xL and FLIP),

proliferation (Cox-2) and tumour invasion (ICAM-1). Apart from lung cancer, HMBA has been shown to repress TNFα-induced activation of *NF*κB target genes in breast cancer cells with various mutation classes; this is especially significant in obtaining a deeper understanding of the mechanism of action of HMBA. The ideal cancer treatment strategy would include a combination of drugs with the same target that synergistically affect different mechanisms. This is considered the optimal method to combat drug resistance and improve the efficacy of chemotherapy. An important problem in cancer drug progression is the discovery of biomarkers of response (Park et al. 2004).

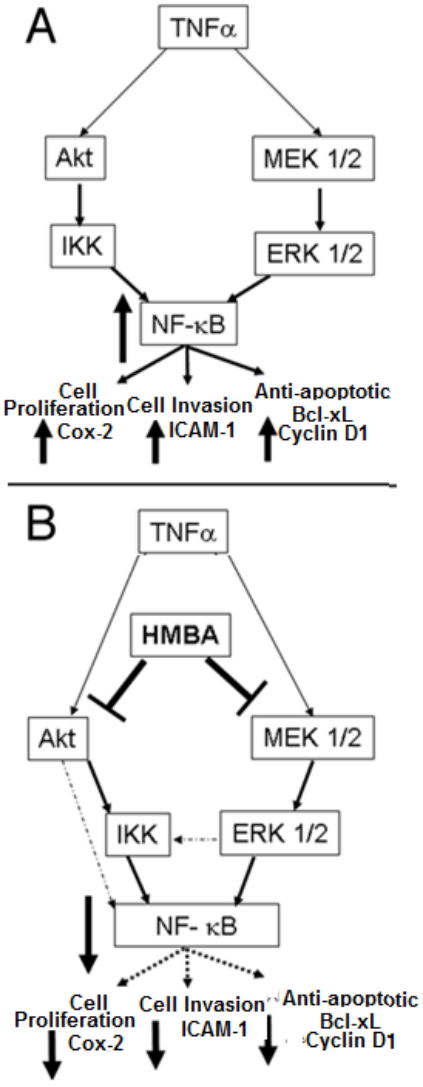



**Figure 5.1. Flowchart illustrating the mechanism of action of HMBA and its inhibition of the NFκB process.** (A) TNFα-induced activation of the NFκB process. (B) HMBA represses both Akt and MAPK processes and also downregulates the NFκB process and its gene products (Dey et al. 2008b).

### 5.2.3 Oct4 as a master key for NTERA2 cells

The strongest evidence suggesting that stem cells are the potential origin of the "cancer" stem cells and that stem cells are targets for carcinogenesis was obtained when studies reported that the transcription factor Oct3/4 (later known as Oct4), required for maintaining "stemness," was found in embryonic stem cells but not in normal tissues (Pesce, Schöler 2001). Later, Oct4 was detected in a few tumours. These authors concluded that Oct4 expression in tumour cells was "restored" by the transformation process (Monk, Holding 2001b, Gidekel et al. 2003). In my experiment, I used this stimulus to determine whether the undifferentiated or differentiated NTERA2 cells were the targets where the cancer stem cell (CSC) process was initiated. Since NTERA2 cell lines were treated with HMBA were available in our laboratory, we evaluated the Oct4 expression in these cells in two ways (differentiated and undifferentiated cells) and all the test samples were treated with four anticancer drugs, namely, camptothecin, aphidicolin, phleomycin and hydroxyurea. The results indicated that Oct4 was expressed in all the CSCs (undifferentiated stage) but not in the differentiated cells. This can be seen in the undifferentiated cells. Our results suggested that 100% of the NTERA2 expressed Oct4.

These findings are consistent with the notion that tumours contain cells with for stem cell biomarkers (Oct4), suggesting that they originate from the normal adult stem cells.

## 5.2 Results

### 5.2.1 Analysis of NTERA2 HMBA induced differentiation

NTERA2 offers a unique model system for studying human stem cell and cancer stem cell biology. NTERA2 cells can be differentiated using various agents to generate populations of proliferating cells which no longer have the markers of stem cells (as Oct4). Our aim in this chapter is to differentiate NTERA2 down a non-stem like lineage and determine whether the cellular response to DNA damage-inducing agents alters during the differentiation period. This provides an *in vitro* system with which to dissect the mechanisms of action of agents which might be applied to differentiation therapies. Use of NTERA2 has the added advantage that the stem-like and differentiated cell populations have an identical genetic lineage.

Here HMBA is to be used of the induction of differentiation. Given this, it was essential to establish that differentiation of NTERA2 can be induced under the laboratory conditions used here. Moreover, it was essential to test that the differentiation potential of the NTERA2 stocks used here has been maintained. For these reasons differentiation experiments were set up in which stem markers were followed, with an expectation that the key marker for stemness, Oct4, would be lost from the cell population upon differentiation.

NTERA2 cell cultures were set up and monitored over eight days by western blotting and immune fluorescence to evaluate levels of Oct4 (with the expectation that they will be reduced). Four independent culture sets were established, one left untreated, one treated with 3 mM HMBA and to others treated with 3 mM HMBA and 1 µl or 2 µl DMSO (as this is the solute for some of the genotoxic agents to be used, such as CPT). Different culture sets of four were established with distinct cell seeding densities (50,000, 75,000, 100,000 cells per 2 ml culture). The data shown are for 75,000 seeded cells. Each experimental set (within the sets of four) was established with 8 different cell cultures, one for each day of the experiment. Each culture was set up on day 0 and every day one culture was taken for western blot analysis and immune fluorescence. All experiments were triplicate to permit statistical analysis of western blot data. Figure 5.2 shows western blot data for Oct4 levels for all four conditions (no HMBA; + HMBA; +HMBA/ 1 µl DMSO; +HMBA / 2 µl DMSO) over a 6 day period (days 7 and 8 are not. The gels are shown use a tubulin loading/normalisation control. The values of Oct4 protein level are taken as a percentage of the untreated control

(no HMBA / no DMSO). Cultures were also immune stained for Oct4 and the proliferation marker Ki-67; Figure 5.3 shows examples of Oct4 stained and Ki-67 stained cells for days 0 – 6 for following HMBA treatment. As can be seen from the figures (5.2 & 5.3), the cells treated with HMBA lose Oct4 protein (as measured by western blot), and Oct4 levels start to diminish on day 1 and are almost undetectable from about 4 days onward. The staining for Ki-67 demonstrates a relatively uniform staining in all cultures indicating that all cultures maintain proliferative potential and activity. These data indicate that the NTERA2 cultures have retained differentiation capability, can be induced to differentiate under the laboratory conditions used here and that proliferation is maintained during and following the differentiation process.

### 5.2.2 Effect of HMBA on proliferation of NTERA2

In conjunction with the analysis of differentiation (above), it was important to establish whether HMBA treated cells maintained a proliferative ability. The Ki-67 staining of the HMBA treated cells indicated the cells had indeed retained proliferative potential. To analyse this in more detail we measured cell proliferation of NTERA2 cells over a four day period without HMBA treatment and with HMBA (3 mM) treatment.

Cells were treated with HMBA for a 24 hour period and then the HMBA containing medium was washed off and replaced with fresh pre-warmed medium. Seeding densities of 50,000 / 2 ml, 75,000 / ml and 125,000/ 2 ml were used. A similar growth response was observed for all three seeding densities. Figure 5.4 shows the 50,000 cell seeding density (data for other cultures is not shown – a similar pattern was seen). As can be seen NTERA2 cells continue to proliferate following HMBA treatment, but the rate of proliferation is slightly less than untreated cells. This indicates that HMBA treatment reduces proliferation rates slightly, but still permits cells to divide, which is essential if the response of cells to DNA replication-dependent DNA damage is to be assessed.

### 5.2.3. Preliminary assessment of CPT on differentiated NTERA2 cells

Having established that NTERA2 cells differentiate and maintain proliferative capacity following HMBA treatment an initial assessment was carried out to determine the response of HMBA differentiated NTERA2 to CPT. Cultures were set up with and without HMBA (see

above) and permitted to proliferate for 8 days, when differentiation should have been completed (see above). At 8 days CPT was added (either 1 µM or 2 µM) and the cells were incubated for 3 hours prior to the medium being changed to fresh pre-warmed medium without CPT. Different cell seeding densities were used (50,000, 75,000 and 125,000) and the data shown in Figure 5.5 are for 75,000 cells per 2 ml well; all seeding densities gave similar patterns indicating that within this seeding range responses are similar. All cultures were carried out in triplicate and mean values presented. Figure 5.5 shows that whilst the HMBA treated cells proliferate more slowly, the percentage of cells killed by 3 hour treatment with CPT is less than that of cells not differentiated with HMBA. Cells untreated with HMBA have approximately 2% and 1.8% survival in response to 1 µM and 2 µM CPT respectively, whereas the HMBA differentiated NTERA2 exhibit approximately 12.5% and 10% survival following 1 µM and 2 µM CPT respectively. This small difference in survival might simply be due to the slightly slower proliferation rate of differentiated, HMBA treated cells (as CPT is only toxic for dividing cells) (see Discussion). When a more chronic CPT exposure time of 24 hours was used both differentiated and undifferentiated cells showed 100% killing (data not shown), indicting there is no major difference in the response to CPT.

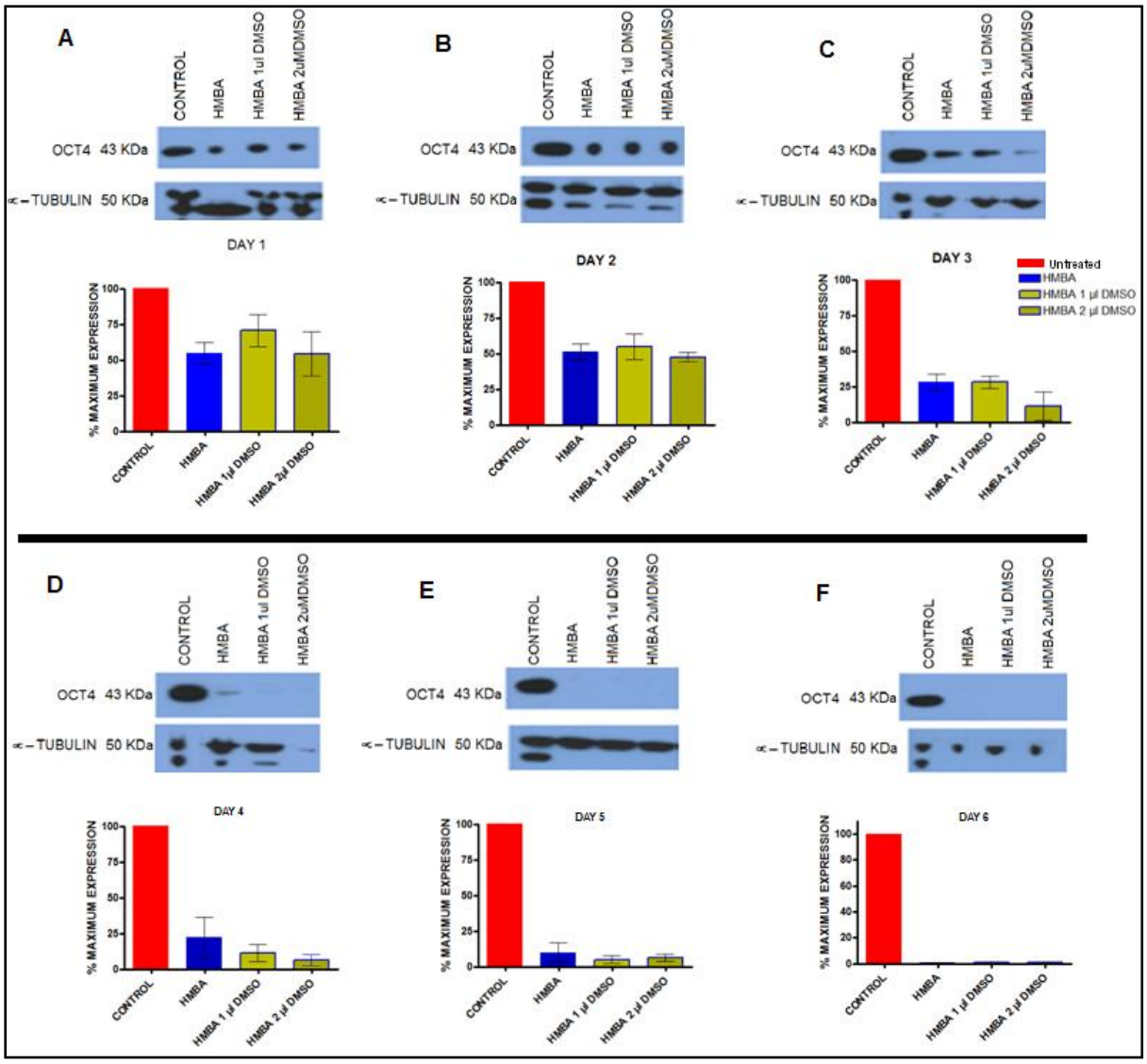



**Figure 5.2 Differentiation status and Oct4 gene expression in NTERA2 with HMBA and without treatment. The expression of markers of pluripotency (Oct4) is shown.** In each case, data represents changes in expression in the differentiate state during six days after exposure the cells to 3 mM HMBA and western blots were scanned by using (Sion Image program). In NTera2 cells, differentiation status is confirmed by decreases in expression of Oct4. Error bars represent Standard Error of Mean and the *p* value is derived by one way ANOVA in all cases. (A) Level of Oct4 after treatment the cells with HMBA for one day. (B) Level of Oct4 after treatment the cells with HMBA for two days. (C) Level of Oct4 after treatment the cells with HMBA for three days. (D) Level of Oct4 after treatment the cells with HMBA for four days. (E) Indicate to level of Oct4 after treatment the cells with HMBA for five days. (F) Level of Oct4 after treatment the cells with HMBA for six days.

