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Aging, Stem Cells and the Cancer Stem Cell Hypothesis

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Aging, Stem Cells and the Cancer Stem Cell Hypothesis

by Dave Comstock

for Dr. David Calhoun, Dr. Karen Hubbard, and Dr. Jerry Guyden, The City College of New York

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A discussion on cancer stem cells and their relationship to aging of an organism.

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Organism Aging and Cellular Senescence

Humans have observed for millennia that aging is primarily characterized by a loss of function in society, special age-linked exemptions from societal responsibility have been typically reserved for individuals in their seventh or eighth decade of life (Shahar 1993).

There are common clinical presentations of aging, easily observed, that indicate disruption of tissue regeneration, chronic inflammation and neoplastic disease (Pettan-Brewer and Treuting 2011). With age, wound healing in the skin slows, hair turns gray or is lost, skeletal muscle mass and strength decrease, the ratio of cellular constituents in the blood is skewed, and there is a decline in neurogenesis (Sharpless and DePinho 2007). By definition, "age-related diseases" display an age-dependency, with increasing frequency as an organism ages, and peak incidence occurring in the later decades of life. Common human age-related diseases include diabetes (Tollefsbol 1987; Franceschi, Bonafe *et al.* 2000; Thunander, Petersson *et al.* 2008), atherosclerosis (Franceschi, Bonafe *et al.* 2000; Vasto, Candore *et al.* 2007), sarcopenia (Baumgartner, Koehler *et al.* 1998; von Haehling, Morley *et al.* 2010), osteoarthritis (Ladislas 2000; Horton, Bennion *et al.* 2006) and cancer (SEER 2010; Howlader, Noone *et al.* 2012).

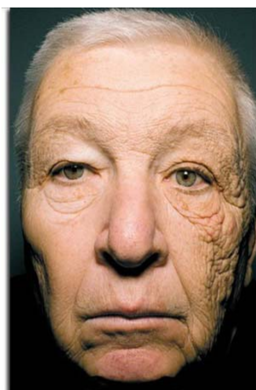

Figure 1 Favre–Racouchot syndrome. A 69 year-old man with unilateral dermatoheliosis (Gordon and Brieva 2012). Dermatoheliosis (Favre–Racouchot syndrome), occurs by ultraviolet A (UVA) rays penetrating the epidermis and upper layers of dermis, and can result in thickening of the corneal layer and epidermis, as well as destruction of elastic fibers (Patterson, Fox *et al.* 2004). UVA has also been shown to induce substantial DNA mutations and direct toxicity, sometimes leading to melanoma (Patterson, Fox *et al.* 2004).

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The phenotype of aging is characterized by the loss of the ability to maintain homeostasis of tissue structure and function, and depends on the capability of the specific tissue cells to regenerate (reviewed in Rando, Thomas A and Chang 2012).

Chronological vs Replicative Aging

All cells experience chronological aging, as a function of existing in time. Tissues composed of continuously dividing cells, such as the bone marrow, intestine or skin, also experience replicative aging with each passage through the cell cycle and the accompanying risk of genetic and epigenetic changes, telomere shortening, and DNA damage (Wang, Jurk *et al.* 2009; Liu and Rando 2011).

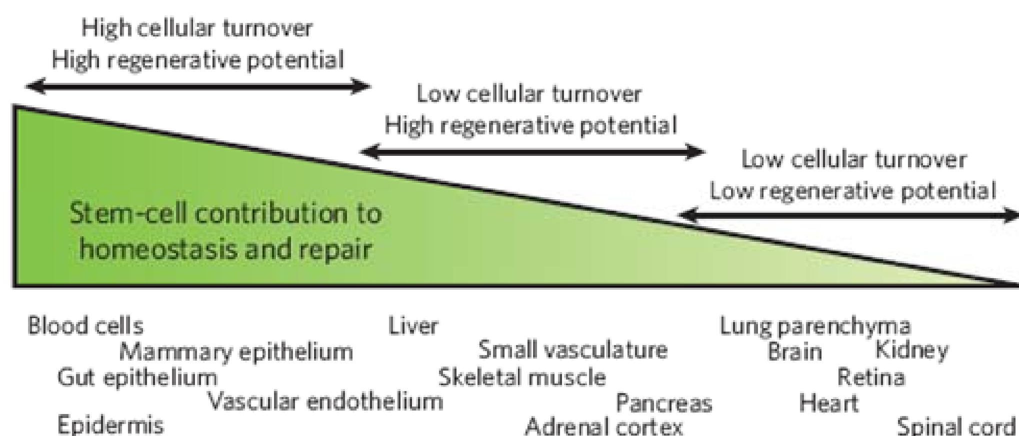

Figure 2 Tissue regeneration and cellular turnover rates by tissue type. The spectrum of tissue types and the compartmental contribution of stem cells in maintaining self-renewal and homeostasis. (Rando, Thomas A. 2006)

The accumulation of somatic damage is considered a main cause of the aging process (Hasty, Campisi *et al.* 2003) Cellular stress from reactive oxygen species, DNA damage, telomere shortening, and deformation of the extracellular matrix have all been proposed as aspects, either causal or resultant, of somatic damage associated with aging.

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Hayflick Limit and Replicative Senescence

All cells experience chronological aging, which is characterized by changes in membrane composition, damage accumulation, or malformation, of key macromolecules resulting in structural and functional changes (Stadtman 2001; Rando, Thomas A and Chang 2012). In the mid-twentieth century, it was found that somatic cells in culture have an average maximum lifespan of ~50 population doublings, now known as the Hayflick Limit (Hayflick and Moorhead 1961). Cells from different fetal tissues were sub-cultivated for different lengths of time and number of passages, lung being the greatest, undergoing 55 passages over eight months, until they reached a point whereby the cells were viable and metabolically active, but permanently removed from the cell cycle, described as " an expression of aging or senescence at the cellular level'' (Hayflick and Moorhead 1961).

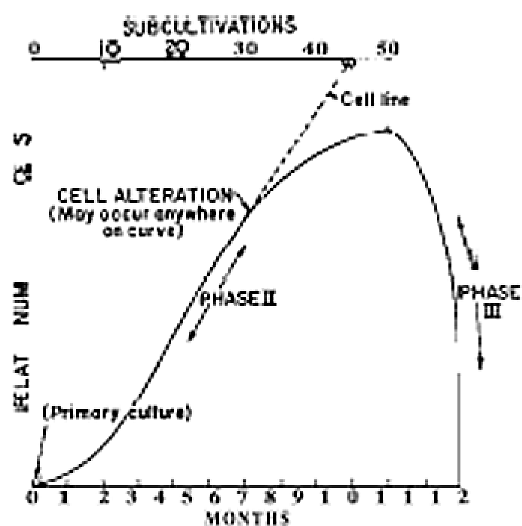

Figure 3 Fetal fibroblast culture growth curve. Three phases of human fibroblast proliferation in culture are: phase I, establishment of the culture, a period of little proliferation; phase II, rapid cell proliferation; and phase III, proliferation declines and stops. At any point, immortalization can occur. (Hayflick and Moorhead 1961)

The "end replication problem" of DNA synthesis (as part of the mitotic process), proposed by Olovnikov in the early 1970s, provides an explanation for the Hayflick Limit by suggesting that the binding properties of the DNA polymerase force the loss of DNA telomeric regions with each passage through the cell cycle (Olovnikov, A. M. 1973; Olovnikov, Alexey M. 1996). Olovnikov also predicted the existence of an enzyme that would synthesize and replace the telomeric caps, anticipated to be found in

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germ line cells, and cancer cells (Olovnikov, Alexey M. 1996). In the late 1970s, Elizabeth Blackburn and Jack Gall identified the telomere-shortening mechanism, and later described the structure of telomeres, which appears to set a fixed number of divisions, and hence the lifespan of cells (Allsopp, Vaziri *et al.* 1992; Cech 2000), and the enzyme telomerase that synthesizes and rebuilds telomeric caps (Blackburn, E H and Szostak 1984; Blackburn, Elizabeth H. 1991). Subsequently, telomerase was found in most stem cell and cancer cell populations (Thomson, Itskovitz-Eldor *et al.* 1998; Maser and DePinho 2002).

Cell Cycle Mechanics

After receiving a mitogenic signal, cells enter the G1 phase where initiation and progression depends on the cell type and growth signal. These signals come from autocrine, paracrine or endocrine sources, and reflect the metabolic state, DNA damage, physical and chemical stress, or oncogenic stimuli (Massague 2004). Continually dividing intestinal crypt stem cells and target antigen-bound lymphocytes are driven by different G1 network signals and developmental programs, and each carries a different risk and mechanism of malignant transformation (reviewed in Massague 2004).

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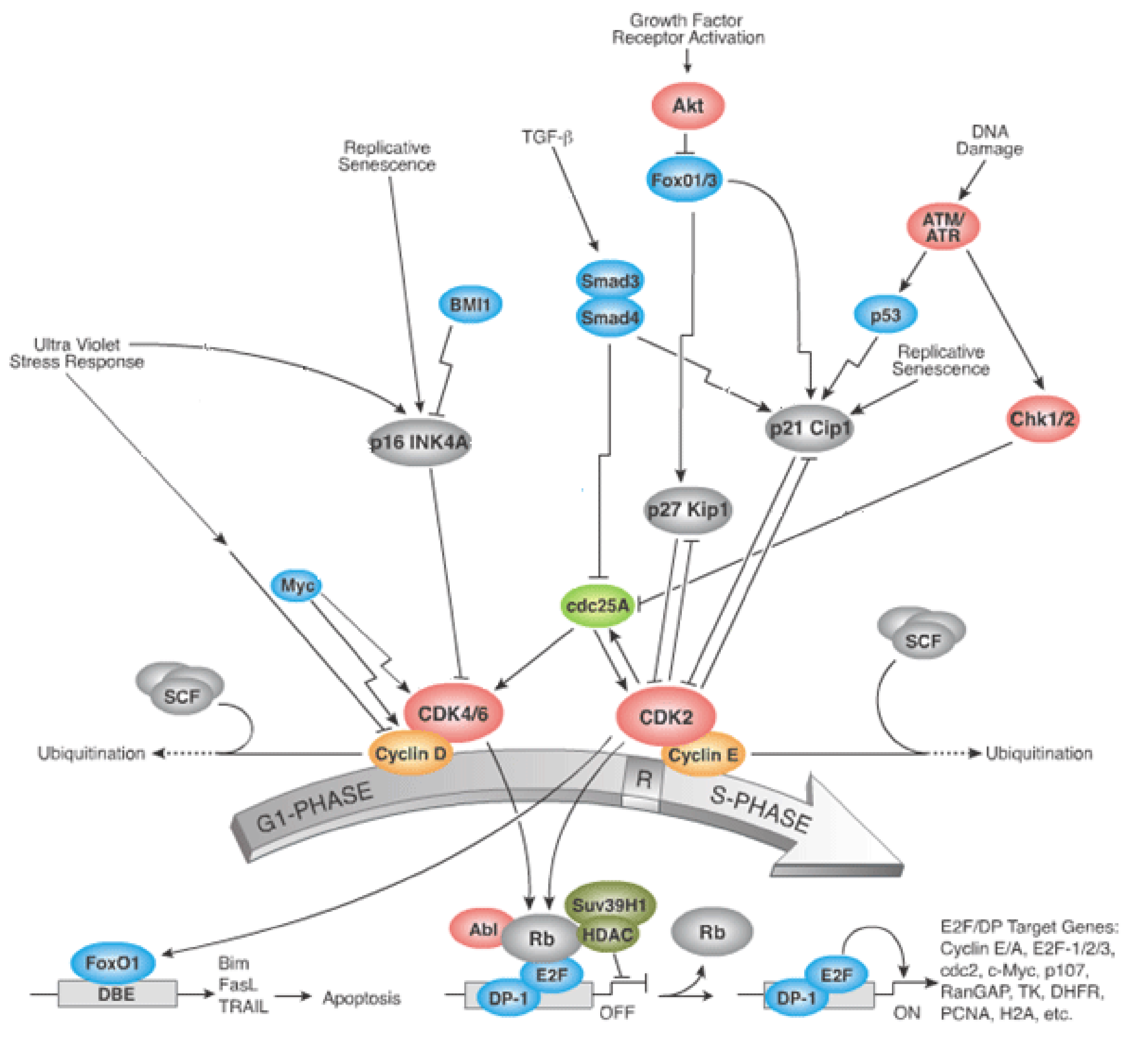

Figure 4 G1 Phase Cell Cycle Controls (Cell Signalling Technology 2010)

Stem Cells

There is direct evidence for stem cells in the hematopoietic system, epidermis, intestinal epithelium, the male germ line, the adult nervous system, and cardiac and skeletal muscle tissue (Gnecchi, Zhang *et al.* 2008; Jones and Fuller 2009; Liu and Rando 2011; Hsu and Fuchs 2012), and there is significant variation in cell types and the molecular factors that maintain homeostasis within individual compartments (Fuchs, Tumbar *et al.* 2004; Hsu and Fuchs 2012). The most extensively characterized stem cells are embryonic (ESC), neural (NSC), and hematopoietic (HSC) stem cells (Ramalho-Santos, Yoon *et al.* 2002).

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It is presumed that a stem cell compartment exists for every type of tissue or organ in the mammalian system. Stem cells are primarily characterized by the capacity for unlimited or prolonged periods of self-renewal, and the ability to produce one (unipotent) or more (oligopotent) highly differentiated descendants (Thomson, Itskovitz-Eldor *et al.* 1998; Watt, Hogan *et al.* 2000; Rando, Thomas A. 2006; Hsu and Fuchs 2012). In 1998, James Thomson and colleagues isolated human embryonic stem cells from blastocysts (Thomson, Itskovitz-Eldor *et al.* 1998), and these cells exceeded the Hayflick Limit by at least a 10-fold factor (James Thomson, 2012 - personal communication). The main features of stem cells are a cell cycle status of either prolonged arrest in G1 phase or actively cycling with *expression of telomerase*; adherence to the extracellular matrix (ECM) via integrin, ADAM and bystin proteins ; active JAK/STAT, TGF-1, and Notch signaling (Conboy, Conboy *et al.* 2003; Gouti and Gavalas 2008); a capacity to sense growth hormone and thrombin; and a high resistance to stress, with up-regulated DNA repair mechanisms, protein folding, ubiquitin system, and detoxification mechanisms, such as ABC transporters (Bodnar, Ouellette *et al.* 1998; Ramalho-Santos, Yoon *et al.* 2002; reviewed in Jones and Fuller 2009).

Data from the work of Miguel Ramalho-Santos and Douglas Melton shows that a subset of approximately 230 genes are commonly enriched in adult SCs, including 35 signaling proteins, 4 for DNA repair, 13 regulating cell cycle, 8 protein folding chaperones, and 6 proteins for toxic stress response (Ramalho-Santos, Yoon *et al.* 2002). These stem cell characteristics appear similar to those of yeast cells under oxidative stress; up-regulation of chaperones, protein degradation genes, DNA repair, and detoxifying enzymes (Ramalho-Santos, Yoon *et al.* 2002). The primary function appears to be the maintenance of tissue homeostasis, and provide replacement for the turnover of cells that are lost during normal function, or as the need arises for wound repair (Watt, Hogan *et al.* 2000; Coussens and Werb 2002; Jones and Wagers 2008; Walter, Wright *et al.* 2010); although they appear to lose their effectiveness at maintaining homeostasis with increasing age and the disruption or disorder of this

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homeostasis may serve to promote the transition from health to tumorigenesis (Rando, Thomas A. 2006; Wicha, Liu *et al.* 2006; Rosen and Jordan 2009; Bates 2010).

Stem Cell Niche

The stem cell niche is a specialized microenvironment that is required to maintain stem cell self-renewal. These fixed anatomical compartments provide a tightly controlled mechanism to regulate and direct stem cell division, the rate of proliferation, progeny differentiation, protection from parasites and viral infections, and against proliferative exhaustion (Zhang, Niu *et al.* 2003; Jones and Wagers 2008; Levi and Morrison 2008; Kiefer 2011; Neal, Richardson *et al.* 2011; Hsu and Fuchs 2012). Key components of the stem cell niche include cell-cell and cell-matrix contacts, and these help control environmental pH, oxygen tension, and soluble growth factor gradients (Engler, Sen *et al.* 2006; Discher, Mooney *et al.* 2009).

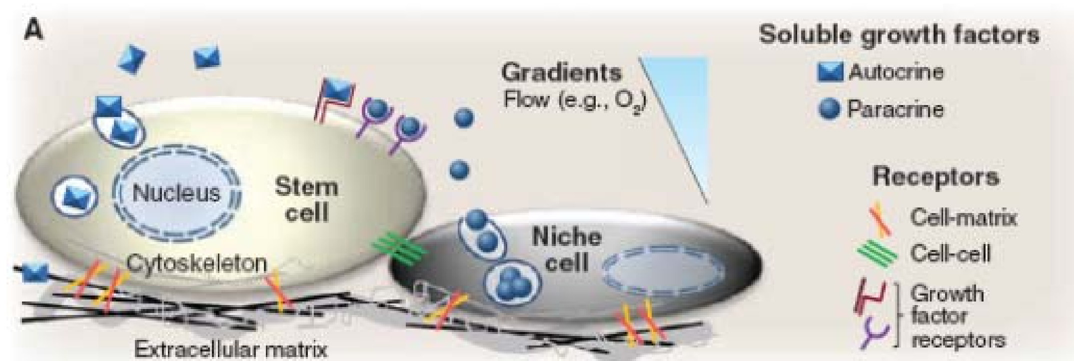

Figure 5 - The stem cell microenvironment. Cell fate and maintenance of the stem cell pool are controlled through a combination of soluble and matrix-bound factors, intercellular contact, cell-matrix adhesions, and chemical gradients *(Discher, Mooney et al. 2009)*

Maintenance of the stem cell nature within the niche is directed by the stromal cells; cell cycling, division, and population count are all tightly regulated (reviewed in Spradling, Drummond-Barbosa *et al.* 2001; reviewed in Drummond-Barbosa 2008; Levi and Morrison 2008; reviewed in Morrison and Spradling 2008; reviewed in Voog and Jones 2010). Clinical studies have long proven that stem cells can

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be transplanted, and depleted niches can be replenished - bone-marrow transplants have been successfully conducted for over 40 years since the pioneering work of George Mathe and E Donnall Thomas (Buckner, Epstein *et al.* 1970). *In vitro* studies have shown that neural cells can be derived from skin precursors (Toma, Akhavan *et al.* 2001), skeletal muscle satellite cells can replenish hematopoietic niches (Jackson, Mi *et al.* 1999), and bone marrow stem cells can produce lung tissue (Kotton, Ma *et al.* 2001; Krause, Theise *et al.* 2001). Stromal cells vary among different niche types, but commonly include fibroblasts, fat cells, mesenchymal stem cells and their progeny, for example the hematopoietic system niche also includes chondrocytes and osteoblasts (Minguell, Erices *et al.* 2001).

Inomata and colleagues showed that lack of maintenance of the melanocyte stem cell pool by niche stromal cells may result in premature differentiation of stem cells, leading to irreversible graying of hair, an indicator in the aging phenotype (Inomata, Aoto *et al.* 2009). The display that age-dependent alterations in gene expression in hematopoietic stem cells results in a decline in immune function further bolsters the concept that maintenance of the stem cell niche is a significant component in aging (Rossi, Bryder *et al.* 2005).

Extracellular Matrix

In addition to stem cells and their progenitor cells (also called transit amplifying cells), the stem cell niche includes the ECM which provides a structural scaffolding for cells, endothelial vasculature for providing nutrients and oxygen, macrophages and other immune cells to combat pathogens and remove apoptotic cells, and the epithelial cells which organize into sheets of polarized cells (Bissell and Radisky 2001). Cells secrete a variety of proteins that form the extracellular matrix (Weinberg 2007). Individual components of the ECM include collagens and glycoaminoglycans, proteoglycans, and glycoproteins (eg, fibronectins, elastins, hyaluronic acid), and the mechanical and bioinductive properties of these

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scaffolds play an important role in cellular proliferation and migration, largely the result of collagen fiber architecture and kinematics. (Romberger 1997; Labat-Robert 2004; Badylak 2007).

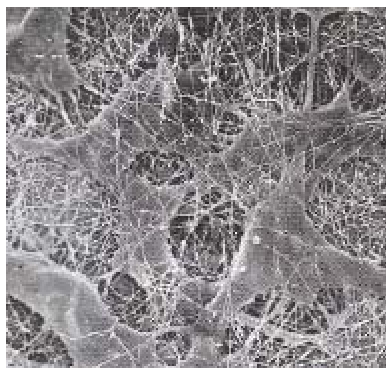

Figure 6 The Extracellular Matrix. The complex meshwork of collagen fibers, glycoproteins, hyaluronan, and proteoglycans, in which fibroblasts (connective tissue cells) are embedded. (Nishida, Yasumoto *et al.* 1988)

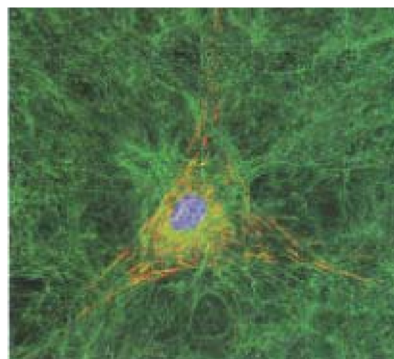

Figure 7. A 3T3 (fibroblast) cell amid an ECM network of fibronectin fibers *(green)*. Integrin receptors on the cell surface (orange, yellow) mediate the points of attachment to ECM fibronectin. (Cukierman, Pankov *et al.* 2002)

Signaling factors, secreted or induced by stromal cells or the stem cells themselves, directs stem cell fate through precise signaling pathways, though these pathways may differ for each tissue type (Jones and Fuller 2009). Stem cell regeneration reiterates the process of embryonic organogenesis and regulatory signal transduction networks, such as Notch, Wnt, TGF-β, and Hedgehog pathways (Carlson, Hsu *et al.* 2008; Jones and Wagers 2008), though they appear to be maintained by different self-renewal programs at different ages in response to changes in growth and repair demands (Levi and Morrison 2008). The Notch-1/Delta-1 and Wnt/β-catenin transduction pathways have been identified in a number of stem cell types for maintaining non-differentiated status and initiating self-renewal, while the mitotic spindle formation axis, oxygen tension, and other biochemical and biophysical forces influence division type (symmetric or asymmetric), while cytokine gradients may serve as a guide for progenitor cells to required location (Fuchs, Tumbar *et al.* 2004; Carlson, Hsu *et al.* 2008; Tottey, Johnson *et al.* 2011). Experimental control of Notch signaling in both young and old muscle tissue dramatically affects muscle regeneration (Conboy, Conboy *et al.* 2003), and the balance between Notch activation and TGF-β

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activation changes with age, resulting in under-expression of Notch/Delta signaling and the subsequent replicative senescence of satellite cells in the muscle tissue of aging mice. (Conboy, Conboy *et al.* 2003).