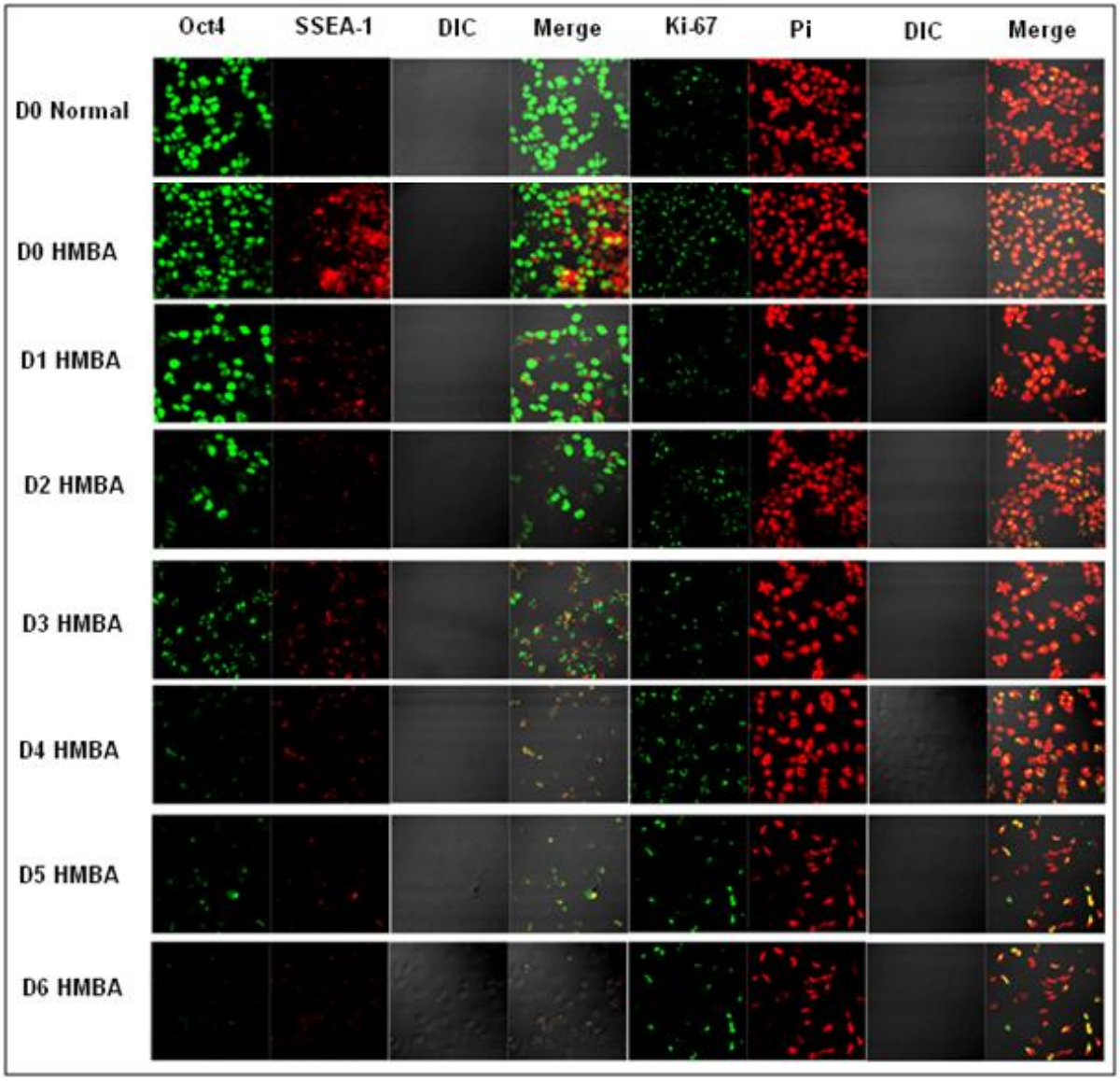



**Figure 5.3. Immunoflurescent staining shows decreased Oct4 expression in NTERA2 cells during six days after treatment with HMBA indicating loss of self-renewal during six days.**(A). It indicates of Oct4 expression . (B) It indicates phase contrast. (C) Indicates Ki-67. (D) Pi nucleus stain. (E) It indicates phase contrast. (F) It indicates to merge between Ki-67 and Pi stain.

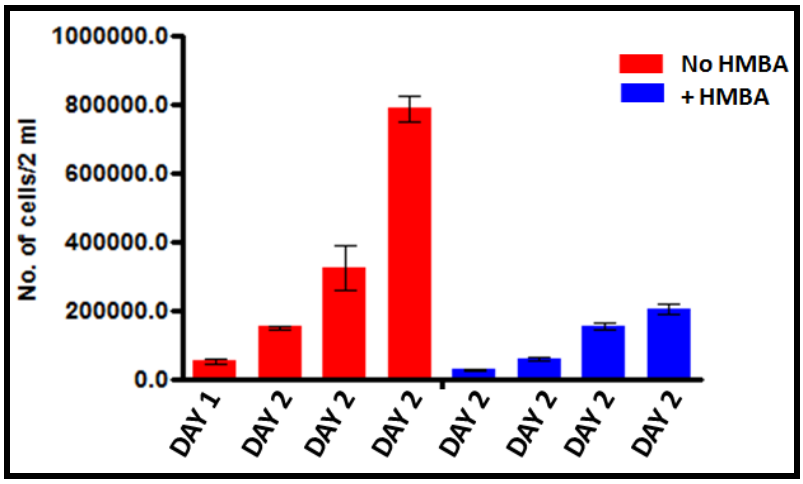



**Figure 5.4. The effect HMBA as potential inducer on the proliferating of NTERA2 cells during four days.** Untreated cells (red) were seeded at density of 50,000 cells/ well in 24-wellplate in DMEM medium without treatment. HMBA treated cells were also seeded at dendity of 50,000 cells/ well were with DMEM medium containing with 3 mM HMBA. Mean cells numbers were generated from three independent experiments. Statistical significance in cellular response to HMBA was assessed by the two-way ANOVA with comparison test and Student's *t* test. NTERA2 cells were significantly reduction with HMBA comapsion without HMBA ($p < 0.001$) for all day 4.

### 5.2.4 NTERA2 response to DNA damaging agents during differentiation

Following the preliminary analysis of differentiated and undifferentiated cells to CPT the analysis was extended by studying the response to DNA damaging agents during the differentiation processes. NTERA2 cultures were set up so that the sensitivity to the agents could be tested on every day of an 8 day differentiation period. At the start of each experimental set 8 cultures of undifferentiated NTERA2 cells were set up in duplicate (all experiments were also triplicate to obtain data which could be subjected to statistical analysis). All cultures were induced to differentiate using HMBA (see page 108). On each day, one duplicate pair of cultures was removed for analysis, one being exposed to a DNA damaging agent and one being left without DNA damage, both cultures were then incubated in the DNA damaging agent for a short period (3hours) and then incubated to 24 hours. Both cultures were then analysed by western blot for the presence of Oct4 and cell numbers were counted by haemocytometer. Using this method the cell survival levels were determined for each day of the 8 day differentiation period.

#### 5.2.4.i Response to CPT

1 µM and 2 µM CPT exposure was used and both concentrations resulted in similar sensitivities. Figure 5.6 shows the response to 2 µM CPT. As can be seen at day 0 (with no HMBA) the cells show a survival of about 2%, as had previously been shown (see above). During differentiation the percentage of the cells surviving increases slightly, peaking on day 3. These data suggest a subtle resistance to CPT developing during the differentiation process; as indicated above, this might be due to the reduced proliferation rate following HMBA treatment (see Discussion). The western blot data are shown for the CPT treated cells (after 24 hours incubation); for days 0 to day 2 cellular survival levels were too low to obtain significant whole cell extract material, as measured by the loss of α-tubulin (control) signal (Figure 5.6 A). However, it is clear that from day 3 onwards there is no detectable Oct4 present indicating that differentiation had occurred in the HMBA + CPT treated cells.

#### 5.2.4.ii Response to Aphidicolin

Cell cultures were treated as above for CPT, but the CPT was replaced by 1 µM aphidacolin, a DNA polymerase inhibitor. As can be seen from Figure 5.7A, significant levels of Oct4 are no longer detected from about day 3 to day 4 onward, indicating that differentiation was

occurring in these cultures. A relatively uniform response to aphidicolin is observed. This indicates there is no measurable change in the response to DNA replication inhibition during NTERA2 differentiation in response to HMBA.

### 5.2.4.iii Response to hydroxy urea

Cell cultures were treated as above for CPT, but the CPT was replaced by 1 μM hydroxy urea (HU), a ribonuclease reductase inhibitor which reduces cellular levels of nucleotides and thus inhibiting DNA replication. As can be seen from Figure 5.8A, significant levels of Oct4 are no longer detected from day 3 onward, indicating that differentiation was occurring in these cultures. As for aphidicolin, as relatively uniform sensitivity is observed for HU, indicating there is no inherent difference in the response to this drug during differentiation.

### 5.2.4.iv Response to phleomycin

In addition to CPT two agents capable of inducing DNA damage were tested (Aphidicolin and HU; see above), both of which inhibit DNA replication. Both these agents differ to CPT in that they do not necessarily generate double-stranded DNA breaks (although they are capable of this). Given the fact that CPT seems to cause a subtle increased sensitivity to Oct4 expressing NTERA2 cells than these two agents (see above) another agent was tested which is known to generate DSBs directly, phleomycin. Figure 5.9 shows the response to pheleomycin. As for the other experimental sets Oct4 signal is lost from approximately day 3 onwards, indicating the differentiation programme is proceeded as in these cultures. As can be seen, there is a slight difference in the sensitivities to phleomycin, with day 0 and day 1 showing slightly higher levels of sensitivity to this agent relative to the other days (days 2-8). The correlation between Oct4 levels and sensitivity are not exact, but there is a subtle pattern (i.e. high Oct4 correlates with increased phleomycin sensitivity).

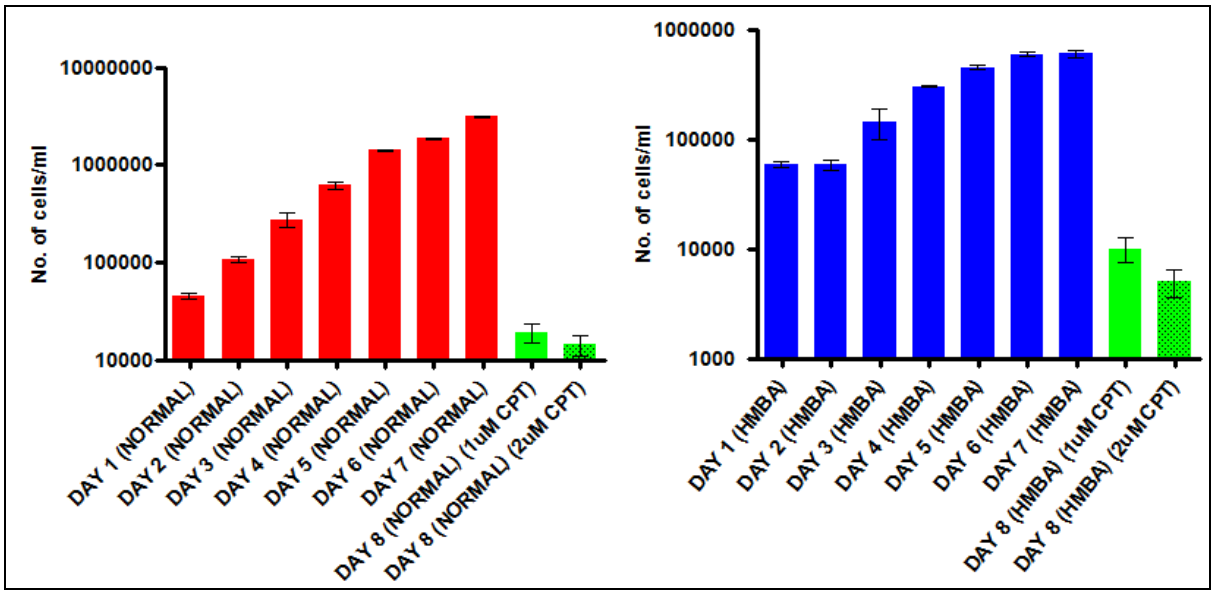



**Figure 5.5. Two charts display the effect of two different concentrations 1µM and 2 µM camptothecin on undifferentiated and differentiated NTERA2 cells after day 7.** (A) 75,000 NTERA2 cells were seeded and treatment by anti-cancer drugs 1µM and 2 µM CPT after day 7. (B) 50,000 NTERA2 cells were seeded in medium containing 3 mM HMBA and treated by anti-cancer drug 1µM and 2 µM CPT after day 7. Cytotoxic responses to camptothecin were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to different concentrations of camptothecin was assessed by the two-way ANOVA with comparison test and Student's t test. NTERA2 cells were significantly hypersensitive from 1µM and 2 µM CPT ($p < 0.001$) for all days and three stars were donated on the columns.

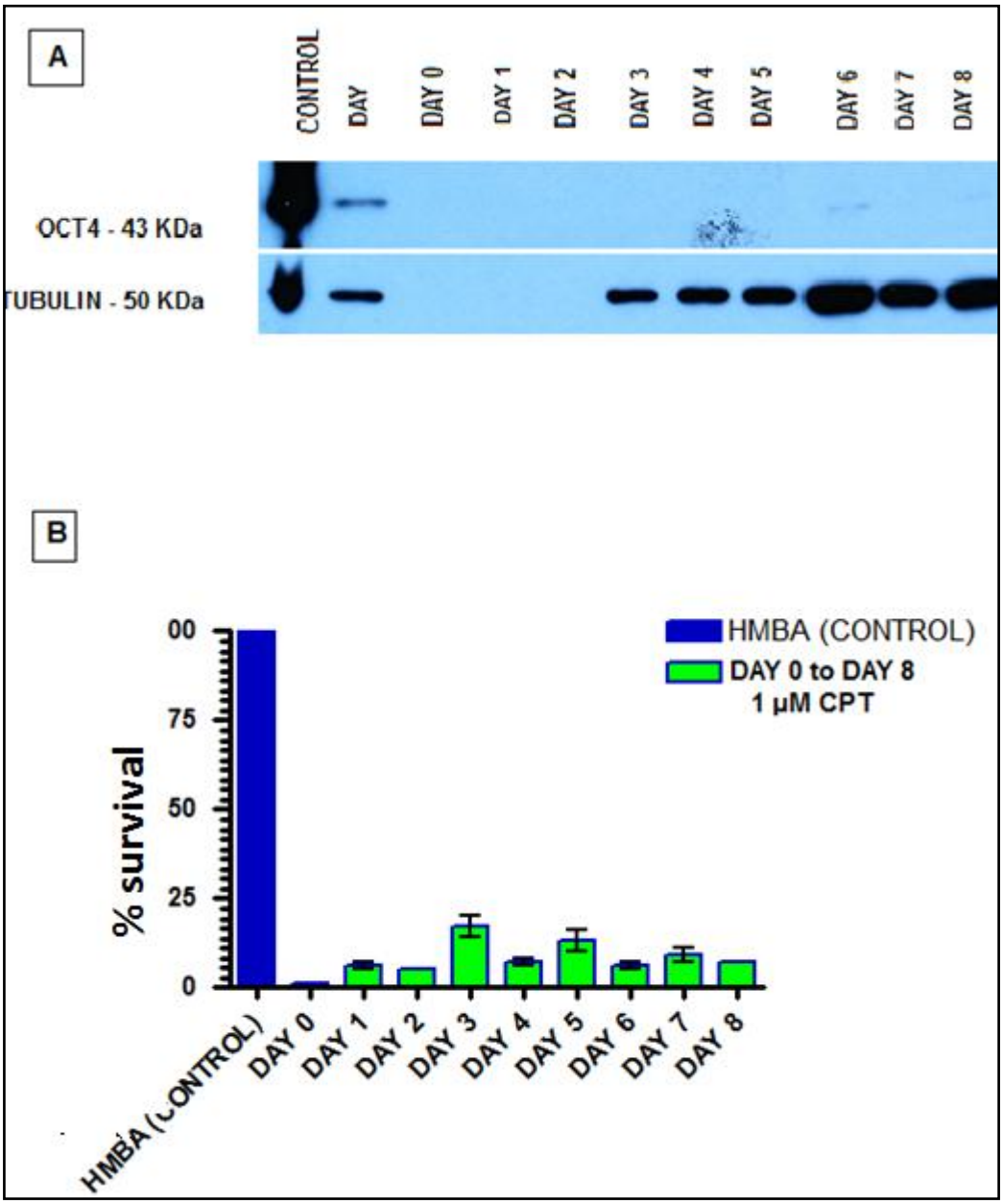



**Figure 5.6. The effect camptothecin on differentiated NTERA2 cell after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days and alph-tubulin was control. (B) Percentage of survival cells for eight days and cytotoxic responses to 2 µM CPT were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to 2 µM CPT was assessed by the two-way ANOVA with comparison test and Student's t test. NTERA2 cells were significantly sensitive to 2 µM CPT ($p < 0.01$) for all days comparing with control.