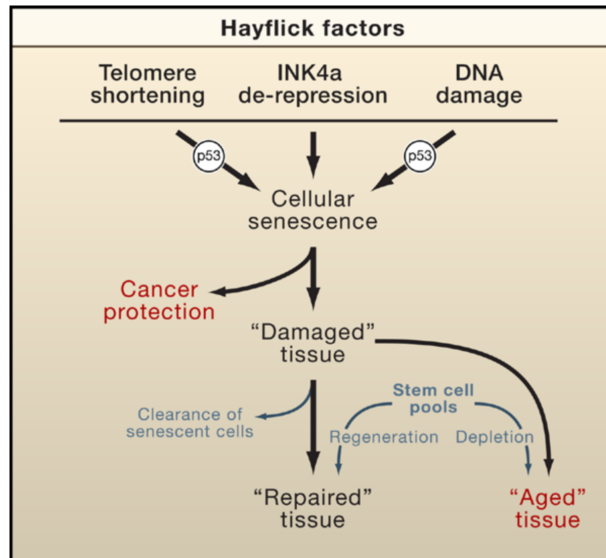

Figure 8 The classical view of cancer as the body's protection from aging; or conversely, senescence (cellular aging) as protection from developing cancer (from Collado, Blasco *et al.* 2007)

Cellular senescence

Cellular senescence is typically marked by permanent exit from the cell cycle, morphological enlargement, alterations in chromatin structure, expression of senescence-associated β-galactosidase activity, and profound changes to the transcriptome (Campisi, Judith 2005). Cellular senescence has been shown to occur in response to telomere malfunction, DNA damage, or oncogenic activation and oxidative stress (Kuilman, Michaloglou *et al.* 2010; Kosar, Bartkova *et al.* 2011). While some cells acquire a spindle shape, a flat cell phenotype is commonly seen and cells may become large, flat, and multinucleated, or display an increased number of vacuoles, depending on the cell type and the activator of senescence (Kuilman, Michaloglou *et al.* 2010). A classic characteristic of senescence is the activation of the p53 and p16INK4a–RB pathways, and a rise in levels of DDR elements and cell cycle inhibitor proteins (Campisi, Judith 2005; Kuilman, Michaloglou *et al.* 2010; Coppe, Rodier *et al.* 2011; Kosar, Bartkova *et al.* 2011). A DNA damage response (DDR) induces a temporary arrest of the cell cycle, a pause in the replication process and provides time for repair. If the DNA damage exceeds a threshold,

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cells undergo either apoptosis or proceed into a senescent state (Massague 2004; Kuilman, Michaloglou *et al.* 2010). The gene product p53 is a core pathway mediator of early senescence signals resulting from oncogene activation, telomere dysfunction, DNA damage, and increased levels of reactive oxygen species (ROS) (Massague 2004; Campisi, Judith 2005). The RB tumor suppressor network includes cyclin-dependent kinase inhibtors such as p16INK4a, p27Kip1, and p21Cip1/WAF1, which serve as G1/S cell cycle checkpoint regulators to inhibit E2F transcriptional proteins (Massague 2004). A punctate DNA staining pattern has been observed for many years, senescence-associated heterochromatic foci (SAHF), localized to methylated Lysine residues of histone H3, as well as proteins associated with specific promoter regions of E2F cell cycle regulator proteins (Kuilman, Michaloglou *et al.* 2010; Kosar, Bartkova *et al.* 2011).

The transcriptional changes seen in senescent cells include secretion of various cytokines, proteases and growth factors, known as the senescence-associated secretory phenotype (SASP) (Coppé, Patil *et al.* 2008; Kosar, Bartkova *et al.* 2011). Expression of secreted factors, including various cytokines and chemokines, insulin-like growth factor, insulin-like growth factor binding protein 3, and plasminogen activator inhibitor 1, has been shown to be altered in senescent cells, and these changes effect changes in immediate and distant neighbors (Campisi, Judith 2005; Jeyapalan and Sedivy 2008). Further, alterations in proteolysis occur in senescent cells, such that short-lived proteins tend to degrade faster, and long-lived proteins degrade slower (Okada, Annabelle A. and Dice 1984; Okada, Y. and Okada 2000; Eden, Geva-Zatorsky *et al.* 2011).

The SASP and tumorigenesis

In several studies, senescent fibroblasts have been shown to increase the rate of proliferation and the ability to alter epithelial differentiation, leading to tumorigenesis (Krtolica, Ana, Parrinello *et al.* 2001; Krtolica, A. and Campisi 2003; Parrinello, Coppe *et al.* 2005; Coppé, Desprez *et al.* 2010). Analysis of the

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SASP reveals a number of cytokines and chemokines , such as IL-6 (in response to oncogene-induced senescence, eg, BRAF) and IL-8 (in response to telomeric dysfunction) , indicating a strong similarity to the inflammatory response, as seen in wound healing; the C/EBPb and NF-kB transcription factors activate the C-Fos and Jun components of the inflammatory transcriptome (Cichowski and Hahn 2008; Kuilman, Michaloglou *et al.* 2010). While senescence can limit expansion of early malignant cells through cell cycle arrest, they can also promote cancer by stimulating the proliferation of incipient tumor cells that reside in their microenvironment , and this is consistent with the notion that cancer is "wound-healing gone awry" (Cichowski and Hahn 2008; Kuilman and Peeper 2009; Kuilman, Michaloglou *et al.* 2010; Hanahan and Weinberg 2011; Martínez-Corral, Olmeda *et al.* 2012).

ROS

Reactive oxygen species (ROS), the natural by-products of oxidative energy metabolism and cellular respiration, have long been proposed as a primary cause of cellular aging (Harman 1956; Drew and Leeuwenburgh 2002; Hasty, Campisi *et al.* 2003). Oxidative stress in cells and tissues can occur during pathophysiological developments, such as during chronic inflammatory states, prolonged allergic reaction or during ischemic or toxic and hyperglycemic conditions (Bertram and Hass 2008). Given that most tumors undergo a period of hypoxia, prior to angiogenesis, oxidative stresses can apparently worsen the condition, through a positive-feedback system. Oxidative stress also puts cells at risk for senescence or apoptosis, and the organism may be required to replace tissue cells, thereby reducing overall resilience to external stress. Ultraviolet stress, DNA damage and ROS damage to specific proteins and the proteosome, activate cell cycle control and DNA repair mechanisms; activation of ATM/ATR, p53, $p16^{INK4\alpha}$, $p21^{CIP1}$, and other proteins of the apoptosis and senescence pathways regulate cyclins, cyclin-dependent kinases (CDKs), retinblastoma (pRb) and the transcription factor E2F (Massague 2004). Cyclin-dependent kinases associate with different cyclins to regulate a cascade of proteins to control transcription, differentiation, nutrient uptake and other functions by means of

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balancing both positive (growth, survival and mitogenic) and negative (apoptotic and cytostatic; genotoxic, metabolic, oncogenic and oxidative stress) signals (Massague 2004). Cyclin D1 over-expression occurs in 50% of breast cancers (Massague 2004).

Telomere function

For many years the classical view was that chromosomes simply came to an end with telomeric repeats and a linear structure, a view which has changed dramatically in the last 15 years (Greider 1999). If the classical view were accurate, the resulting DNA structure would resemble a double-strand chromosome break in need of repair, and DNA polymerases and repair proteins would identify and recombine these ends with other chromosomal ends; and this is exactly what is observed in various cancers, and also accounts for the difference in chromosome number between humans and all other great ape species (IJdo, Baldini *et al.* 1991; van Steensel, Smogorzewska *et al.* 1998; Griffith, Comeau *et al.* 1999; Sfeir, Kosiyatrakul *et al.* 2009).

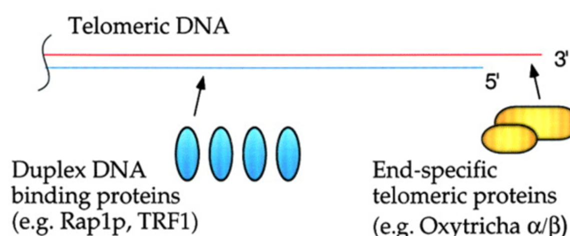

Figure 9 The classical view of telomeres and chromosomal ends (Greider 1999)

The new paradigm of chromatin structure at the terminal ends suggests a complex of telomeric tandem repeats of G-T rich nucleotide sequences (eg, TTAGGG) and associated proteins, approximately 10–15kb long, which loop back and integrate into the DNA forming Displacement loops (D-loops) and telomere loops (T-loops) with a 150–200 nucleotide- long, single-stranded overhang of GT-rich repeat sequences. (Blackburn, E H and Szostak 1984; Harley, Vaziri *et al.* 1992; Blasco 2005). Two main protein complexes are bound to telomeres, the telomere repeat binding factor 1 and 2 complexes, TRF1 and TRF2 which

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assist in the stabilization of the molecule, and structure of T- and D-loop conformations (Greider 1999; Blasco 2005).

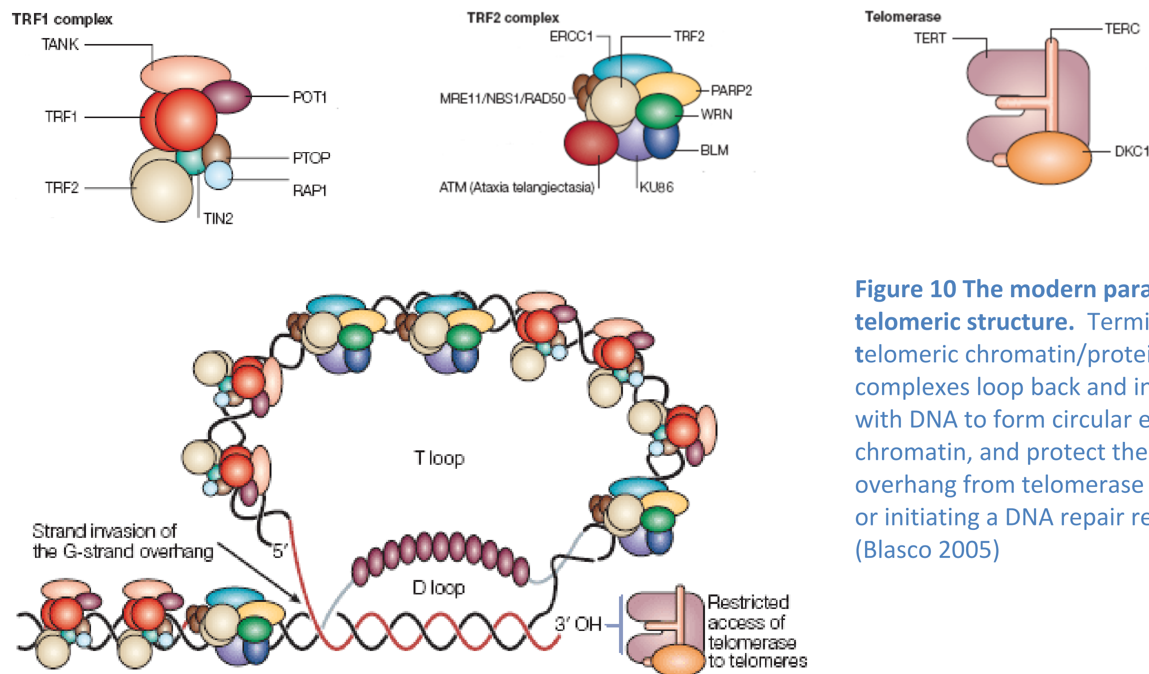

Figure 10 The modern paradigm of telomeric structure. Terminal telomeric chromatin/protein complexes loop back and integrate with DNA to form circular ends to chromatin, and protect the 3'-overhang from telomerase binding, or initiating a DNA repair response (Blasco 2005)

Telomeres serve two major functions: 1) they provide stability from end-joining homologous recombination, and 2) they allow for complete replication of the entire coding structure of DNA by DNA polymerases (Blackburn, Elizabeth H. 1991; Blasco 2005). The position and conformation of the catalytic unit of DNA polymerase, forces a loss of telomeric DNA in differentiated somatic cells with each passage through the cell cycle (Olovnikov, A. M. 1973; Blackburn, Elizabeth H. 1991), and this has been suggested as a mechanism for the molecular clock which explains the Hayflick Limit (Harley, Vaziri *et al.* 1992). When a certain length is reached on one or more telomeres in a dividing cell, a cellular "crisis" occurs, and a DNA damage response is initiated - cell cycle checkpoints and senescence pathways may be activated, resulting in removal from the cell cycle - this can be temporary or permanent (Deng, Chan *et al.* 2008; Fridman and Tainsky 2008; Sahin, Colla *et al.* 2011).

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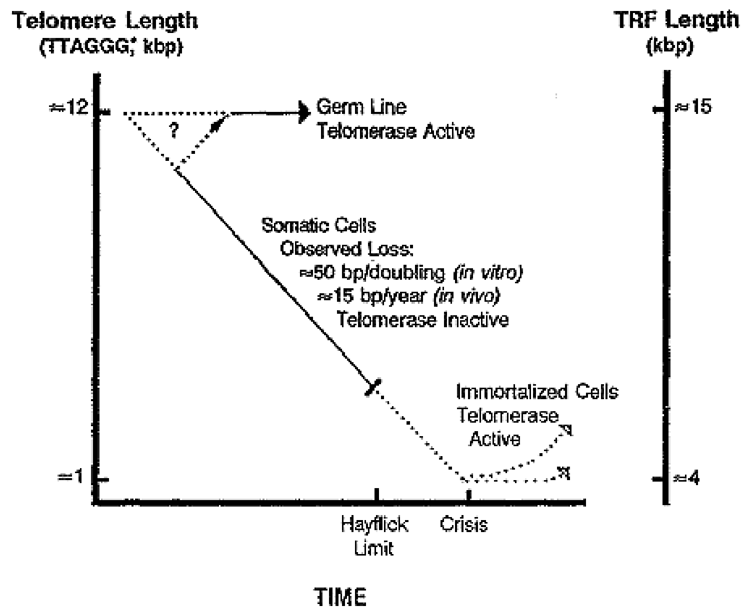

Figure 11 - Relationship of telomere length and the Hayflick Limit of cellular senescence. Stem and germ line cells possess active telomerase which synthesizes telomeres after each passage through the cell cycle. Somatic cells fail to express active telomerase, and as such telomere length decreases until a crisis occurs and the cells enter replicative senescence (the Hayflick Limit) or telomerase is reactivated and the cells become immortalized. (Harley, Vaziri *et al.* 1992)

In embryonic and adult stem cells, a rare enzyme complex, telomerase, serves to synthesize telomeres and extend protection to chromosomal ends (Blackburn, EH 1990; Cohen, Graham *et al.* 2007). Telomerase is an enzyme complex consisting of two core components, telomerase RNA component (Terc), which serves as a template for synthesis of telomeric DNA, and the catalytic protein Telomerase reverse transcriptase (Tert) and a third small nucleolar ribonucleoprotein, dyskerin (Cohen, Graham *et al.* 2007). Active telomerase complexes appear as a 670 kDa homodimer; two 334 kDa protomers, each consisting of an hTERT (127 kDa),a dyskerin (57 kDa) and a 150 kDa telomerase RNA (Cohen, Graham *et al.* 2007).

Telomerase synthesizes telomeres to maintain length in non-differentiated cells, such as stem and germ line cells (Blackburn, EH 1990; Harley, Vaziri *et al.* 1992). The reverse transcriptase (TeRT) recognizes the 3'-OH at the end of the G-strand overhang, and using the RNA molecule (TeRC) as a template, elongates the telomere (Blasco 2005).

The rarity of telomerase has been proposed to be as few as 20 molecules per cell, and binding affinity varies on the specific telomeric repeat sequence; for example, telomerase binds to the telomere more

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strongly if the reading frame sequence is TTAGGG rather than GGGTTA (Scott Cohen, 2012 - personal communication). Further, telomerase transcription is thought to be switched off during cellular differentiation, but is active in stem cells, both embryonic and adult, and cancer cells (Harley, Vaziri *et al.* 1992; Blasco 2005).

Telomere shortening activates the DNA damage response signaling pathways, mediated by the ATM and ATR kinases (d'Adda di Fagagna 2008; Sfeir, Kosiyatrakul *et al.* 2009). In functional telomeres, TRF2 and Protection of Telomeres Protein 1 (POT1) blocks the activation of ATM signaling and the action of ATR kinase, respectively (de Lange, Titia 2005; de Lange, T. 2010). Consequently, without active telomerase, shortened telomeres initiate p53-mediated cellular growth arrest, senescence or apoptosis and drives tissue atrophy and functional decline in high-turnover organs (Sahin, Colla *et al.* 2011).

In cells that are deficient in p53, persistent shortening of telomeres can result in a prolonged DNA damage response, an extended G2 phase due to an ATM-mediated cyclin inhibition, and bypass mitosis; a second S-phase duplication of the genome results in tetraploidy and a provides a plausible mechanism toward the aneuploidy seen in cancer (Gisselsson, Jonson *et al.* 2001; Davoli, Denchi *et al.* 2010).

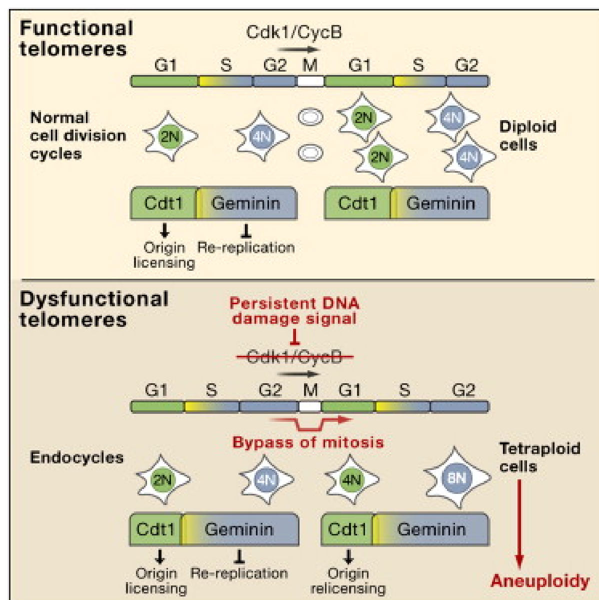

Figure 12 Dysfunctional telomeres may initiate a chronic DNA damage response, resulting in cyclin disruption. Bypassing mitosis, these cells then re-enter S-phase and become tetraploid, continuing the cycle and developing more pronounced aneuploidy. (Davoli, Denchi et al. 2010)

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Recently, an association was observed between paternal age and the telomere length of offspring across multiple generations. In a longitudinal study, grandchildren of older *paternal grandfathers* (regardless of their father's age) have longer telomeres, and passed elongated telomeres to the second generation, suggesting that life extension in the population could be achieved through late-life paternal reproduction (Eisenberg, Hayes *et al.* 2012).

Cancer

Cancer is an age-related genetic disease characterized by extensive disruption of cellular homeostasis, affecting the genome, the proteome, and the metabolome (Lengauer, Kinzler *et al.* 1998; Massague 2004; Rajagopalan and Lengauer 2004; Diehn, Cho *et al.* 2009; Eden, Geva-Zatorsky *et al.* 2011; Hanahan and Weinberg 2011; Boisvert, Ahmad *et al.* 2012). The risk of developing cancer increases with chronological age (Fridman and Tainsky 2008; Howlader, Noone *et al.* 2012).

The lifetime risk of developing cancer increases with chronological age (Fridman and Tainsky 2008; Howlader, Noone *et al.* 2012). According to the National Cancer Institute, over 40% of will be diagnosed with cancer at some time during their lifetime, and over half of them will develop cancer between age 50 and 70 (Howlader, Noone *et al.* 2012).

It is estimated that 60 percent of newly diagnosed cancers and 70 percent of cancer deaths occur in those over 65, equating to a 10-fold increase in the risk of acquiring cancer, and a 15-fold increase in the risk of death (SEER 2010). Current pharmaceutical therapies for treating cancers are, generally, ineffective in over 66% of patients (Bell 2012).

Cancers arise through a sequential acquisition of genetic mutations occurring in specific genes (Rajagopalan and Lengauer 2004). Karyotype analysis of cancer cells typically shows extensive chromosomal aberrations, loss of chromosomes, fusion of chromosomal arms, rearrangement and

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polyploidy (Knudson 2001; Weinberg 2007). Normal cells contain a full complement of 46 chromosomes, while cancer cells often contain from 60 to 90 chromosomes, and individual cells within the tumor will differ in number (Rajagopalan and Lengauer 2004).

Genetic alterations in cancer fall into four major categories: subtle sequence changes, alterations in chromosome number, chromosome translocations, and gene amplifications (Lengauer, Kinzler *et al.* 1998).

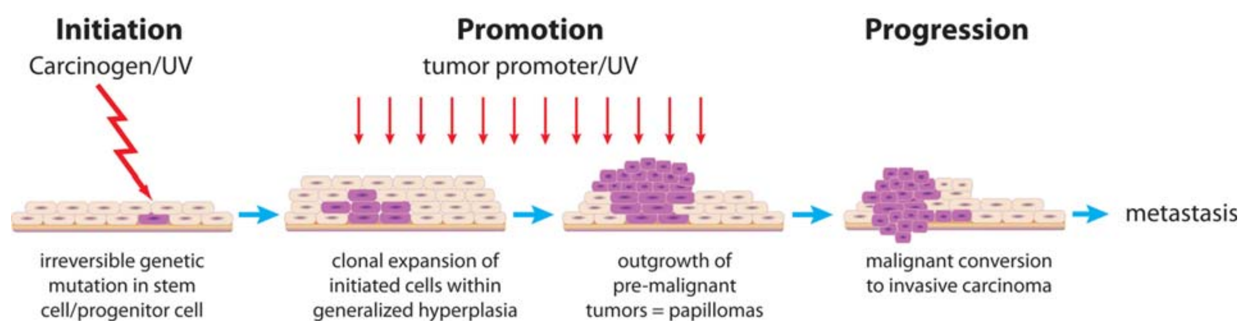

Figure 13 - Three stages of cancer development. Cells exposed to UV light, carcinogenic environmental chemicals and endogenous toxins, that continue through the cell cycle are subject to increased damage and DNA alteration, which DNA synthesis can fix as a mutation, resulting in the initiation of tumorigenesis, the promotion of the tumor growth, and progression to a malignant state that can invade distant tissue. (Rundhaug and Fischer 2010)

Changes to normal cellular mechanisms, termed the "hallmarks of cancer" by Hanahan and Weinberg, include uncontrolled, sustained proliferation, evasion of growth suppressors, resistance to apoptosis, replicative immortality, expression of angiogenic factors, and increased motility and ability to metastasize (Hanahan and Weinberg 2000). Two important characteristics also include 'genomic instability' and 'inflammation', and there is debate within the medical and research community as to whether these are causative, or a result (reviewed in Rajagopalan and Lengauer 2004; Hanahan and Weinberg 2011).

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Cancer stem cell hypothesis

The cancer stem cell (CaSC) hypothesis is based on widespread observation of cellular and genomic heterogeneity within tumors, and that the majority of tumor cells are rapidly proliferating cells or postmitotic, terminally-differentiated cells, neither of which possess the capacity for self-renewal and long-term sustainability (Quintana, Shackleton *et al.* 2008). The CaSC hypothesis proposes that the growth of tumors is fueled by limited numbers of dedicated stem cells that are capable of self-renewal, recapitulating normal developmental processes seen in rapidly proliferating epithelial tissue such as bone marrow, intestine and skin (Clevers 2011).

Healthy stem cells are protected from mutagens and carcinogens by their replicative quiescence, high expression of ATP-binding cassette (ABC) transporters (drug pumps), anti-apoptotic proteins and DNA damage repair mechanisms, and these same characteristics are observed in cells that have been shown to regenerate tumors via serial transplantation, suggesting an explanation for the failure of modern chemo- and radiotherapy to successfully eliminate tumors, and prevent recurrence (Voog and Jones 2010; Clevers 2011).

It has long been suggested that permanent removal from the cell cycle, replicative senescence, is a biological mechanism to prevent cancer, however in the last decade, as our understanding of stem cell biology, tissue repair, and developmental cellular induction increases, it is becoming clear that in some cases cellular senescence saves the organism from one particular tumorigenic initiation, only to cause another in a neighboring cell. Cellular senescence is therefore a double-edged sword which can both prevent, and promote cancer (Campisi, Judith 2005).

The tumor-suppressor mechanisms of the p53 network, and retinoblastoma (Rb) family proteins are two primary cell cycle regulators for preventing the synthesis of damaged DNA, and cellular division. Two

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classic results of constitutive activation of these networks are the programmed cell death of apoptosis and permanent removal from the cell cycle through cellular senescence.

ROS double-agent theory

Research into pharmaceutical therapies to combat cancer and old age have failed to deliver an unequivocal clinical breakthrough. Attempts to treat age-related diseases with antioxidant supplements have resulted in Americans having among the most expensive urine in the world; further, there is evidence that supplemental antioxidants actually inhibit expression of superoxide dismutases and result in an increase of ROS in the cell (reviewed in Gutteridge and Halliwell 2000). Nick Lane proposes a "double-agent theory" drawing on flaws in three leading theories of ageing: he argues that a tradeoff exists between oxidative stress as a critical redox signal that induces inflammatory responses such as those orchestrated by NFkB toward infection, and oxidative stress as a cause of aging and age-related disease (Lane 2003). The double-agent theory suggests that aging is a function of increased intracellular oxidative stress, and that there is an increase in free-radical leakage through mitochondrial membranes with the increase of chronological age. According to Lane, this continuous mitochondrial leakage produces a persistent shift in gene expression leading to the chronic inflammation characteristic of old age (Lane 2003).