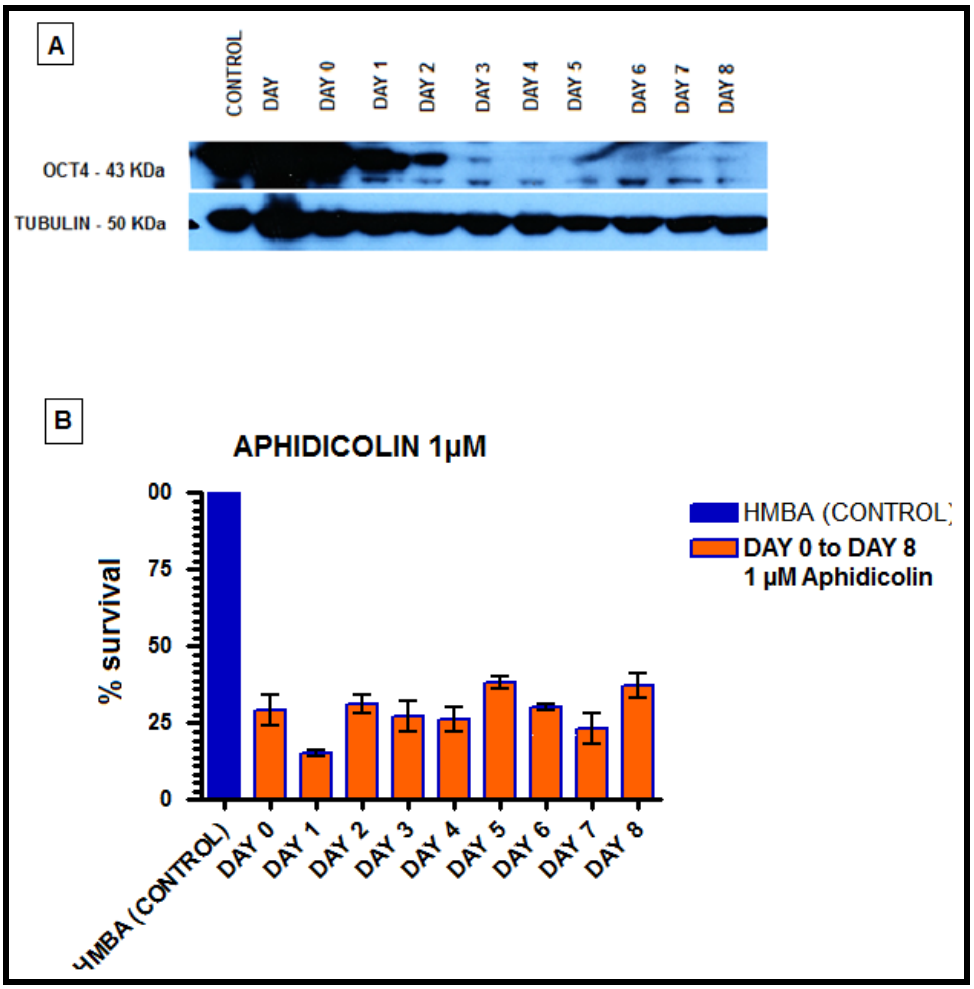



**Figure 5.7. The effect aphidicolin on differentiated NTERA2 cell after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days and alph-tubulin was control. (B) Percentage of survival cells for eight days and cytotoxic responses to aphidicolin were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to aphidicolin was assessed by the two-way ANOVA with comparison test and Student's t test. NTERA2 cells were significantly sensitive to aphidicolin ($p < 0.01$) for all days comparing with control.

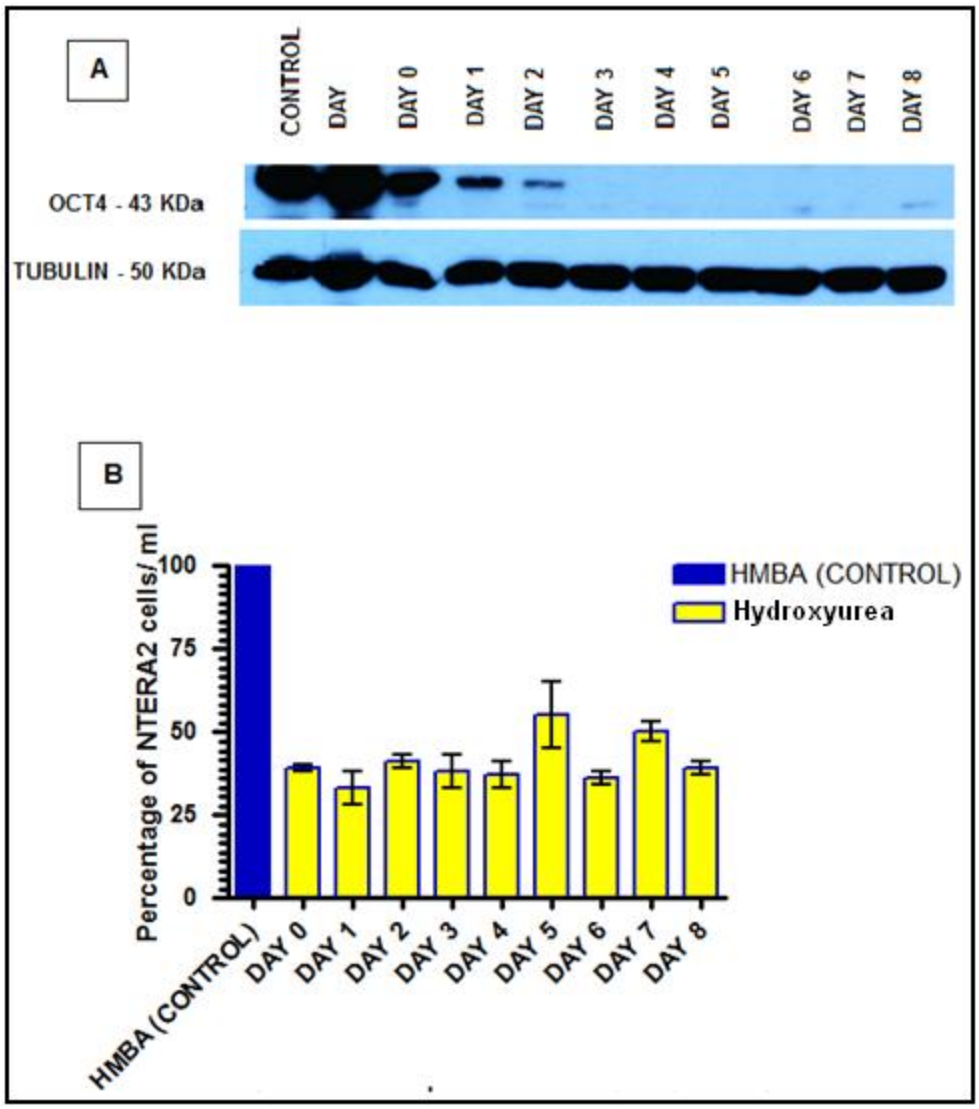



**Figure 5.8. The effect 1 µM hudroxyurea on differentiated NTERA2 cell after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days and alph-tubulin was control. (B) Percentage of survival cells for eight days and cytotoxic responses to 1 µM hudroxyurea were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to 1 µM hudroxyurea was assessed by the two-way ANOVA with comparison test and Student's t test. NTERA2 cells were significantly sensitive to 1 µM hudroxyurea ($p < 0.01$) for all days comparing with control.

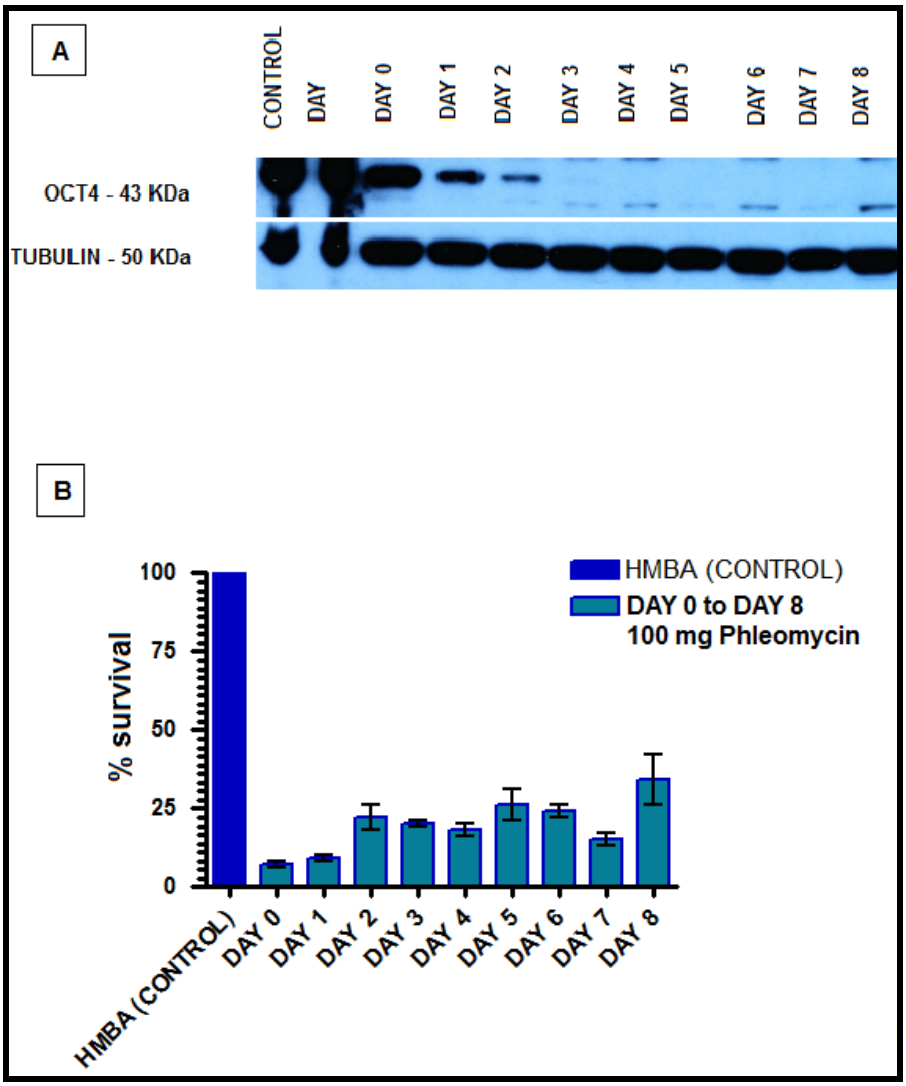



**Figure 5.9. The effect 100 mg phleomycin on differentiated NTERA2 cell after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days and alph-tubulin was control. (B) Percentage of survival cells for eight days and cytotoxic responses to 100 mg phleomycin were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to phleomycin was assessed by the two-way ANOVA with comparison test and Student's t test. NTERA2 cells were significantly sensitive to phleomycin ($p < 0.01$) for all days comparing with control.

## 5.4. Discussion

In the previous chapter we demonstrated that NTERA2 exhibited sensitivity to the topoisomerase inhibitor CPT. NTERA2 was employed in this study as is shares many characteristics with human embryoic stem cells (Pal, Ravindran 2006) and extensive gene expression analysis and profiling of NTERA2 has shown that a strong correlation to validated human ES cells (Schwartz et al. 2005, Sperger et al. 2003). Indeed, the pluripotency regulating transcription factor Oct4 is present in NTERA2 and is required for pluripotency in this cell line (Pal, Ravindran 2006).

The study of NTERA2 was triggered by the preliminary observation that a mouse ES cell line and non-isogenic mouse embryonic fibroblast cells exhibited different sensitivities to CPT (Wakeman and McFarlane, unpublished, personal communication). This implied that ES cells might have distinct mechanisms for regulating their genome stability. Indeed this observation is supported by a variety of other observations which indicates ES and ES-like cells have unique pathways to regulate their genomic integrity (Momčilović, Navara & Schatten 2011). Indeed, recent work has directly linked transcription of Oct4 via the nucleotide excision repair pathway complex XPC suggesting a unique genome stability monitoring process in stem cells, perhaps also including NTERA2 (Fong et al. 2011).

In this study it was observed that NTERA2 cells in the undifferentiated state (no HMBA / expressing Oct4) appeared to be slightly more sensitive to the DNA damaging agent CPT than cells which had been induced to differentiate using HMBA (see page 115). CPT works by inhibiting topoisomerase I. Topoisomerase I normally functions to regulate DNA super coiling and it does so by forming breaking the sugar-phosphate back bone of DNA by forming a transient covalent phosphotyrosyl intermediate between the broken strand and a tyrosine residue on the topoisomerase (Lorence, Nessler 2004, Keck, Berger 1999, Rasheed, Rubin 2003, Pommier 2006, Rothenberg 1997b). CPT acts by stabilising the transient covalent complex between the protein and the DNA resulting in a prolonged unsealed nick in the duplex (Hsiang et al. 1985). These relatively stable nicks can be converted to DNA DSBs during DNA replication when the replisome encounters them, making a one sided DSB which will require recombinogenic repair mediated by homologous recombination factors. This dependence upon DNA replication to generate DSB damage might provide an explanation for

why undifferentiated (no HMBA) NTERA2 cells show more sensitivity to CPT than the differentiated. This work has demonstrated that HMBA treated, differentiating / differentiated cells proliferate (they express the proliferation marker Ki-67; Figure 5.3) and the cell number continues to increase (Figure 5.4). However, their rate of proliferation appears to be lest than undifferentiated NTERA2. This reduced proliferation rate might simply indicate that the levels of DNA damage generated by CPT may be less in cell populations which divide more slowly (HMBA treated NTERA2), or alternatively these cells have more time to recover from the damage acquired (or a combination of both). This possibility cannot be dismissed by studying these data, but it remains possible that there is a subtle distinction in the mechanistic pathways governing the response to CPT (possibly DSBs) between differentiated and undifferentiated NTERA2 (see below).