Senescence and Cancer

The development and progression of many tumors results from the coordinated activity of stem cells and neighboring fibroblasts and inflammatory cells and the loss of molecular fidelity (Romberger 1997; Ohashi, Kiehart *et al.* 1999; Beacham and Cukierman 2005; Zigrino, Löffek *et al.* 2005; Hanahan and Weinberg 2011). Modifications in ECM surrounding stem cells can occur as a result of senescent fibroblasts in the stem cell microenvironment, and alterations in fibronectin expression and changes to fibronectin properties alter stem cell signaling, affecting cellular communication, cell adhesion, and

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proteolysis (Romberger 1997; Beacham and Cukierman 2005; Zigrino, Löffek *et al.* 2005; Chondrogianni and Gonos 2010; Boisvert, Ahmad *et al.* 2012). Changes in fibronectin properties alter maintenance of the stem cell population, and signals directing differentiation, expression of growth factors, cytokines and proteases, and has been shown to impact promotion, progression and metastasis (Romberger 1997; Beacham and Cukierman 2005; Zigrino, Löffek *et al.* 2005).

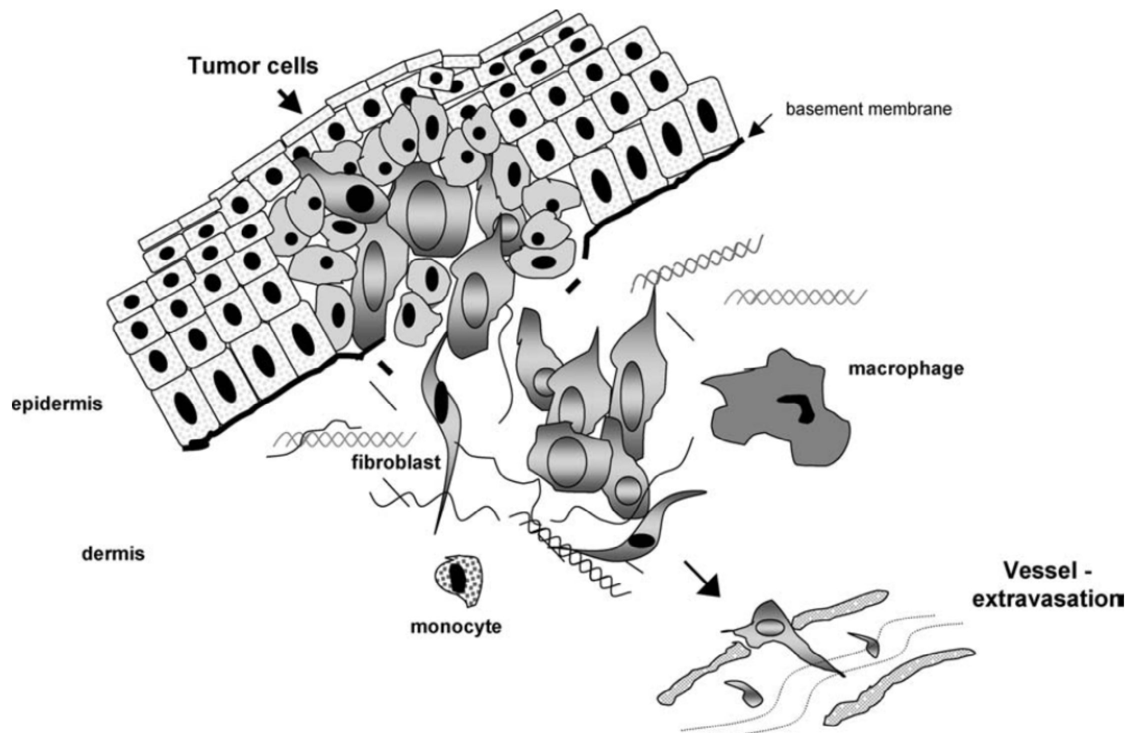

Figure 14 Metastasis of epidermal tumors. The first barrier of tumor migration from the primary site is the connective tissue of the ECM. Escaping it depends on adhesion, degradation, and migration. Protein structural changes resulting from the SASP of fibroblasts plays a critical role in each step. Altered fibronectin and over-expression of matrix-degrading proteases, are primary drivers of the process (Romberger 1997; Beacham and Cukierman 2005; Zigrino, Löffek *et al.* 2005). Image from Zigrino et al, 2005 (Zigrino, Löffek *et al.* 2005).

Stem cell depletion through apoptosis versus senescence

The Cancer Stem Cell (CSC) hypothesis provides explanation for several clinically observed phenomena in tumorigenesis and cancer treatment. CSCs are nearly identical to normal stem cells, in that they possess the capability of self- renewal, resilience to radiological and chemical treatment, exist in a prolonged state of quiescence, and are designed to migrate to and colonize in distant sites. The CSC

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hypothesis addresses what is commonly seen in the clinic: even when response to radiation or chemotherapy is encouragingly robust, we do not consider cancer "cured," as rare tumor cells are able to survive cytotoxic treatment and recurrence is common in most cancers (Bonnet and Dick 1997; Dick 2008; Clevers 2011). The impact of senescent cells on the population of stem cells in an organ microenvironment is best exemplified in the example of an intestinal stem cell population, found in the base layers of the intestinal crypt.

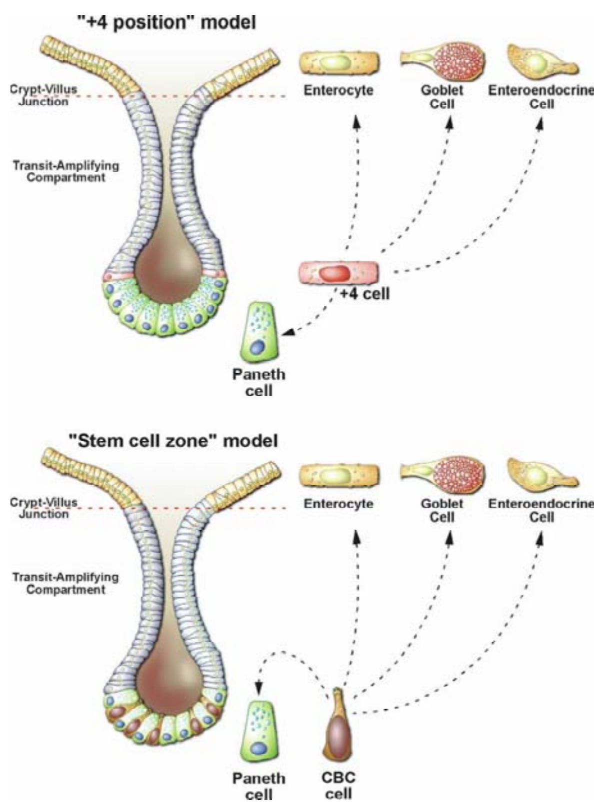

Figure 15 - Two models of Intestinal crypt stem cells. (*Top* panel) The “+4 position” model assumes that that the crypt base is exclusively populated by terminally differentiated Paneth Cells and stem cells are located just above the Paneth cells at the +4 position. This model, suggests that enterocytes, goblet cells, and enteroendocrine cells are derived from +4 cell progeny that differentiate as they migrate out of the crypts onto the villi, while Paneth cells differentiate as they migrate down toward the crypt base (Barker, van de Wetering *et al.* 2008; Barker, Ridgway *et al.* 2009).

(*Bottom* panel) The “stem cell zone” model proposed by Cheng and Leblond (1974) suggested that undifferentiated "crypt base columnar cells," the true intestinal stem cells, are distributed among Paneth cells at the base of the crypt (Cheng and Leblond 1974; Barker, van de Wetering *et al.* 2008; Barker, Ridgway *et al.* 2009).

The epithelium of the human small intestine is replaced every 3-6 days throughout life, originating from a stem cell niche located at the bottom of the intestinal crypt (Barker, van Es *et al.* 2007; Barker, van de Wetering *et al.* 2008; Barker, Ridgway *et al.* 2009; Barker, Bartfeld *et al.* 2010). Intestinal stem cells (ISCs) have been identified through unique expression of a G protein-coupled receptor, Lgr5 (Barker, van Es *et al.* 2007; Haegebarth and Clevers 2009; Merlos-Suárez, Barriga *et al.* 2011; Neal, Richardson *et al.* 2011). The path of their transient amplifying (TA) progeny, supports the 'stem cell zone' model

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proposed by Leblond. TA cells expand through several passages and migrate upwards through a morphogen-like gradient of Wnt signals along the crypt wall. Also present is a gradient of EphB2, a receptor tyrosine kinase member of the NMDA (glutamate receptor) signaling pathway (Merlos-Suárez, Barriga *et al.* 2011). As TA cells approach the intestinal lumen, they undergo cell cycle arrest and achieve terminal differentiation (Barker, van de Wetering *et al.* 2008; Merlos-Suárez, Barriga *et al.* 2011).

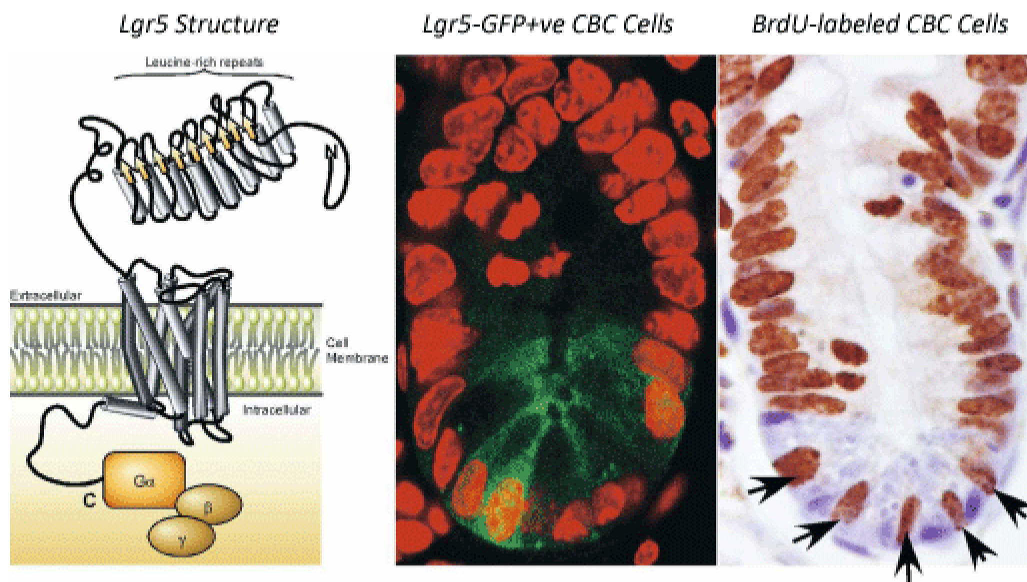

Figure 16 The G-protein-coupled receptor (GPCR) Lgr5. The structure of the Leucine-rich GPCR (left). Confocal image of Lgr5-GFP+ Crypt-Base-Columnar (CBC) cells at the base of an intestinal crypts, interspersed between Paneth cells (middle). The right panel shows a 24-hour label-retention of BrdU-stained Lgr5^{+ve} CBC cells, indicating that all are actively cycling (Barker, van de Wetering *et al.* 2008).

Fibronectin

Among the components of the SASP are metallomatrix proteases, which increase the number of proteolytic fragments of matrix macromolecules (Labat-Robert 2004). Fibronectin fragments have been shown to increase tissue degradation, recruitment of inflammatory cells and tumor progression (Labat-Robert 2004). Cell–matrix interactions are critically important in tissue homeostasis (Labat-Robert 2004).

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In the 1980s De Petro and Barlati showed that fibronectin fragments bind with G-protein-coupled receptors (GPCRs) in tumor patient plasma cryoprecipitate, can transduce extracellular stimuli to intracellular signals and effect transformation of chicken embryo fibroblasts infected with Rous sarcoma virus (De Petro, Barlati *et al.* 1981; Labat-Robert 2004) (Armstrong and Armstrong 2000; Short, Boyer *et al.* 2000; Kroeze, Sheffler *et al.* 2003; Labat-Robert 2004; Barker, van de Wetering *et al.* 2008).

Proteins of the ECM connect cell membranes, and provide a tensile conduit through the membrane, across the cytoplasm, and into the nucleus, providing a signal transduction channel seemingly for the purpose of controlling transcription, cell motility mechanisms, and differentiation (Hynes 1999; Ohashi, Kiehart *et al.* 1999; Armstrong and Armstrong 2000; Attwell, Roskelley *et al.* 2000; Labat-Robert 2004; Discher, Janmey *et al.* 2005; Engler, Sen *et al.* 2006; Discher, Mooney *et al.* 2009; Lodish, Flygare *et al.* 2010). As fibronectin is degraded in the ECM, cryptic fragments bind to cell-surface receptors and initiate transcription; specifically, *fibronectin fragmentation is a primary signal to the cell to produce more fibronectin* (Labat-Robert 2004).