Following this two DNA replication inhibitors were employed to determine whether or not NTERA2 responded differently to this agent during differentiation. These agents should result in failures in DNA replication, which have the potential to generate failed DNA replication forks without causing DSBs (McFarlane, Al-Zeer & Dalgaard 2011 ), although it can result in DSBs (Liu, Kuo & Melendy 2003). Aphidicolin was shown to repress growth of human cultured cells over 30 years ago (Pedrali-Noy, Spadari 1979, Bucknall et al. 1973c) and it has been shown to be an inhibitor of polyα-like DNA polymerases which generates regions of un-replicated DNA (Brox, Hunting & Belch 1984, Wang et al. 1999, Hellman et al. 2000, Hellman et al. 2000). HU inhibits ribonucleotide reductase and thus exhausts nucleotide pools resulting in failures in DNA replication progression (Mirkin, Mirkin 2007a, Feng et al. 2006, Bianchi, Pontis & Reichard 1986, Matsumoto, Rey & Cory 1990, Koç et al. 2004).

Treatment of differentiated and undifferentiated NTERA2 with these two agents resulted in a relatively uniform sensitivity. This is clearly distinct from the response shown to CPT and the subtle increased sensitivity observed in undifferentiated NTERA2 to CPT was not reflected upon treatment with these two agents. This observation challenges the postulate that the subtle difference in sensitivities observed in response to CPT was due to cell proliferation, as one might have anticipated a similar response in the case of these two agents if this was correct. The uniformity of sensitivity to aphidicolin and HU suggests that the distinction between differentiated and undifferentiated NTERA2 in response to CPT might indeed reflect an inherent difference in the way these cells deal with genomic insult, most likely DSBs. It is

possible that CPT generates DSBs, whereas the levels of aphidicolin and HU employed here might not be sufficient to induce DSBs, rather they generate failed forks which do not get processed to DSBs and forks are reactivated effectively without a DSB intermediate.

The data obtained for phleomycin go some way to supporting this latter proposal. Phleomycin is a copper-containing protein acquired form *Streptomyces verticillus* (Maeda et al., 1956) and has been used for many years and an anti-tumour agent (Bradner, Pindell 1962a). Whilst the exact mechanism of action for phleomycin has not been elucidated carefully, it is widely accepted to be an agent that directly generates DSBs given the similarity of action to the DSB inducer bleomycin (Saito, Andoh 1973). Phleomycin treatment also resulted in a slightly elevated sensitivity of the undifferentiated (no HMBA) NTERA2 relative to cells which were differentiating (reduced Oct4) or differentiated (no Oct4). This observation supports the possibility that there is an inherent difference in the mechanism of action between stem-like cells (undifferentiated NTERA2) and differentiated cells in the way they process and respond to significant levels of DSBs, but not failures in DNA replication which result in fork collapse with no increased associated DSB formation.

Previous studies have demonstrated that there are distinctions between multipotent haematopoietic stem cells and proliferating myeloid cells (Francis, Richardson 2007) and that mouse stem cells have distinct homologous recombination regulatory systems relative to isogenic mouse embryonic fibroblast (MEF) cells (Cervantes et al. 2002). The preliminary observations of McFarlane and Wakeman, which showed that mouse ES cells and non-isogenic MEFs responded distinctly to CPT also pointed to a difference in DSB repair pathways between ES cells and differentiated cells. The data obtained here do not point to a clear distinction between ES-like human embryonal carcinoma (EC) cells and their differentiated counter parts, but there is a suggestive hint from these data that there is a subtle distinction to the response to DSBs. Recent findings have demonstrated that there are differences between mouse and human ES cells in their response to ionizing radiation-induced DSBs (Banuelos et al. 2008a) and it might be the case that the data generated here indicate that the pathways in humans are not heavily altered between the undifferentiated and differentiated state. However, there are many other factors which have not been least of which is the nature of the model system we have used. NTERA2 does not have a normal human karyotype and the changes generated over the many passages these cells have

undergone might have resulted in NTERA2 developing genome repair mechanisms which are distinct from human ES cells or the NTERA2 progenitor cells.

Despite the lack of a large margin differences in response to DNA damaging agents, the subtle difference observed in NTERA2 might provide a platform for the study of genome stability in human ES like cells. Induced pluripotent human stem cells might also present a model system, but they too appear to have draw backs (Blasco, Serrano & Fernandez-Capetillo 2011). Given the potential clinical importance of human stem cells and the exponentially growing interest in these cells, it is interesting to note that cultured human ES cells take on genomic rearrangements in culture for examples, see (Draper et al. 2003, Spits et al. 2008). To safely employ these cells clinically it is essential we gain a full insight into the mechanisms governing their genomes and comparisons of NTERA2 to other ES and ES-like systems and other differentiation pathways will provide a characterisation of genome stability pathways for clinically important biological cells.

## Chapter 6 Analysis of the effect of HMBA on a non-stem cancer cell line

### 6.1. Introduction

p53 and p21 are stimulated in cells after DNA damage. This transiently arrests cells at the G1 and G2 checkpoints of the cell cycle (Bartek, Lukas 2001, Taylor, Stark 2001, Colman, Afshari & Barrett 2000). These occurrences give the cells enough time to maintain and repair damaged DNA, thus stopping harmful mutations in the cells that would otherwise be eventually transferred to daughter cells (Lane 1992, Levine 1997). DNA damage is sensed by the ataxia-telangiectasia mutated protein, which is a member of the phosphoinositol-3 lipid kinase families (Jeggo, Carr & Lehmann 1998, F. Lavin, Kum Kum Khanna, M. 1999). P53 is one of the key targets that are subjected to activation by ataxia-telangiectasia mutated catalyzed phosphorylation (F. Lavin, Kum Kum Khanna, M. 1999). Activated p53 induces the expression of several proteins involving p21, which is a general inhibitor of the cyclin-dependent kinases (Cdks)1 (Xiong et al. 1991), and is needed to arrest cells at the G1 and G2 checkpoints of the cell cycle following DNA damage (Deng et al. 1995, Bunz et al. 1998, Waldman, Kinzler & Vogelstein 1995).

DNA damaging agents, including γ-irradiation and inhibitors of the nuclear topoisomerases I and II (Top1 and Top2), are extensively incorporated in treatments for cancer patients. The progression of numerous kinds of human cancers (50%) is connected with the loss of p53 or mutations in p53. Hence, the connection between p53 level and the sensitivity of cancer cells to kinds of drugs, specifically DNA damaging agents, has been extensively inspected (Lowe 1995, Morgan, Kastan 1997, Brown, Wouters 1999a).

We used an adherent epithelial cell line, HCT-116, originating from a colorectal carcinoma that has a mutator phenotype. The apoptotic influence of DNA damaging agents on the HCT116 human colon cancer cells was displayed to be obstructed by p21, which was expressed by a p53-dependent process (Waldman et al. 1996, Bunz et al. 1999). The results acquired from a clonogenicity assay indicated that the long-period survival of HCT116 cells following DNA damage was independent of both p53 and p21 (Brown, Wouters 1999b). Hence, these studies show that the necessity for p53 in the mechanism of apoptosis depends on whether the cells are of rodent or human origin. Furthermore, opposite conclusions can be drawn regarding the connection between p53 and drug sensitivity because of variability in the experimental conditions addressed to study the influence of DNA damaging agents (Brown,

Wouters 1999b). Thus, it is probable that the loss of clonogenicity of cancer cells following DNA damage can cause an irreversible arrest of the cell cycle rather than the loss of viability. Apposite is the demonstration that p53 and p21 have significant functions in the senescence progression of normal human cells (Noda et al. 1994, Wynford-Thomas 1996, Brown, Wei & Sedivy 1997, Itahana, Dimri & Campisi 2001). Therefore, it is possible that p53 and p21 are needed for the senescence development of human cancer cells following DNA damage. Regarding this relationship, we examined the long-term effect of four DNA damage agents that were treated with and without HCT116 cells in existing HMBA.

## 6.2 Result

### 6.2. 1 HCT116 response to DNA damaging agents during differentiation

Following the preliminary analysis of the response of differentiated and undifferentiated cells to four poisons with HMBA and RA in the previous chapter, the analysis was extended by studying the response to DNA damaging agents during the differentiation processes by using HCT116 cells, which do not have the properties of cancer stem cells.

#### 6.2.1.1 Response to CPT

To induce Top1-mediated DNA damage, HCT116 cells were treated with CPT. The cells were exposed to high (1 µM and 2 µM) concentrations of CPT, and the effects on cell proliferation and survival were monitored. Treatment of the HCT116 cells with either concentration of CPT (1 µM and 2 µM) for 24 h was sufficient to induce cell cycle arrest. Treatment with 1 µM and 2 µM resulted in apoptosis of HCT116 cells (Figure 7.1).

1 µM and 2 µM CPT exposure was used, and both concentrations resulted in similar sensitivities. Figure 7.1 shows the response to 2 µM CPT. As shown (with no HMBA), the cells show a survival of about 5%, as was previously shown (see above). During differentiation, the percentage of the cells that survived remained at a similar level. These data suggest that a subtle resistance to CPT develops during the differentiation process, which might be due to the reduced proliferation rate following HMBA treatment (see Discussion). The western blot data are shown for the CPT treated cells (after 24 hours incubation). For all days, cellular survival levels were too low to obtain significant whole cell extract material, as measured by the loss of α-tubulin (control) signal (Figure 7.1 A). However, it is clear that for all days, there is no detectable Oct4 present, which indicates that HCT116 cells had occurred in the HMBA + 1 µM or CPT 2 µM treated cells. Thus, HCT116 was not an expression of Oct4.

Our results showed that HCT116 cells did not lose viability; however, they lost clonogenicity. These outcomes suggested that p53 and p21 were needed to block apoptosis of HCT116 cells treated with either 1 µM CPT or 2 µM CPT. The loss of clonogenicity of HCT116 cells treated with 1 µM CPT or 2 µM CPT was not because of the loss of long-term viability; rather, it was the effect of senescence development.

#### 6.2.1.2 Response to Aphidicolin

Cell cultures were treated as above for CPT, but the CPT was replaced by 1 µM aphidicolin and 2 µM aphidacolin (for data of 2 µM aphidicolin see the Appendix), a DNA polymerase inhibitor. As Figure 7.2 A shows, significant levels of Oct4 are not detected on all days, indicating that differentiation occurred in these cultures. A relatively uniform response to 1 µM aphidicolin is observed, which indicates that there is no measurable change in the response to DNA replication inhibition during HCT116 differentiation in response to HMBA.

#### 6.2.1.3 Response to hydroxyurea

Cell cultures were treated as above for CPT, but the CPT was replaced by 1 µM hydroxyurea (HU), a ribonuclease reductase inhibitor that reduces cellular levels of nucleotides and thus inhibits DNA replication. As Figure 7.3 A shows, significant levels of Oct4 are not detected from day 0 onward, indicating that differentiation occurred in these cultures without the effect of HMBA. With regard to aphidicolin, relatively uniform sensitivity is observed for HU, indicating that there is no inherent difference in the response to this drug during HCT116 cells with HMBA.

#### 6.2.1.4 Response to phleomycin

In addition to CPT, two agents capable of inducing DNA damage were tested (Aphidicolin and HU; see above), both of which inhibit DNA replication. Both agents differ from CPT in that they do not necessarily generate double-stranded DNA breaks (although they are capable of this). Given the fact that CPT does not cause a subtle increased sensitivity to Oct4 expressing HCT116 cells more than these two agents (see above), another agent, phleomycin, was tested, which is known to generate DSBs directly. Figure 7.4 shows the response to pheleomycin. With regard to the other experimental sets, the Oct4 signal is lost from all days onwards, indicating that the differentiation programme proceeds as in these cultures. As shown, there is a slight difference in the sensitivities to phleomycin; days without HMBA show slightly higher levels of sensitivity to this agent relative to the other days (days 0-8). There is no correlation between Oct4 levels, and the sensitivities are not exact because HCT116 is not an expression of Oct4 protein.

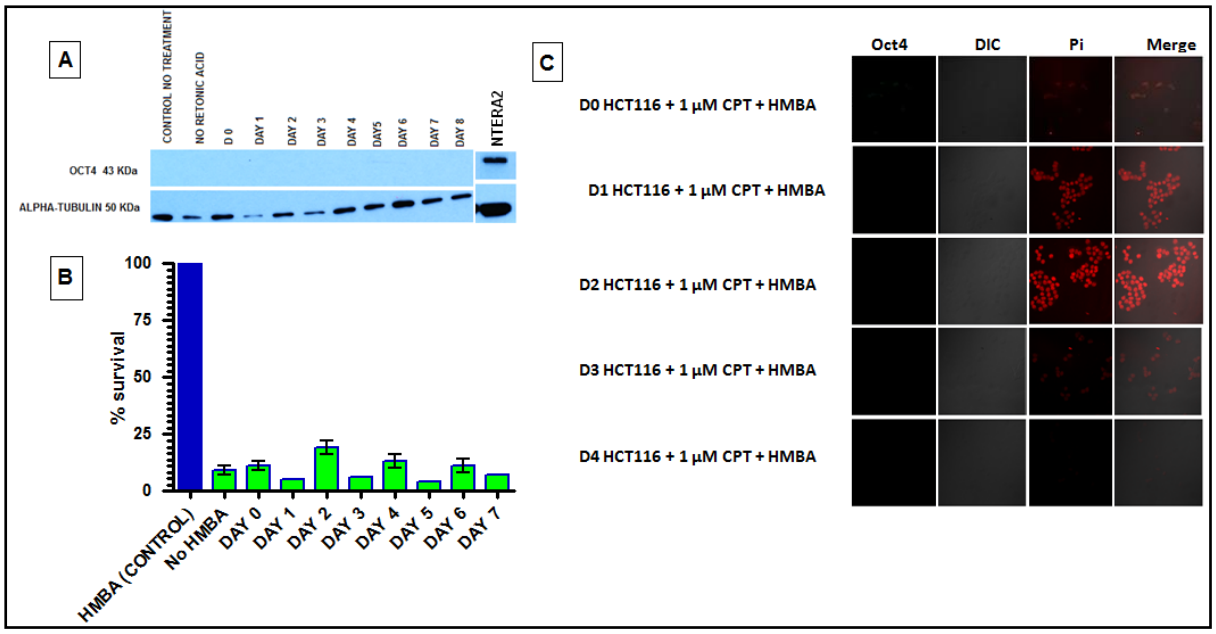



**Figure 6.1. The effect of camptothecin on differentiated HCT116 cells after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days; alph-tubulin was the control. (B) Percentage of survival cells for eight days; cytotoxic responses to 1 µM CPT were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in cellular response to 1 µM CPT was assessed by the two-way ANOVA with a comparison test and Student's t test. HCT116 cells were significantly sensitive to 1 µM CPT ($p < 0.01$) for all days compared with the control. (C) Immunoflurescent staining shows decreased Oct4 expression in HCT116 cells during seven days after treatment with HMBA, indicating loss of self-renewal during seven days. (Green) indicates Oct4 expression. (Black) indicates phase contrast. (Red) indicates Pi nucleus stain. (Merge) indicates the merging of Oct4 and Pi stain.