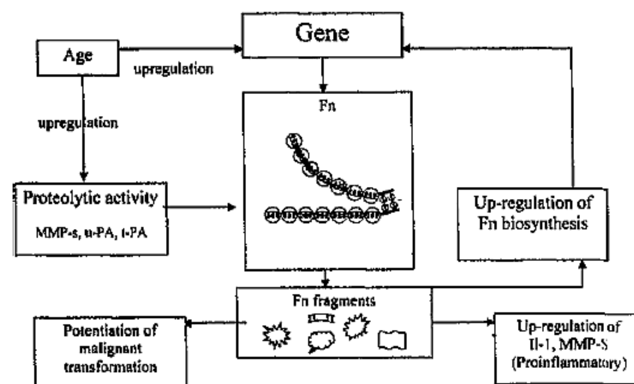

Figure 17 Proteolytic fragmentation of fibronectin induces amplification of fibronectin biosynthesis with age (Labat-Robert 2004).

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The specific properties of collagen and fibronectin change with age, and understanding how tissue cells such as fibroblasts, myocytes, and neurons, sense matrix stiffness is beginning to emerge as quantitative studies of adherent cells and relative elasticity can be measured (Discher, Janmey *et al.* 2005). It has been observed that local matrix stiffness has a direct impact on cell state with regard to development, differentiation, disease, and regeneration (Discher, Janmey *et al.* 2005).

The ECM mediates cell attachment, provides cell signals, binds growth factors (and limits their diffusion), and in 2006, Adam Engler at the University of Pennsylvania showed that ECM matrix protein characteristics *(eg, rigidity/elasticity, atomic force)* have a direct impact on lineage specification (Engler, Sen *et al.* 2006; Discher, Mooney *et al.* 2009). Engler observed that marrow-derived mesenchymal stem cells have the capacity to differentiate into discrete cell types, based on the impact of matrix forces corresponding to the stiffness of matrix network for each tissue type (Engler, Sen *et al.* 2006). Using an vitro gel system to allow for control of matrix elasticity, he showed conclusively that **stem cell plasticity is guided by physical surface tension**: that is, soft matrix resulted in differentiation into neuronal tissue, medium-tension matrix resulted in myoblasts, and high-tension resulted in osteoblasts (Engler, Sen *et al.* 2006; Discher, Mooney *et al.* 2009).

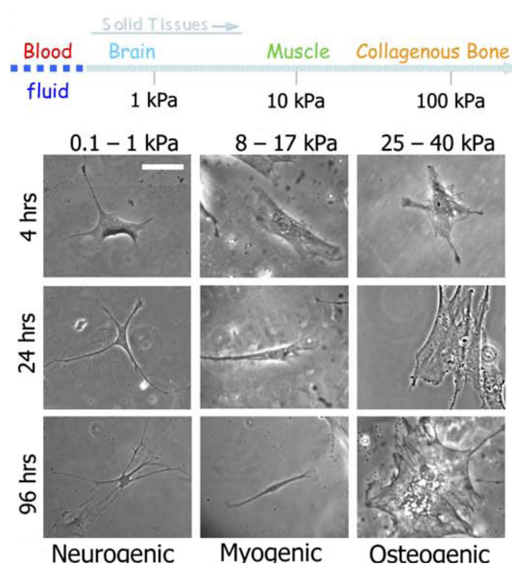

Figure 18 - Solid tissues exhibit a range of stiffness,

An in vitro ystem controls crosslinking, and cell adhesion, allowing for discrete changes of force exhibited by the ECM. Standard phenotype, naive MSCs develop increasingly branched, spindle, or polygonal shapes when grown on matrices representing specific tissue types: Brain (0.1–1 kPa), Muscle (8–17 kPa), or bone (25–40 kPa). Scale bar is 20 mm.

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Summary

It is widely accepted that cellular senescence serves as an anti-cancer mechanism, the resulting consequence of which is aging. Because tumorigenesis is the result of multiple genetic alterations or genetic 'hits' it is reasonable to conclude that stem cells are among the few cell populations that would survive long enough to accumulate these mutations.

Ostensibly, stem cells exhibit increased DNA repair mechanism activity and greater volume of ABC transporters to extrude toxins, to protect against accumulation of damage, and when these mechanisms fail they are occasionally removed through apoptosis or senescence when cellular injury or dysfunction occurs (Knudson 2001; Lynch, Magnus D. 2006; Hanahan and Weinberg 2011).

In the case of mutation, both the deletion from the stem cell pool or the maintenance of dysfunctional stem cells within the microenvironment present significant risks (Campisi, J., Kim *et al.* 2001). If a stem cell is deleted via apoptosis, companion stem cells in the niche will receive a signal to replenish the number, and at least one of those cells will divide symmetrically, restoring the niche number. The niche now contains the full complement of stem cells, but the genomic diversity has been reduced, such that the probability for occurrence of another mutation increases; If the stem cell enters a senescent state, the overall niche function is strained, as fewer cells are left to maintain tissue homeostasis (Lynch, M. D. 2004; Lynch, Magnus D. 2006). The result is a choice - increased risk of cancer, or decreased stem cell capacity and development of the aging phenotype, and cellular senescence plays a direct role in both cases.

The discrete numbers of stem cells are tightly regulated through interaction with stromal cells and the extracellular matrix (Jaenisch and Bird 2003; Boyer, Lee *et al.* 2005; Discher, Mooney *et al.* 2009). As fibroblasts age, changes in their secretory phenotype affect the production and efficacy of proteins that are involved in micro-environmental homeostasis. Changes in exerted mechanical force between

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extracellular matrix proteins and stem cells can directly affect cellular differentiation, and under stress, fragments of the ECM proteins have been shown to promote cellular division, proliferation and migration - a hallmark of cancer (Engler, Sen *et al.* 2006).

Applying de-cellularized ECM proteins to chronic wounds dramatically improve outcomes, and appears to reset the cycle of cellular behavior in aging or damaged tissue environments. Conversely, cancer cells have been shown to degrade the ECM of their own environment. While cell differentiation and tissue regeneration have both been shown to be directly affected by ECM proteins, it would be interesting to see the affect of ECM proteins on cancerous cells *in vitro*.

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Intrathymic thymopoiesis and the induction of T cell self-recognition

by Dave Comstock

for Dr. David Calhoun, Dr. Karen Hubbard, and Dr. Jerry Guyden, The City College of New York

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The basis of the immune response is the ability of T cells to distinguish self from foreign antigens. Explain how this process occurs in the thymus (i.e. explain T cell development and how mature cells are selected). What cells of the thymus are involved? What is positive and negative selection? Explain the role of the T cell antigen receptor in this process.

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Thymic lymphocytes (T cells) play a key role in cell-mediated immunity, possessing the ability to recognize and eliminate cells infected with bacteria, viruses and some tumor cells by secreting a variety of cytokines or by directly inducing cellular apoptosis in aberrant cells.

T cells arise from hematopoietic stem cells in the bone marrow that migrate to the thymus, and develop through a coordinated process of interaction with thymic stromal cells in a highly specific, step-wise developmental pathway.

Early in development a divergence of two distinct lineages occurs in thymocytes, based on T cell receptor configuration, identifying them as either $\gamma{:}\delta$ or $\alpha{:}\beta$ TCR cells. Within the $\alpha{:}\beta$ lineage, cells diverge further into subsets based on secretory factors and expression of CD4 or CD8 co-receptors, these subsets include T_H1 and T_H2, T_H9, T_H17, T_H22 or T_{REG} cells. Approximately 95% of T cells commit to the T_H1 or T_H2 cell type (Itoh, Takahashi *et al.* 1999; Janeway Jr, Travers *et al.* 2004; Weaver, Harrington *et al.* 2006; Dong and Martinez 2010).

The development pathway of T cell progenitors is delineated by a number of specific events including somatic rearrangement of the T cell receptor (TCR) gene loci, "double-positive" (DP) expression of CD4 and CD8, a process that restricts their 'identity' to binding either class-I or class-II of the major histocompatiblity complex, and a selection process that regulates survival based on their affinity for self-peptides (Janeway Jr, Travers *et al.* 2004).

This paper will focus on these canonical developmental stages and markers of early thymic progenitor (ETP) from their entry into the thymus through their exit as a naive mature $\alpha{:}\beta$T cell.

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The thymus represents the primary site for T cell lymphopoiesis, or thymopoiesis, and provides a microenvironment that coordinates a discrete set of factors that induce and support lineage commitment, and the development, differentiation and survival of T cells (Miller 1961; 1992).

Jacques Miller and colleagues performed a number of post-natal thymectomy experiments in the late 1950s which resulted in repeated atrophy of the lymphoid system, increased susceptibility to foreign infection, and failure to reject xenografts (Miller 1961).

T lymphoctyes, or T cells, are part of a group of white blood cells identified as those lymphocytes that develop within the thymus and express a unique surface molecule complex known as a T cell receptor (TCR); and it is these cells that perform a central role in cell-mediated immunity (Allison and Lanier 1987; Miller 1992).

Research over the last several decades have shown that the thymus and the particular orientation and organization of various stromal cell types orchestrate a specific interaction process with developing thymocytes; secreting chemokines to induce proliferation, differentiation and protein expression, and lead thymocytes through the cortex to the subcapsular region and then back to the medulla of the thymic lobes before exiting the thymus and migrating to peripheral lymphoid organs (Miller 1992; 2002; Janeway Jr, Travers *et al.* 2004; Miller 2011).

TCRs are disulfide-linked, membrane-anchored heterodimers consisting of highly variable alpha (α) and beta (β) chains expressed as part of a complex with an invariant set of CD3 chain molecules; also referred to as α:β T cells, a minority of T cells (~5%) express an alternate receptor formed by variable γ and δ chains, and these are known as γδ:T cells (Janeway Jr, Travers et al. 2004).

The individual alpha and beta chains that form the TCR contain a Variable (V), Joining, and Constant (C) region, while the β-chain contains an additional Diversity (D) region. Each chain of the TCR is formed

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individually through a rearrangement of the genetic loci of the TCR genes which are located separately on chromosomes 14 and 7, facilitated through a variety of transcription factors and proteins that enable chromatin remodeling, recombination of DNA sequences, random insertion of nucleotides to those rearranged gene segments, and ligation of the double-strand structure (Collins, M. K. L., Goodfellow *et al.* 1984; Collins, M. K. L., Goodfellow *et al.* 1985; Dudley, Petrie *et al.* 1994; Livak, Petrie *et al.* 1995; Nakajima, Menetski *et al.* 1995; Fehling, Hans Jörg and Von Boehmer 1997; Janeway Jr, Travers *et al.* 2004).

In the fully formed receptor, the constant region anchors the TCR to the cell membrane, including a short cytoplasmic tail, while the extracellular Variable region contains a number of binding sites for recognition of antigens bound to MHC complexes (San José, Sahuquillo *et al.* 1998).

The α:β TCR heterodimer associates closely with a CD3 complex of peptide chains, and together they are responsible for recognizing antigens bound to MHC molecules, and serve as a transducer for T cell signaling (Miller 1992; San José, Sahuquillo *et al.* 1998).

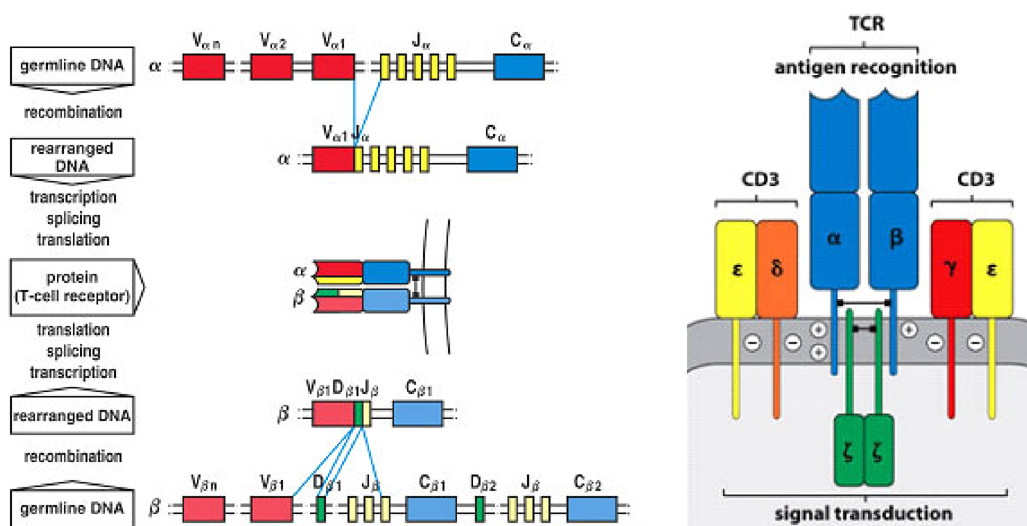

Figure 1 Rearrangement of the a/b chains to form the TCR. Association with CD3 complex allows the structure to recognize antigens and transduce intracellular signals (Janeway Jr, Travers *et al.* 2004)

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The TCR/CD3 complex is constructed in the endoplasmic reticulum through an initial formation of dimers of CD3e with either CD3γ or CD3δ, and these dimers then aggregate with either TCRα and TCRβ chains, before finally adding a CD3ζ homodimer, then transferred to the surface (San José, Sahuquillo *et al.* 1998). Based on the expression of co-receptors CD4 or CD8, α:βT cells are divided into two primary types of cell: helper T cells (T_H) or cytotoxic (or cytolytic) T cells (T_C or CTLs) (reviewed in Janeway Jr, Travers *et al.* 2004; Vallejo, Davila *et al.* 2004; Dong and Martinez 2010).