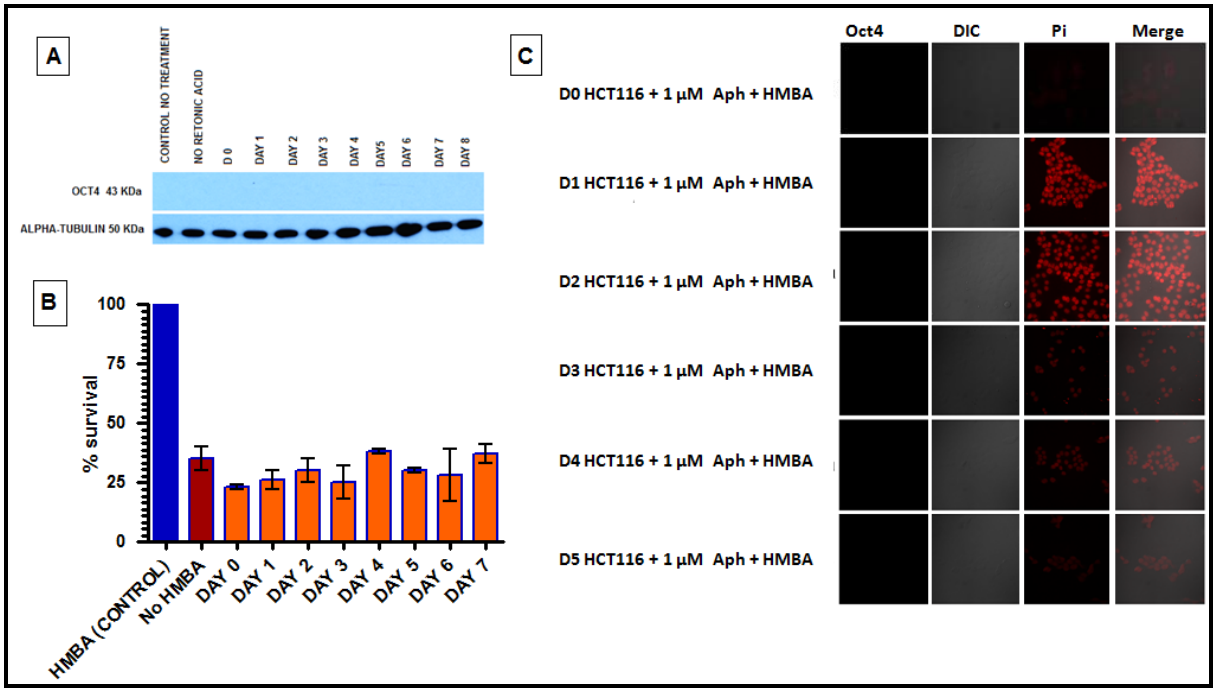



**Figure 6.2. The effect of aphidicolin on differentiated HCT116 cells after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days; alph-tubulin was the control. (B) Percentage of survival cells for eight days; cytotoxic responses to aphidicolin were analyzed by cell accounting using haemocytometer. Columns were generated from three independent experiments. Statistical significance in the cellular response to aphidicolin was assessed by the two-way ANOVA with a comparison test and Student's t test. HCT116 cells were significantly sensitive to aphidicolin ($p < 0.01$) for all days compared with the control. (C) Immunoflurescent staining shows decreased Oct4 expression in HCT116 cells during seven days after treatment with HMBA, indicating loss of self-renewal during seven days. (Green) indicates Oct4 expression. (Black) indicates phase contrast. (Red) indicates Pi nucleus stain. (Merge) indicates the merging of Oct4 and Pi stain.

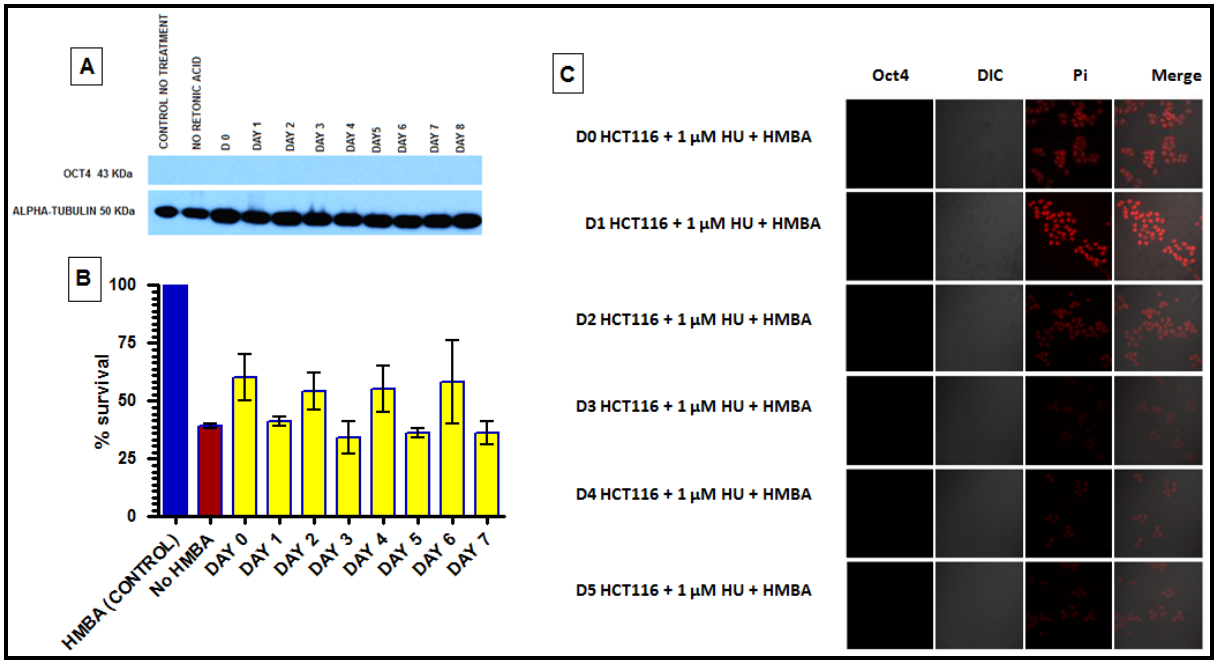



**Figure 6.3.The effect 1 µM hudroxyurea on differentiated HCT116 cells after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days; alph-tubulin was the control. (B) Percentage of survival cells for eight days; cytotoxic responses to 1 µM hudroxyurea were analyzed by cell accounting using a haemocytometer. Columns were generated from three independent experiments. Statistical significance in the cellular response to 1 µM hudroxyurea was assessed by the two-way ANOVA with a comparison test and Student's t test. HCT116 cells were significantly sensitive to 1 µM hudroxyurea ($p < 0.05$) for day 6, day 0, and ($p < 0.01$) for other days compared with the control. (C) Immunoflurescent staining shows decreased Oct4 expression in HCT116 cells during seven days after treatment with HMBA, indicating loss of self-renewal during seven days. (Green) indicates Oct4 expression. (Black) indicates phase contrast. (Red) indicates Pi nucleus stain. (Merge) indicates the merging of Oct4 and Pi stain.

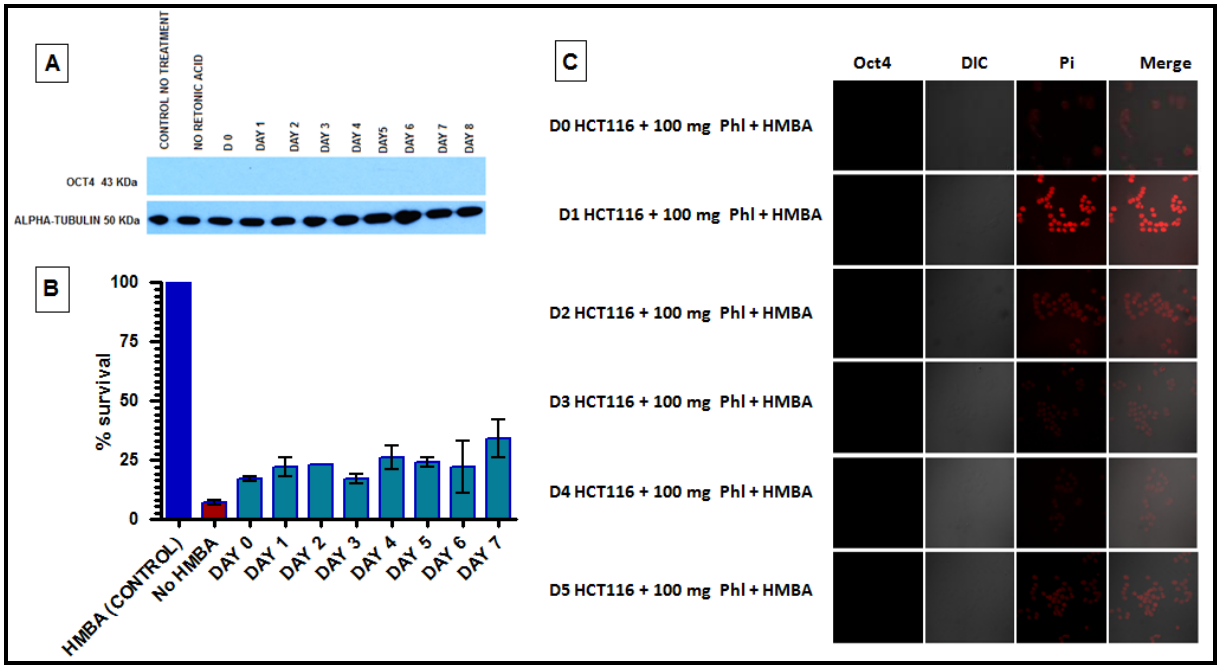



**Figure 6.4. The effect of 100 mg phleomycin on differentiated HCT116 cells after treatment with 3 mM HMBA for eight days.** (A) Western blot analysis for level of Oct4 during eight days; alph-tubulin was the control. (B) Percentage of survival cells for eight days; cytotoxic responses to 100 mg were analyzed by cell accounting using a haemocytometer. Columns were generated from three independent experiments. Statistical significance in the cellular response to phleomycin was assessed by the two-way ANOVA with a comparison test and Student's t test. HCT116 cells were significantly sensitive to phleomycin ($p < 0.01$) for all days compared with the control. (C) Immunoflurescent staining shows decreased Oct4 expression in HCT116 cells during seven days after treatment with HMBA, indicating loss of self-renewal during seven days. (Green) indicates Oct4 expression. (Black) indicates phase contrast. (Red) indicates Pi nucleus stain. (Merge) indicates the merging of Oct4 and Pi stain.

### 6.3 Discussion

TOP1 mutations that confer resistance to camptothecin and derivatives have been known in both mammalian cells and yeast (Chrencik et al. 2004, Arakawa et al. 2006, van der Merwe, Bjornsti 2008, Losasso et al. 2008).

This chapter determined the connection between HCT116 cell differentiation and its relative sensitivity to four poisons, as described above. HCT116 cells were used as a controllable model of colon cancer. We found that the sensitivity of HCT116 cells to four poisons during cell differentiation + or - HMBA was minimal and did not significantly change with culture time. Proliferating HCT116 cells were significantly more sensitive to the four poisons; however, HCT116 cells appeared to become more resistant to camptothecin treatment as they differentiated with HMBA. This was illustrated by a 2- to 4-fold increase in the growth inhibition induced by the four poisons in differentiated cells compared with proliferating cells without HMBA. It is important to note that in the differentiated cells, CPT still displayed a strong topo I complex signal. A CPT cleavable complex was usually very weak and did not change appreciably through the differentiation of these cells (see Figures 7.1, 7.2, 7.3 and 7.4). In addition, CPT-induced cytotoxicity did not change significantly throughout the differentiation system.

## Chapter 7 Analysis of NTERA2 retinoic acid induced differentiation

### 7.1. Introduction

Cancer relapse is the primary cause for the failure of cancer treatments. Cancer stem cells play a prominent role in the growth, relapse, and metastasis of cancers. These cells are analogous to normal stem cells, and possess features of self-renewal and differentiation. Unlike normal cells, cancer stem cells may result in two or more cancer stem cells as well as non-tumourigenic cancer cells, resulting in a perpetual increase in the total number of cancer cells, leading to neoplastic relapse (Hadnagy et al. 2006). Breast cancer stem cells with the CD44+/CD24− phenotype exhibit a strong ability for tumour induction. Cells with this phenotype were found to create fresh tumours even when present in small numbers, while large number of non-stem cells with additional phenotypes were required to elicit tumour formation in SCID mice (Ponti et al. 2005).

However, cancer stem cells are normally quiescent, while cancer chemotherapy normally targets only those cells that are dividing. Therefore, cancer stem cells survive chemotherapy and then recreate the tumour, resulting in a relapse (Woodward et al. 2005, Marx 2003). Therefore, while chemotherapy may be able to destroy the majority of non-cancer stem cells, they do not affect cancer stem cells (Huff et al. 2006). Since the cancer stem cells that remain after chemotherapy may lead to possible relapse, it is necessary to improve the approach for eliminating cancer stem cells by a non-apoptotic or non-necrotic process. Inducing division of cancer stem cells (Soltysova, Altanerova & Altaner 2005) via chemotherapy may provide an opportunity to overcome the resistance of cancer stem cells to cytotoxic agents.

In this chapter, we explored the hypothesis that all-trans retinoic acid (ATRA) could differentiate cancer stem cells using NTERA2 as a model. We investigated this hypothesis by using ATRA concomitantly with a cytotoxic agent for eliminating cancer stem cells, thus preventing relapse.

If the tumour cells are cancer stem cells (Sell & Pierce 1994, Reya et al. 2001, Pardal, Clarke & Morrison 2003, Bonnet & Dick 1997, Singh et al. 2003), then the cancer may be treated by inducing differentiation of the stem cells, i.e. differentiation therapy (Pierce 1983). Tumour stem cells of teratocarcinomas can be affected by the conditions of the growing embryo to differentiate into normal adult tissues. If malignant cells can be constrained to differentiate and to break off proliferation, then their malignant ability will be restrained and controlled. Although many agents have been investigated in the past (Spira & Carducci 2003), most

studies have comprehensively examined and clinically studied retinoic acid (RA, Vitamin A), particularly ATRA, as a differentiating agent.