MHC structure and antigen presentation

Mature T cells in the periphery serve to recognize antigenic determinants, or epitopes, presented by terminally differentiated or antigen presenting cells (APCs) such as macrophages or dendritic cells (Boehmer 1988; Townsend and Bodmer 1989; Miller 1992; Janeway Jr, Travers *et al.* 2004; Vallejo, Davila *et al.* 2004; Nicholson, Hahn *et al.* 2005). The human MHC, or HLA, contains approximately 21 highly polymorphic genes, and 5000 different alleles, located within the 6p21.3 region of the short arm of human chromosome 6, that contribute immensely to the diversity within the immune system (Horton, Wilming *et al.* 2004; Robinson, Mistry *et al.* 2011).

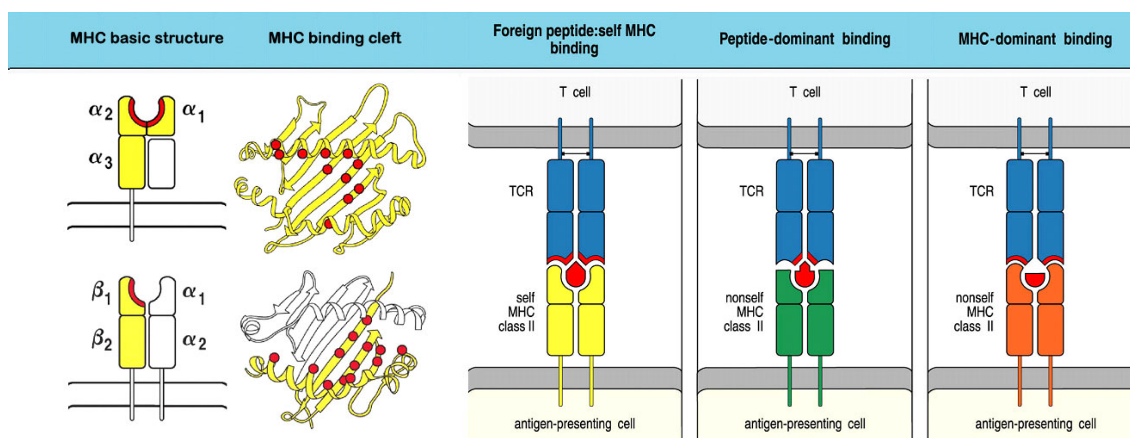

Figure 2 MHC Structure. A groove on the upper surface of the MHC molecule binds peptides of 8–10 amino acids in length (Hewitt 2003; Janeway Jr, Travers et al. 2004). These peptide fragments are derived from either endocytic vesicles (binding to MHC class II) or fragments normally derived from the cell's defective ribosomal translation products, cytosolic (binding to MHC class I); in an infected cell, peptides may be derived from bacterial or viral proteins (Boehmer 1988; Hewitt 2003; Janeway Jr, Travers *et al.* 2004; Nicholson, Hahn *et al.* 2005).

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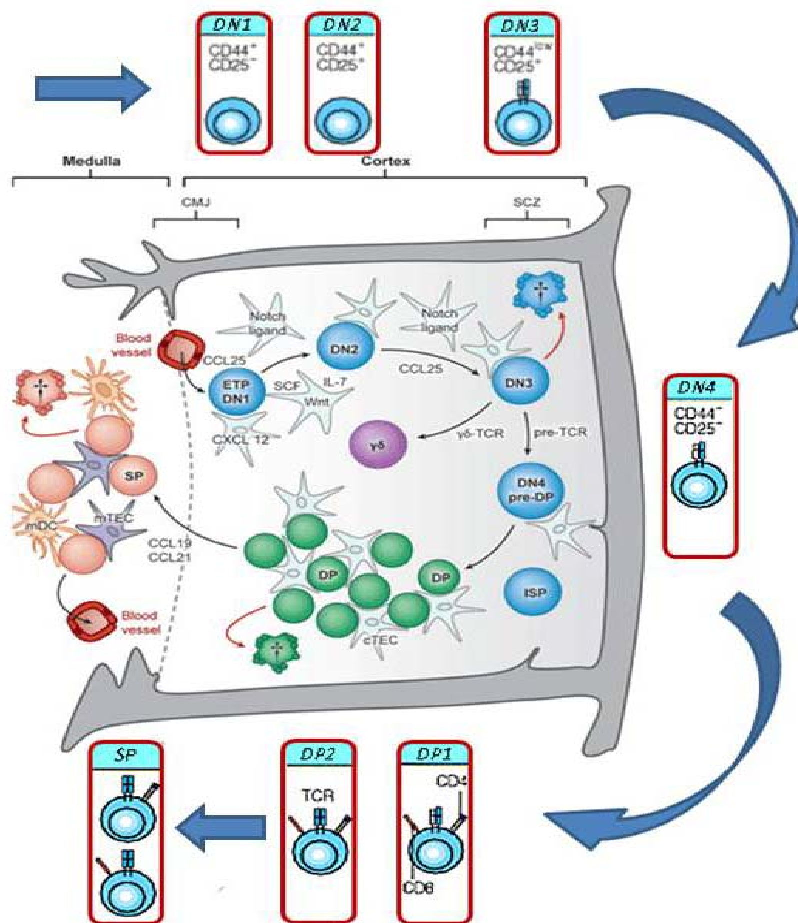
Intrathymic development

T cell development occurs in discrete steps in a compartmentalized fashion within specific microenvironments of the thymus, through contact with different types of stromal cells in the cortex and medulla (Fehling, Hans Jörg and Von Boehmer 1997; Janeway Jr, Travers *et al.* 2004).



Entering at the corticomedullary junction region of the thymus from the circulatory system, thymocytes are drawn to the cortex, and induced to proliferate and undergo changes in genetic expression, through a variety of cytokines and protein signaling pathways (reviewed in Fehling, Hans Jörg and Von Boehmer 1997; Wu 2006; Schlenner and Rodewald 2010).

As they migrate through specific regions of the thymus, they engage with thymic stromal cells such as dendritic cells (DCs), cortical thymic epithelial cells (cTECs) and thymic nurse cells (TNCs) that provide

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other developmental cues as they develop, and reciprocally, the developing thymocytes provide a "cross-talk" set of signals which is critical in the development of stromal cells (Hikosaka, Nitta *et al.* 2008; Love and Bhandoola 2011; Nitta, Ohigashi *et al.* 2011).

Distinct phases of thymocyte development are marked by changes in surface moleucle expression, specifically: TCR beta-chain selection, expression of co-receptors CD4 and CD8, MHC class restriction, TCR alpha chain determination, single-positive expression of either CD4 or CD8 co-receptor, and negative selection against cells reactive to peripheral tissue antigen (Clevers, Alarcon *et al.* 1988; Mombaerts, Clarke *et al.* 1992; Fehling, Hans Jörg and Von Boehmer 1997; Janeway Jr, Travers *et al.* 2004).

Double Negative Stages

Bone marrow-derived early thymic progenitor (ETP) cells, *(surface marker phenotype SCA-1$^+$, Thy-1$^+$, c-Kit$^+$, CD27$^+$ and CD44$^+$ CD4$^-$ CD8$^-$ CD25$^-$)* are drawn into the thymus by p-selectin glycoprotein ligand 1 (PSGL-1), and enter thymic lobes by way of high endothelial venules (reviewed in Schmitt, de Pooter *et al.* 2004; Yang, Jeremiah Bell *et al.* 2010; Koch and Radtke 2011).

Because these cells express neither CD4 nor CD8, they are termed double negative(DN), a designation that has four subsequent stages, DN1 through DN4. More specific identification of these cells is additionally based on differential surface expression of the CD25, CD44, and c-Kit molecules (Koch and Radtke 2011).

Upon entering the thymus, DN1 thymocytes reside in the corticomedullary junction region, and may continue proliferating for up to 10 days; these cells still retain the differentiation capacity to become eitherT cells, B cells, macrophages, dendritic cells, monocytes, or NK cells (Wu, Vremec *et al.* 1995; Schmitt, de Pooter *et al.* 2004; Yang, Jeremiah Bell *et al.* 2010; Koch and Radtke 2011).

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Notch-Delta signaling is a crucial signaling pathway is a prime determinant in B-cell (CD19+) or T cell (CD25+44+) differentiation, driving proliferation and lineage commitment at the DN1 stage until β-chain selection occurs or γ:δ selection occurs (Schmitt, de Pooter et al. 2004; Rothenberg 2012). Chemotactic cytokines, low molecular weight soluble proteins, serve to promote activation and migration (Ibelgaufts 2012). DN cells advance through gradients of chemokines CCL7, and CXCL4 and CCL9 toward the cortex and subcapsular zone of the thymic lobule; while IL-7 concentrations promote thymocyte proliferation (Misslitz, Pabst et al. 2004; Rossi, Corbel et al. 2005; Takahama 2006; Rothenberg, Moore et al. 2008; Gossens, Naus et al. 2009; Carpenter and Bosselut 2010). After DN1 cells migrate into the cortex they receive signals from cortical thymic epithelial cells (cTECs) and fibroblasts which promote changes in surface molecule expression as they differentiate into DN2 cells (Fehling, Hans Jörg and Von Boehmer 1997; von Boehmer and Fehling 1997; Koch and Radtke 2011).

At the DN2 stage, cells that express the phenotype CD4-,CD8-, CD25+, CD44+, have lost the capacity to differentiate into B-cells, however they maintain the potential to develop into NK-cells, dendritic cells, and T-cells (Wu et al, 1996; Schmitt et al, 2004).

Cues from stromal cells alter transcription factor and protein expression in developing thymocytes, particularly influencing random rearrangement of the TCR gene locus (Petrie, Livak *et al.* 1995; Misslitz, Pabst *et al.* 2004; Webb, Kelly *et al.* 2004; Huseby, White *et al.* 2005; Gleimer and von Boehmer 2010).

Genes that encode the β-chain of the TCR heterodimer undergo recombination of their variable (V), diversity (D), and joining (J) segments from the germline DNA sequence (Allison and Lanier 1987; Petrie, Livak *et al.* 1995).