After exposure of NTERA2 EC cells to retinoic acid, which results in cell differentiation, neural markers are manifested and neurons expressing neurofilament proteins and a characteristically neuronal morphology appear, most of which usually appear during the second week after the first exposure to retinoic acid (Andrews 1984a, Lee & Andrews 1986). Neurons derived from NTERA2 following retinoic acid treatment probably comprise only 2–5% of all differentiated cells; however, they are the most evident and prominent cells among all cell populations. These neurons express tetrodotoxin-sensitive sodium channels (Rendt, Erulkar & Andrews 1989). The neurons may be purified from the cultures by using techniques involving differential trypsinisation and treatment with mitotic inhibitors (Pleasure, Page & Lee 1992). Recently, it has been suggested that these NTERA2-derived neurons could be implanted into the central nervous system to correct neural deficits resulting from various diseases. Thus, such neurons have been reported to survive and integrate functionally to correct partial defects resulting from experimentally induced stroke in rats (Borlongan et al. 1998, Hurlbert et al. 1999, Muir et al. 1999, Philips et al. 1999).

The differentiation of NTERA2 cells into neurons occurs in several ways to summarise the stages through embryonic progression of the nervous system (Przyborski et al. 2000). For instance, nestin, a gene that encodes an intermediate neurofilament protein distinctive of proliferating neuroprogenitors, is quickly upregulated shortly after NTERA2 EC cells are exposed to retinoic acid.

## 7.2 Result

### 7.2.1 Analysis of NTERA2 retinoic acid induced differentiation

Thus far, all-trans-retinoic acid (ATRA) is the most widely used agent to induce EC cell differentiation. NTERA2 EC cells vary from other types of human EC cells due to their sensitivity to differentiation induced by retinoic acid (Andrews 1984b) and to distinct inducing agents such as hexamethylene bisacetamide (HMBA) (Andrews et al. 1986, Andrews et al. 1990) and bone morphogenetic proteins (Andrews et al. 1994). These agents induce differentiation in disting ways; however, researchers have thoroughly studied retinoic acid-induced differentiation, which resulting the formation of neurons and other cell types (Andrews, 1984). However, several other human EC cells do not respond to retinoic acid (Matthaei, Andrews & Bronson 1983).

Here, RA was used for induction of differentiation. A stock solution (10–2 M) was prepared by dissolving RA in dimethyldioxirane (DMDO) at 3 mg/ml. A solution containing 0.1% DMSO was obtained at the highest commonly used RA concentration (10–5 M). While DMSO at this concentration is acceptable, at higher concentrations, DMSO itself may induce differentiation, depending upon the cell line used. Therefore, a control culture was made (see Chapter 4). The control was essential to establish that NTERA2 differentiation can be induced under the laboratory conditions used here. Moreover, it was essential to test that the differentiation potential of the NTERA2 stocks used in this experiment had been maintained. To this end, we set up differentiation experiments in which stem markers were followed, with the expectation that the key marker for stemness, Oct4, would be lost from the cell population upon differentiation.

NTERA2 cell cultures were set up and monitored over eight days by western blotting and immunofluorescence to evaluate Oct4 levels (expecting a reduction in the Oct4 levels following differentiation). Four independent culture sets were established: one set was untreated and the other, treated with 3 mM HMBA. Figure 6.1 shows western blot data for Oct4 levels every day over a 7-day period. The gels shown in the figure contain tubulin as the control. The Oct4 protein levels of the untreated sample (without RA) were used for comparison. Cultures were immunohistochemically stained for Oct4 and the nuclear marker Pi.

### 7.2.2 Response of NTERA2 to DNA-damaging agents during differentiation

Following the analysis of the sensitivity of differentiated and undifferentiated cells to four poisons with HMBA in a previous chapter, the analysis was extended by studying the response of cells to DNA-damaging agents during the differentiation processes initiated by exposure to RA. Treatment with RA results in loss of Oct4 signal (Figure 7.1).

#### 7.2.2.i. Response to CPT

Differentiating cells were exposed to both 1 and 2 µM camptothecin (CPT), and the cells exhibited similar sensitivity to both concentrations of CPT. Figure 7.2 shows the response of the cells to 2 µM CPT. As shown in the figure, the cells show no survival on day 0 (with no RA), as shown previously . During differentiation, the percentage of surviving cells increases slightly, peaking on day 6. These data suggest a resistance to CPT developing during the differentiation process. This might be due to the reduced proliferation rate following RA treatment (see Discussion). The western blot data for the CPT-treated cells (after 24 h incubation); for days 0–2, show that survival levels were too low to obtain significant whole cell extract material, as measured by the loss of the α-tubulin (control) signal (Figure 7.2 A). However, it is clear that from day 3, there is no detectable Oct4 present, indicating that differentiation had occurred in the RA + 1 or 2 µM CPT-treated cells.

#### 7.2.2.ii. Response to Aphidicolin

Cell cultures were treated with aphidicolin in the same manner as described for CPT, but the CPT was replaced by the DNA polymerase inhibitor aphidacolin at concentrations of 1 and 2 µM (for data of 2 µM aphidacolin, see Appendix). As shown in Figure 7.3A, significant levels of Oct4 could no longer be detected from days 3–4, indicating that these cultures were undergoing differentiation. A relatively uniform response to 1 µM aphidicolin is observed, al after day 3 cells appear to become more resistance to aphidicolin.

#### 7.2.2.iii. Response to hydroxyurea

Cell cultures were treated as above for CPT, but CPT was replaced by 1 µM of the ribonuclease reductase inhibitor hydroxyurea (HU), which reduces cellular levels of nucleotides, thus inhibiting DNA replication. As shown in Figure 7.4A, significant levels of Oct4 are no longer detected from day 3, indicating that these cultures were undergoing differentiation. Similar to that observed in response to aphidicolin, relatively uniform sensitivity was observed in response to HU, with an increased resistance correlating to loss of Oct4 level.

### 7.2.2.iv Response to phleomycin

In addition to CPT, two agents capable of inducing DNA damage were tested (aphidicolin and HU), both of which inhibit DNA replication. Both these agents differ from CPT in that they do not necessarily generate double-stranded DNA breaks. Since CPT seems to cause a strong increased sensitivity in Oct4-expressing NTERA2 cells than these two agents (see above), we tested phleomycin, which is known to directly generate double-strand breaks (DSBs). Figure 7.5 shows the response of NTERA2 cells to phleomycin. As for the other experimental sets, the Oct4 signal disappears from approximately day 3 following exposure, indicating that the differentiation programme proceeds similarly in these cultures. There is a slight difference in the sensitivities of the culture to phleomycin: day 0 and day 1 culture showed slightly higher sensitivity to this agent as compared to the other days (days 2–8), when a decreased sensitivity is appearent. As for HU, resistance increases, peaking at day 5, to drop again at day 7.

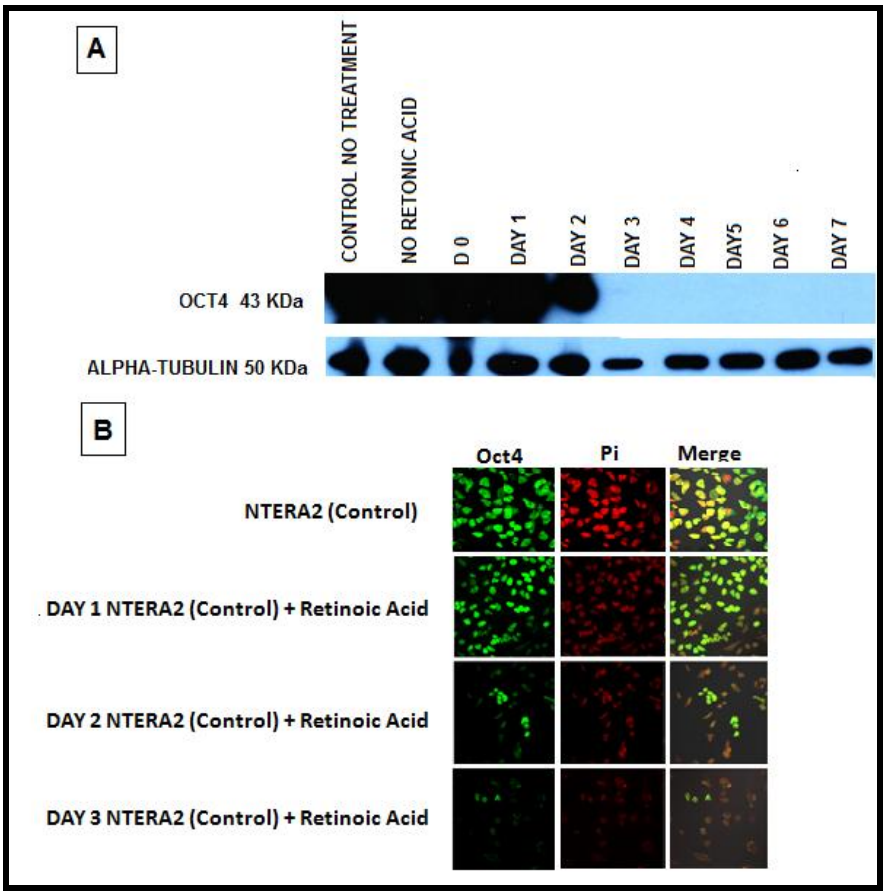



**Figure 7.1. Differentiation status and Oct4 gene expression in NTERA2 treated with retinoic acid (RA) and without treatment.** The expression of the Oct4 marker is shown. The data represent changes in expression in the differentiate state during seven days after exposure the cells to $10^{-2}$ M RA and western blots were achieved. In NTERA2 cells, the differentiation status is confirmed by the decreased Oct4 expression. (A) Oct4 level after RA treatment for seven days. (B) Immunofluorescence staining shows decreased Oct4 expression in NTERA2 cells for seven days after RA treatment, indicating loss of self-renewal during this period. Green indicates Oct4 expression. Black represents phase contrast. Red represents Pi nucleus stain. Merge indicates a merged image of the Oct4 and Pi staining.

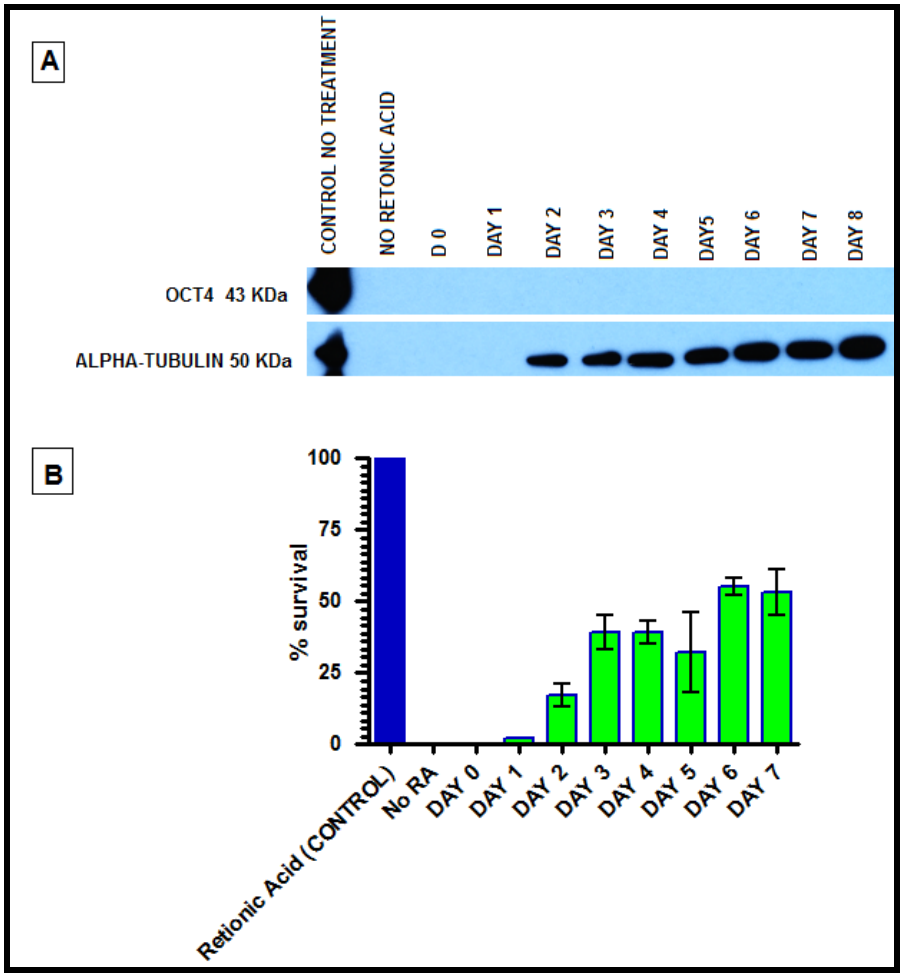



**Figure 7.2. The effect of camptothecin on differentiated NTERA2 cells after treatment with $10^{-2}$ M RA for eight days.** (A) Western blot analysis for Oct4 levels. α-Tubulin was used as the control. (B) Percentage of cells that survived for eight days and the cytotoxic responses to 1 μM CPT were analysed by cell counting in a haemocytometer. Columns were generated from three independent experiments. Statistical significance of the cellular response to 1 μM CPT was assessed by the two-way ANOVA with the comparison test and Student's t test. NTERA2 cells were significantly sensitive to 1 μM CPT ($p < 0.01$) for all days as compared to the control.

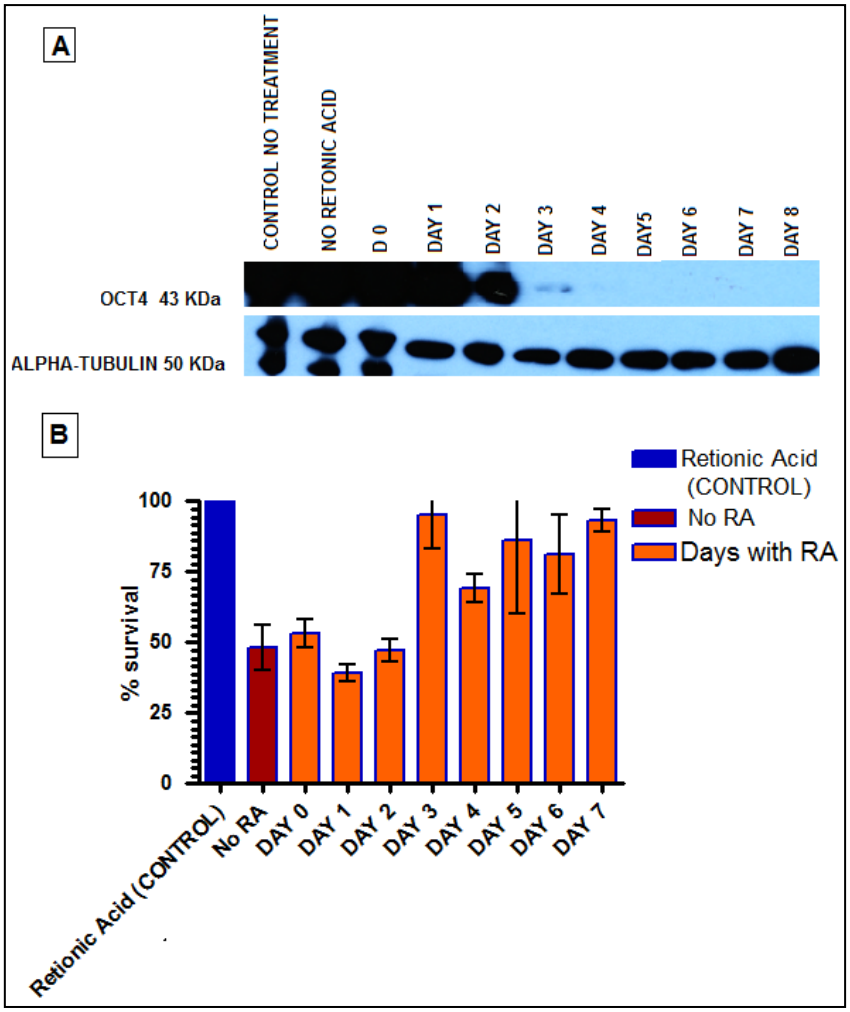



**Figure 7.3. The effect of aphidicolin on differentiated NTERA2 cells after treatment with RA for eight days.** (A) Western blot analysis for Oct4 levels. α-Tubulin served as the control. (B) Percentage of the cells surviving for eight days and the cytotoxic responses to aphidicolin were analysed by cell counting in a haemocytometer. Columns were generated from three independent experiments. Statistical significance of the cellular response to aphidicolin was assessed by the two-way ANOVA with the comparison test and Student's t test. NTERA2 cells untreated with RA were significantly sensitive to aphidicolin ($p < 0.01$) on day 1 as compared to the control on day 0 as compared with control ($p < 0.05$ in both cases).

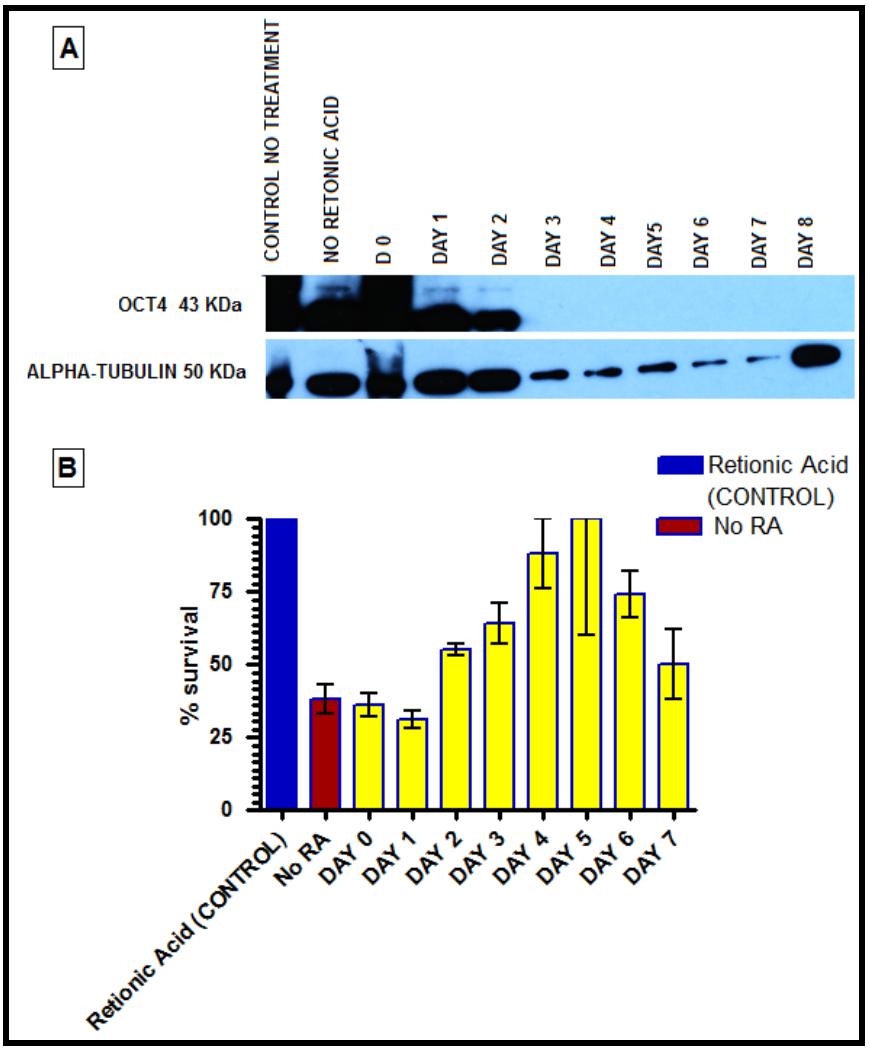



**Figure 7.4. The effect of 1 µM hudroxyurea on differentiated NTERA2 cells after treatment with 10–2 M RA for eight days.** (A) Western blot analysis of Oct4 levels. α-Tubulin served as the control. (B) The percentage of cells surviving for eight days and the cytotoxic responses to 1 µM hudroxyurea were analysed by cell counting in a haemocytometer. Columns were generated from three independent experiments. Statistical significance of the cellular response to 1 µM hudroxyurea was assessed by the two-way ANOVA with the comparison test and Student's t test. NTERA2 cells untreated with RA were significantly sensitive to 1 µM hudroxyurea ($p < 0.05$) on days 0 and 1 as compared to the control.

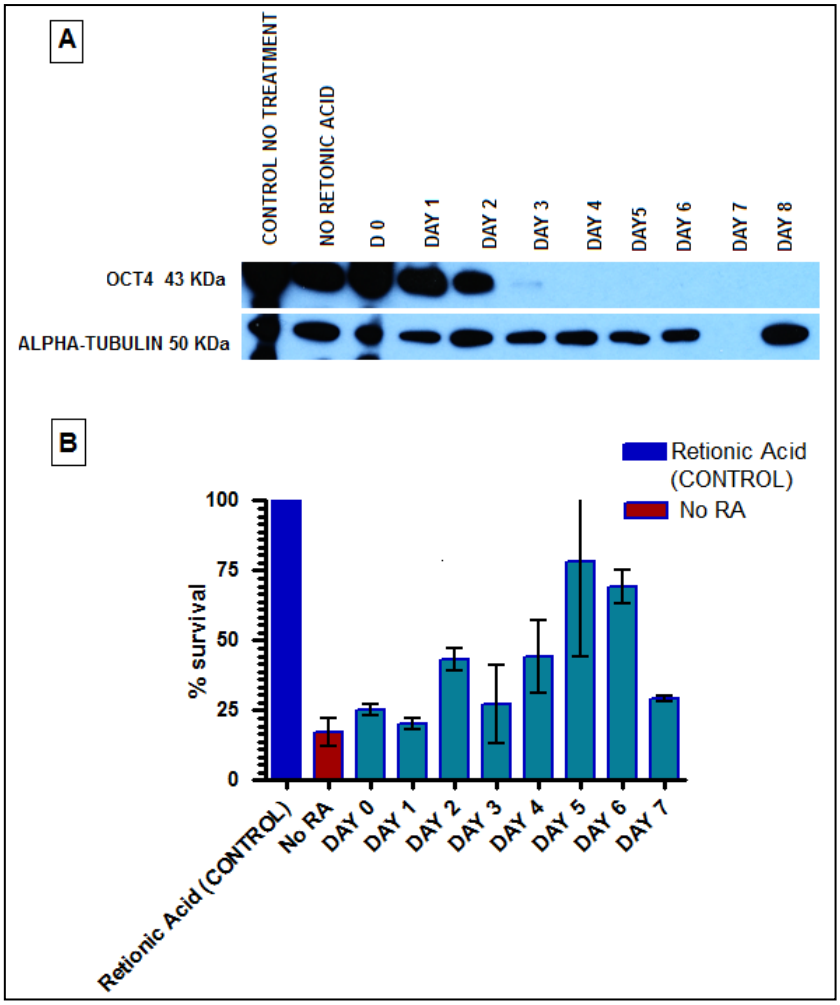



**Figure 7.5. The effect of 100 mg phleomycin on differentiated NTERA2 cells following treatment with 10–2 M RA for eight days.** (A) Western blot analysis of the Oct4 levels. α-Tubulin served as the control. (B) The percentage of cells surviving for eight days and the cytotoxic responses to 2 µM CPT were analysed by cell counting in a haemocytometer. Columns were generated from three independent experiments. Statistical significance of the cellular response to phleomycin was assessed by the two-way ANOVA with the comparison test and Student's t test. NTERA2 cells without RA treatment were significantly sensitive to phleomycin ($p < 0.01$) on days 0 and 1 as compared to the control.

## 7.3. Discussion

A complicated and intricate process of signalling pathways decides if a cell will proliferate, differentiate, die, or survive. Since retinoids have a high impact on differentiative ability, they have been extensively used for both cancer prevention therapy and cancer induction (Sporn & Suh 2002).

As described in the previous chapter, HMBA plays a role in the differentiation of NTERA2 cells as cancer stem cells or stem cells. Further, we observed that undifferentiated NTERA2 cells (no RA/expressing Oct4) appeared slightly more sensitive to CPT than cells that had been induced to differentiate using RA (see above). CPT works by inhibiting topoisomerase I. Topoisomerase I normally regulates DNA supercoiling by forming breaks in the sugar-phosphate backbone of DNA and creating a transient covalent phosphotyrosyl intermediate between the broken strand and a tyrosine residue on the topoisomerase (Keck & Berger 1999, Lorence & Nessler 2004, Rothenberg 1997, Rasheed & Rubin 2003, Pommier 2006). CPT acts by stabilising the transient covalent complex between the protein and the DNA, resulting in a prolonged unsealed nick in the duplex (Hsiang et al. 1985). These relatively stable nicks can be converted to DNA DSBs during DNA replication when the replisome encounters them, making a one-sided DSB that requires recombinogenic repair mediated by homologous recombination factors.

In this study we observed a more startling response of the cells to CPT following RA treatment, than had been observed for HMBA (see Chapter 5). As for the HMBA experiment we observed a relatively strong sensitivity of NTERA2 cells to CPT without treatment (down to about 2%). However, after day 3 cell survival increased to approximately 50% and was retained at this close to this level during the remained of the differentiation process. This is significantly different from what was observed for HMBA differentiation, when a subtle increase in survival levels was observed, not this larger increase. This increased resistance is concomitant with the timing of the loss of Oct4 (see Figure 6.2), suggesting that it is linked directly to the differentiation process. No significant reduction in cell proliferation levels were noted upon RA-induced differentiation relative to the HMBA-induced differentiation and cell numbers continued to increase during the RA-induced differentiation period. Given this, it seems unlikely that the increased resistance to CPT is due to decreased proliferation

levels. However, this scope of this study did not permit for a detailed analysis of the cell division cycle kinetics during the differentiation process and there may be differences in the cell division cycle which could account for this observation; for example, S-phase period might be altered.

These data could suggest that there is a mechanistic distinction in how undifferentiated and differentiated cells deal with CPT damage. What this difference might be remains unclear, but these observation could provide the platform for further analyses; for example, studies into the levels of DSB formation during S-phase using histone δ-H2AX phosphorylation would determine whether the same levels of breakage occur during S-phase in differentiated cells as in undifferentiated cells. Determining this would enable us to distinguish between distinct levels/types of DNA damage being formed verses distinct mechanistic pathways dealing with similar levels of damage.

Treatment with HMBA did appear to give a subtle mechanistic change, but this was not as pronounced as the difference previously seen when comparing the sensitivities of mouse ES cells with non-isogenic MEFs (McFarlane and Wakeman, personal communication). The results obtained with NTERA2 following RA-induced differentiation match the pattern seen previously with the mouse cells. This could indicate that there is a difference in cellular responses to CPT-induced DNA damage, but it is dependent upon the lineage down which cells are induced to follow. This possibility opens up the intriguing question of whether or not there are lineage-specific DNA repair pathways in complex metazoans. This would have considerable implications for how we view cancer development and how we might treat cancer-specific cancer stem cells.

NTERA2 induced to differentiate with RA also showed changes in the patterns of sensitivity to the other DNA damaging agents used here. The changes were not as marked and the increased resistance was not maintained in the longer run (see phleomycin and HU result). However, these data indicate that there may be more global changes to the DNA damage response, although the CPT response is more pronounced, as was the case in the mouse study.

The data here have provided a human model for the further analysis of the mechanistic causes of increased sensitivity to CPT in cells with stem-like characteristics (Oct4 positive). They cast some questions over the dogma that differentiation therapy has a place in cancer therapy, as we find that the differentiated cells become more resistant to the therapeutic agent CPT relative to stem-like cells. Further work is needed to elucidate the molecular basis of these findings and the relevance to chemotherapeutic strategies.

## Chapter 8 Preliminary analysis of mouse stem cells.

### 8.1. Introduction

Mouse embryonic stem cells (mESCs) are obtained from the inner cell mass of pre-implantation blastocysts (Evans, Kaufman 1981b, Martin 1981b). The defining characteristic of mESCs is their capability to accompany multiple differentiation mechanisms, both *in vitro* and *in vivo* (Smith 2001a). mESCs have been widely used for forming mutant mice as a dominant reverse-genetics access to recognise *in vivo* biological events of genes of interest (Smith 2001b, Evans, Kaufman 1981a, Martin 1981a, Kaufman et al. 1983, Rossant 2001). mESCs can also be used to comprehend molecular processes that control primitive organogenesis using *in vitro* analysis procedures (Smith 2001c). mESCs are known for their inability to arrest in the G1 phase after DNA damage. Instead, mESCs with damaged DNA are preferentially eliminated through apoptosis (Aladjem et al. 1998). Furthermore, in addition to cell type-specific differences in the expression of repair elements, there are also species-specific dissimilarities. XRCC-4, for instance, is present in equivalent amounts in mESCs and MEFs, but in hESCs its expression is raised in contrast to differentiated cells (Momcilovic et al. 2010, Tichy et al. 2010). Furthermore, DNA ligase IV is present in higher amounts in hESCs compared to differentiated human cells (Momcilovic et al. 2010), but its expression is lower in mESCs contrasted to differentiated cells (Tichy et al. 2010). In comparison, DNA-PKCS exists in lower lots in mESCs contrasted to MEFs (Banuelos et al. 2008b), while its expression is higher in hESCs in comparison to differentiated human cells (Momcilovic et al. 2010). Furthermore, expression of breast cancer 1 (BRCA-1), a tumour suppressor protein which is implicated in DNA repair regulation, transcription and cell cycle , is often lower in mESCs than in differentiated murine cells, but it exists in higher lots in hESCs than in differentiated human cells (Momcilovic et al. 2010).

Studies show that mESCs are more resistant to oxidative stress and ionizing radiation (IR)-induced DNA damage than differentiated mouse fibroblasts (Saretzki et al. 2004). However, mESCs are hypersensitive to several DNA damaging agents and readily undergo apoptosis (Park, Gerson 2005, Roos et al. 2007, Van Sloun et al. 1999). Furthermore, the efficiency of repair of UV-induced DNA damage is decreased when mouse embryocarcinoma stem cells are induced to differentiate (Rasko et al. 1993).

In this part, we address the issue of whether mESCs switch on the DNA damage response mechanism following exposure to four poisons compared with cancer stem cells (such as NTERA2) and cancer cells (such as HCT116).

## 8.2. Results

### 8.2.1. mESCs exposure to different DNA damage

In our previous chapters, we explained two models of cancer stem cells (NTERA2) and cancer cells (HCT116 as control) and treated them by two inducers (HMBA) and (ATRA) and exposed them to four poisons: camptothecin, aphidicolin, phleomycin and hydroxyurea. We know that the most common response of cells to DNA damage is perturbation of progression through the cell cycle. In this part we examine the response of mouse embryo exposed (E14) cells to those poisons during 24 hours. Four poisons were used for exposure and were seeded in the same densities and four poisons were added at the same time for 24 hours. Figure 8.1, (A) shows the Oct4 signal expression in all samples; but the very low expression with 2 µM CPT indicates that cells were undifferentiated as well as E14 cells having resistance to all poisons in different proportions. The greatest poison effect was with CPT especially the 2 µM CPT. DMSO had no effect compared with the control.

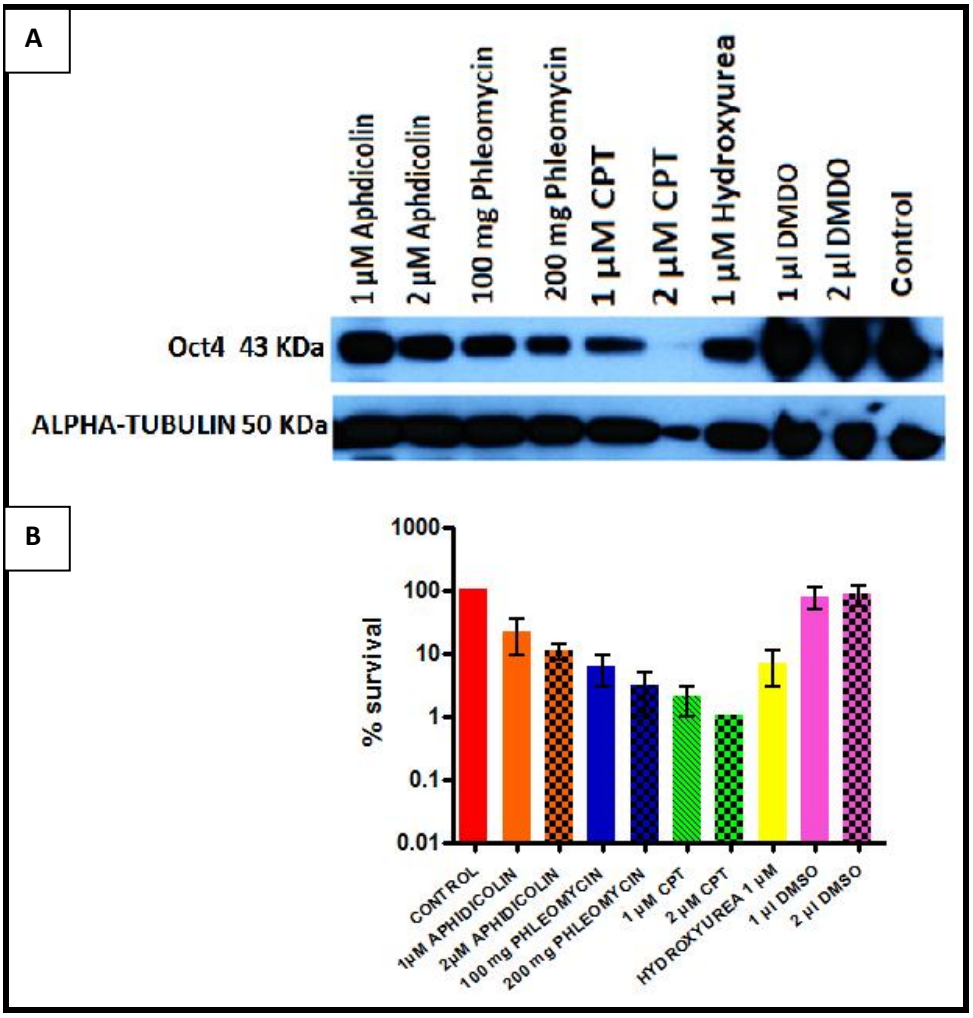



**Figure 8.1. Analysis of cell survival in response to four DNA damaging poisons: CPT, aphidicolin, phleomycin and hydroxyurea.** (A) Western blot analysis for level of Oct4 during 24 hours with four DNA damaging poisons and alpha-tubulin as control. (B) Total number of mESc cells following exposure to camptothecin. E14 cells were exposed to four DNA damaging poisons separately in 6 well plates, 2 ml/ well for 24h. The total number of cells remaining at each time point was determined by cell counting using a haemocytometer. Statistical significance in cellular response to four DNA damaging poisons was assessed by a two-way ANOVA with comparison test and Student's t test. E14 cells were significantly sensitive to all poisons ($p < 0.01$) but DMSO was not sensitive ($p < 0.05$) compared with the control.

### 8.3 Discussion

We described the effect of the four poisons on two types of cells, (1) Cells that have the property of stem cells, which are called NTERA2, as cancer stem cells and showed the effect the four poisons had on these cells in two ways: undifferentiated and differentiated. (2) Cells that do not have the property of stem cells, which are originally differentiated. Then we found there are different responses in those cells to different types of DNA damage. In this part, using E14 cells in the same way experimentally, it was shown that E14 cells have low resistance similar to undifferentiated NTERA2 cells and it was indicated that they appeared to be slightly more sensitive to the DNA damaging agents. This inspection supports the probability that there is a similarity in the process of activity between cancer stem-like cells NTERA2 and mESCs cells in the way they process and respond to significant levels of DSBs.

## Chapter 9 Final Discussion

Genomic instability is a threat to the normal proliferation and homeostasis of cells, and it can cause cancer as well as many kinds of degenerative and ageing effects. DNA receives large amounts of damage from the endogenous metabolites of cells as well as from exterior factors, such as UV radiation and chemicals. In order to prevent malignant transformation and the transmission of damaged genomic material, cells have evolved a DNA damage response (DDR). This response includes an assembly of mechanisms that 1) sense and identify the damage, 2) promote cell cycle arrest, and 3) permit the repair of the damaged DNA or stimulate apoptosis if the damage is extreme (Jackson et al. 2009). In malignant cells, this response is frequently modified, and many anti-cancer drugs that take advantage of this to specifically target cancer cells.

### 9.1. Does the DDR change as cell undergo differentiation?

The bulk of the work in this thesis is aimed at addressing this key question, with an aim of gaining insight into the behaviour of human cells. There is a developing body of literature which indicates there are differences in DDRs between differentiated and undifferentiated cells; for example, the changes in levels of some DDR proteins when comparing differentiated and undifferentiated cells (for example, see Momcilovic et al., 2010 and Chapter 8). Moreover, there is a growing interest in experimentally exploring this question in more detail. The work in this current programme is set against this backdrop of increased studies in this area and the increase in interest in human embryonic stem cells and induced pluripotent stem cells.

The initial observation which triggered this work was from a preliminary study from Wakeman and McFarlane (personal communication). As previously mentioned in the thesis, they studied the cellular survival levels of mouse ES cells with MEF, albeit MEFs from a non-isogenic source. They found that in most cases mES cells exhibited similar sensitivities to drugs which induced DNA damage, mostly through inhibition of DNA replication or in a DNA replication-dependent fashion; however, the response to CPT was markedly different in that the mES cells were significantly more sensitive relative to the terminally differentiated MEFs. Given these findings, in conjunction with the evidence that there are DDR distinctions between differentiated and undifferentiated cells, much of which has been revealed during the time frame of the work presented here, we set out to chose an appropriate model for studying

DDR responses during human differentiation. Obtaining and differentiating human ES cells is both difficult and contentious; indeed, there is current controversy as to whether *bona fide* human ES which are equivalent to the well characterised mES cells have actually been isolated, or whether current so called hES cells actually represent cells from a later developmental period (for example, see Nichols and Smith, 2011). Given this, we chose to use the well characterised teratocarcinoma cell line NTERA2, which can form teratomas and can be induced to differentiate upon treatment with distinct molecular differentiating agents, including HMBA and RA. Upon treatment with HMBA we found that at best there is a subtle change in the cellular DDR during the differentiation process. This does not offer substantive evidence to indicate that there is a major change in the molecular programme underpinning the DDR during differentiation of a more naive human cell (Oct4 positive) and a differentiated state (Oct4 negative). However, when NTERA2 cells were differentiated with RA a much greater difference was observed between the sensitivity of the cells in the undifferentiated state relative to the differentiated state. This difference was only seen for CPT treatment and was not as apparent for the other damaging agents. Taken at face value, this observation appears to indicate that there are changes to the DDR upon differentiation, but that these depend upon which specific differentiation pathway is followed (in this case RA or HMBA-induced). If correct, this observation could have implications for the development of tissue-specific DDR pathways. Taking this further, one could speculate that this reflects distinct oncogeninc / tumour suppressor pathways in distinct tissues.

The data presented here also appear to indicate a damage-specific change which appears to relate to CPT-induced DNA damage. CPT has been reported to have a highly specific mode of action in inhibiting the topoisomerase I reaction, which is thought to result in chromosomal breakages as topoisomerase I-induced nicks are converted to DSBs upon being encountered by the replication machinery (Hsiang and Liu, 1988). Given this, the data here might indicate that there is a distinct change to the pathway(s) related to DNA replication-associated DSBs. However, prior to making too strong a set of conclusions the fact that no detailed cell cycle analysis was carried out on RA-induced NTERA2 should be taken into account. Whilst cells appeared to continue to divide and proliferate, this was not studied in enough depth to fully exclude the possibility that the change in sensitivity in response to CPT upon RA-induction was due to differentiation being paralleled by a significant reduction in proliferation. If this were the case then the loss of sensitivity might simply reflect the fact that

CPT-induced damage, which is dependent upon DNA replication, might be reduced. Further detailed analysis of cell division (e.g., Ki-67 analysis) would be required.

### 9.2. Are human and mouse ES cells / ES-like cells different?

If the data for HMBA-induced NTERA2 are compared to the preliminary data from Wakeman and McFarlane, where mouse ES cells are more sensitive to CPT than non-isogenic MEF, but NTERA2 show only a slight decrease in CPT sensitivity in response to HMBA induction, then it could be argued that mouse cells behave in a different fashion. There are examples in the literature where there are differences in the components of the DRR in mouse and humans; for example, XRCC-4 is present in equal amounts in mES cells and MEFs, whereas human ES cells have higher measurable levels than observed in differentiated cells (Momcilovic et al., 2010, Tichy et al., 2010). However, in the case of CPT response this has undergone little in the way of detailed investigation. One route to address this might be to examine directly the levels of DSBs induced in differentiated and undifferentiated NTERA2 *vs.* mES cells and MEFs. This might provide insight into whether these differences arise due to distinct levels of DNA damage being generated or the way in which they are processed. This however does not address the fundamental question of whether there are inherent differences between mouse and human systems. In addition to this, the fact that the response to RA-induced differentiation appears to indicate commonalities, i.e. sensitivity in the undifferentiated state and reduced sensitivity upon induction, implies that this is not a question which can be resolved with a simple study of this nature. This study reveals the possibility of different differentiation pathways (see above) behaving in distinct ways. To try and then make inter species comparisons using these data is potentially flawed and future independent studies should focus the questions relating to how distinct differentiation pathways result in distinct DDRs.

With this in mind, what can we take from this work? If there are distinct DDR pathways dependent upon which differentiation inducing agent one uses, then this could indicate there is a complex network of DDR programmes for distinct lineages. However, there is one common feature of both RA- and HMBA-induced pathways for differentiation of NTERA2 and that is that both pathways result in Oct4 being lost from the cells. This indicates that what ever factors govern the distinct DDR mechanisms following differentiation induction, the role of Oct4 is not the differentiator (as it is lost in both cases). This is an important observation as

recent work has demonstrated a role for the nucleotide excision repair (NER) XPC complex in Oct4-mediated gene transcription activation (Fong et al., 2011). This work has linked the association of XPC with Oct4 to a novel genome stability control mechanism which proposes that DNA damage which requires XPC repair (NER) will sequester XPC to sites of damage and prevent the Oct4-mediated transcription of genes needed for stem cell maintenance (e.g. Nanog) thereby triggering differentiation or apoptosis in response to DNA damage. This suggestion implicates Oct4 directly in control of at least one DDR programme. Here we have provided evidence that distinct DDR mechanisms which might occur in response to distinct differentiation pathways (HMBA- or RA-induced) are independent of direct Oct4 control.

### 9.3 Closing remarks

In this work the link between genome stability maintenance mechanisms and developmental programmes have been investigated, with particular focus on DNA replicative stress scenarios. Firstly, the question of whether meiotic recombination hotspots can be activated in situations where the DNA replication machinery is compromised or the cell is under a given stress condition. A model system, the fission yeast, was employed, as studies of this nature in humans are only now becoming possible as human meiotic recombination hotspots become identified, which was not the case at the onset of this work. Intriguingly, we did not find any conditions in which the *S. pombe* developmentally related hotspot could be activated in mitotically proliferating cells. This leaves open the question of whether this can occur. This might reflect the fact that the developmental programme of meiosis requires many novel genes to be expressed for hotspot activation, such as the meiosis-specific nuclease Spo11 (Rec12). It might be the case that a tight transcriptional and post transcriptional regulation of the activation of meiotic factors is central to preventing meiotic recombination hotspot becoming the sites of genomic rearrangements in mitotically dividing cells. Interestingly in human cancer many testis-specific genes do become deregulated, and may provide a sufficient platform for human recombination hotspots to be more readily activated to drive oncogenic changes.

Finally, this work has taken a preliminary view of how human cells with pluripotent potential respond to DNA damage before, after and during the differentiation process, with a particular focus on DNA damage associated with DNA replication. One key observation comes from this work, one which might indicate that the response to DNA damage can differ significantly

between the differentiated and undifferentiated state, but that this is dependent upon which pathway of differentiation is followed. If this observation should hold up upon further more extensive scrutiny (for example, more extensive cell cycle analysis of RA-induced NTERA2), then it has extensive implications which could ultimately impinge upon how we view distinct tissue specific cancers and distinct tissue-specific regenerative clinical approaches using stem cells.

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