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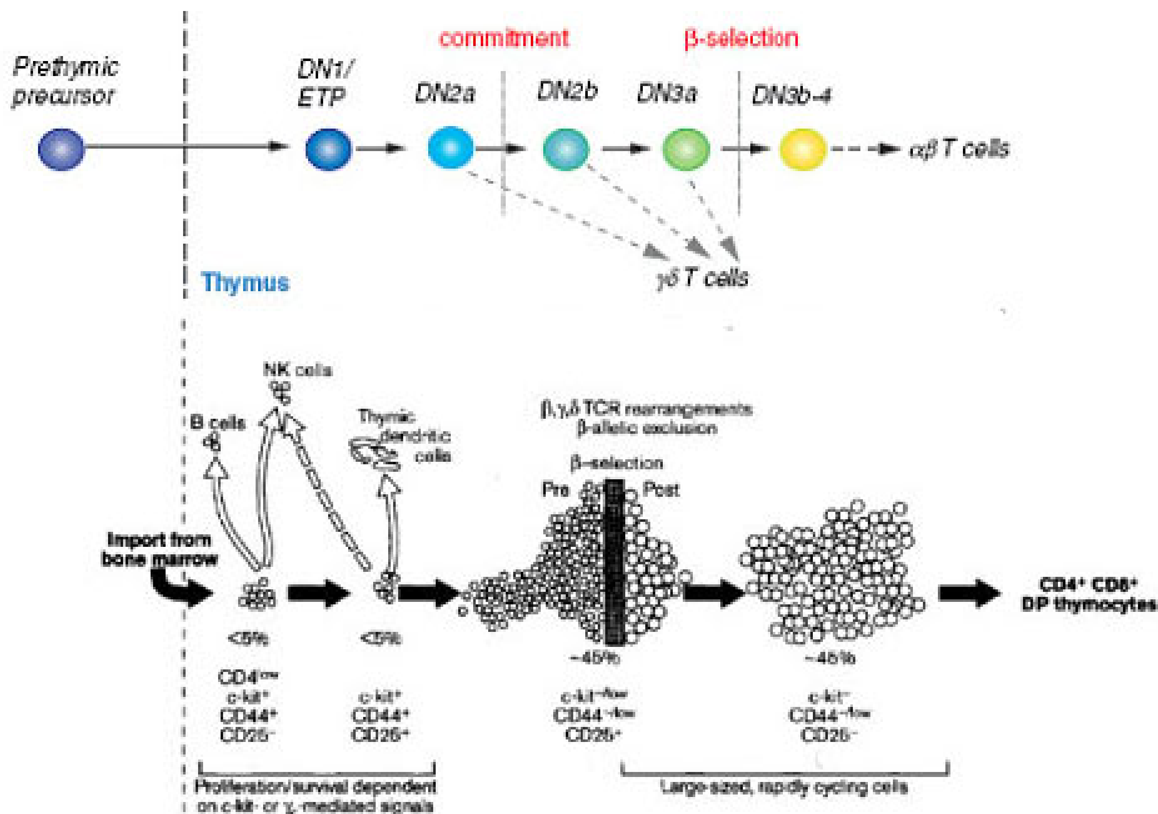

Figure 3 Double-negative stages of early thymocyte development. Progression through initial double-negative phases is identified by specific expression of c-Kit, CD25, CD28 and CD44. Percentages give the approximate proportion of each respective subset that survives the stage (adapted from Fehling, Hans Jörg and Von Boehmer 1997; Rothenberg 2012).

A variety of transcription factors participate in altering chromatin structure and provide an environment permissive for survival, differentiation and rearrangement of the TCR β–chain gene, include Runx1, CBFβ, Bcl-11b, TCF-1 and GATA-3 (Ting, Olson *et al.* 1996; Schmitt, de Pooter *et al.* 2004; Rothenberg 2012).

Diversity of the α:βTCR repertoire is generated through this transcription-mediated V(D)J recombination (Boehmer 1988; Petrie, Livak *et al.* 1995; Fehling, Hans Jörg and Von Boehmer 1997; von Boehmer and Fehling 1997).

A key set of proteins involved in this recombination are the Recombinant Activation Genes (RAG), specifically variants RAG-1 and RAG-2, along with high mobility group (HMG) proteins, and another enzyme critical to thymocyte development, terminal deoxynucleotidyl transferase (Tdt), a DNA

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polymerase that catalyses addition of nucleotides to a DNA molecule without a template (Collins, M K, Tanigawa *et al.* 1985; Turka, Schatz *et al.* 1991; Mombaerts, Iacomini *et al.* 1992; Petrie, Livak *et al.* 1995; Hernandez-Munain, McMurry *et al.* 1999; Mahajan, Gangi-Peterson *et al.* 1999; Janeway Jr, Travers *et al.* 2004). Recombinant activating genes (RAG), RAG-1 and RAG-2 are up-regulated, along with surface expression of CD25, the alpha chain of the IL-2 receptor molecule, which serves as a marker that the cell has progressed to the DN2 stage (Rothenberg, Moore *et al.* 2008; Rothenberg 2012).

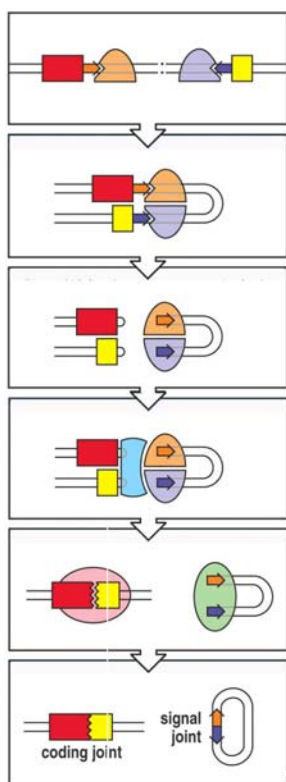
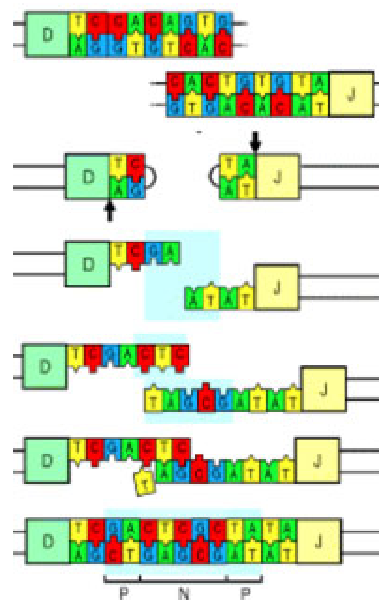


Figure 4 The action of RAG, Tdt and DNA ligase proteins in somatic recombination of TCR chain loci.

The process of somatic recombination is facilitated by Recombination Signal Sequences (RSS) that are 12 and 23bp in length.

RAG proteins bind at these loci and bind to each other, creating a hairpin DNA structure. DNA-dependent protein kinases (Ku 70/80) bind to the hairpins and the RSSs now serve as a point of random chromosomal cleavage.

Exonucleases may eliminate nucleotides or base pairs, while Tdt inserts random nucleotides into the sequences. Finally, DNA ligase IV seals the genetic recombination, adds to the diversity of TCR epitope recoginition (Janeway Jr, Travers et al. 2004).



Differentiation between α:β and γ:δ T cells occurs during the DN2 to DN3 transition, and is primarily based on expression of IL-7Rα (Koch and Radtke 2011). DN3 cells express the c-Kit$^-$ CD25$^-$CD44$^-$ CD4$^-$CD8$^-$ phenotype, and having migrated to the thymic subcapsular zone, their commitment to the T cell lineage is complete (Bhandoola, von Boehmer *et al.* 2007; Rajewsky and von Boehmer 2008; Kreslavsky, Gleimer *et al.* 2010; Koch and Radtke 2011). At this point, having reached the subcapsular zone, they turn around and head back toward the medulla (Koch and Radtke 2011).

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Factors that influence β-chain rearrangement, also control upregulation of expression of the preTα chain which serves as surrogate TCR α-chain, and these chains associate with CD3 complexes to form the preTCR (Schmitt, de Pooter *et al.* 2004; Rothenberg and Taghon 2005; Gleimer and von Boehmer 2010; Kreslavsky, Gleimer *et al.* 2010; Yang, Jeremiah Bell *et al.* 2010; Koch and Radtke 2011; Rothenberg 2012). The productive genetic recombination of the TCR β-chain locus is both critical and sufficient to drive the formation of a preTCR in DN thymocytes; association of a TCRβ with a preTα chain and CD3 complex not only defines the DN4 stage, but drives the expression of CD4 and CD8 which define the DP stage (Mombaerts, Iacomini *et al.* 1992; Hoffman, Passoni *et al.* 1996). This is the definition of "beta selection" (Hoffman, Passoni *et al.* 1996). Cells positively selected for β-chain formation continue to proliferate, and clonal deletion of the others occurs at a massive rate, approximately 35-45% of the cells survive this process (Petrie, Livak *et al.* 1995; Hoffman, Passoni *et al.* 1996; Fehling, Hans Jörg and Von Boehmer 1997).

Double Positive Stage (c-Kit^{+}CD25^{-}CD44^{-} preTCRβ^{+}CD4^{+}CD8^{+})

Successful rearrangement and prodution of a TCRβ-chain initiates signals to begin expression of the CD4 and CD8 TCR co-receptors, and rearrangement of the TCRa-chain (Fehling, Hans Jorg, Krotkova *et al.* 1995; Petrie, Livak *et al.* 1995). These cells are phenotypically identifiedby the up-regulation and surface expression of CD4 and CD8. Repeated rearrangements can rescue nonproductive Vα–Jα joins. Initial nonproductive rearrangements may result in subsequent rearrangements that bypass the nonproductive VJ gene segments, possibly undergoing multiple rounds of rearrangement until a functional α chain is generated or the cell dies.

Positive selection is the process by which developing α:βTCR expressing, DP thymocytes, when exposed to MHC binding, display low to moderate affinity for self-peptide/MHC complexes as presented by cTECs

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or dendritic cells in the thymic cortex, and are then induced to continue differentiation and the thymocytes mature into single-positive cells (Klein, Hinterberger *et al.* 2009).

In cells expressing a preTCRb, TCRα-chain recombination continues until positive selection for MHC restriction occurs, or the cell undergoes apoptosis (Petrie, Livak *et al.* 1995; Fehling, Hans Jörg and Von Boehmer 1997). Additionally, this stage of development results in a burst of proliferative activity accounting for as much as 98% of cells in the thymus (Petrie, Livak *et al.* 1995).

Chun and Schatz et al (1991) analyzed intrathymic expression of RAG-1 and RAG-2 mRNA transcripts in cortical thymocytes. Their experiments showed that DP TCR+ thymocytes continued RAG mRNA expression, but neither RAG-1 nor RAG-2 transcripts were detectable in single positive thymocytes (Turka, Schatz et al. 1991). The fact that RAG proteins are expressed in DP but not SP thymocytes is consistent with the observation that the TCR α-chain locus has the ability to recombine after failed receptor formation, a process that repeats for several days until a successful TCR is formed, or the cell undergoes apoptosis through neglect (Turka, Schatz et al. 1991; Benoist and Mathis 1992; Krangel 2009; Seitan, Hao et al. 2011). The positively selected DP cells then commit to either CD4 or CD8 single-positive then migrate(SP) expression (Koch and Radtke 2011).

Single Positive ($TCR^+CD4^+CD8^-$ or $TCR^+CD4^-CD8^+$)

Following MHC (positive) restriction in the cortex, single-positive (SP) cells migrate to the medulla where they encounter medullary thymic epithelial cells (mTECs) (Anderson, Harman *et al.* 2000; Anderson and Takahama 2012). A critical mechanism of development is a selection process by which T cells that recognize and bind moderately or strongly to self-antigens are eliminated, known as negative selection (Kyewski and Derbinski 2004).

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Negative selection is the apoptosis that is induced following a high affinity binding between the newly formed TCR and self-antigen: MHC complexes presented by mTECs (Ohashi 2003; Koch and Radtke 2011). In order for this to occur, the thymic environment must present peptides from cellular processes that occur in peripheral tissue, peptides referred to as tissue-specific antigens (TSA), and it is thought recognition of these TSAs is what drives the negative selection process (Heino, Peterson *et al.* 1999; Anderson, Venanzi *et al.* 2005; Derbinski, Gäbler *et al.* 2005; Anderson and Su 2011).

Medullary thymic epithelial and dendritic cells have been shown to engage in a unique behavior known as 'promiscuous gene expression' (Derbinski, Gäbler et al. 2005). Promiscuous gene expression is defined in regards to our knowledge of cell type-specific gene expression; that is, expression of genes that are not known to play a role in the physiological phenotype of a given cell type (Derbinski, Gäbler *et al.* 2005; Tykocinski, Sinemus *et al.* 2010). For example, an mTEC expressed gene would be considered tissue restricted (or tissue-specific) if it is commonly expressed in less than 10% of peripheral tissue; using this operational definition, Derbinski et al (2005), showed that approximately 28% of the overexpressed genes in mTECs (152 out of 545 genes) are peripheral tissue-specific. Moreover, they found that promiscuous gene expression correlates with expression of CD80 (B7-1), which serves as a ligand for CD28, a T cell co-stimulatory signaling molecule (Nunès, Truneh et al. 1996; Derbinski, Gäbler et al. 2005).

The **Autoimmune Regulator** (AIRE) gene has been shown to be a major regulator of promiscuous gene expression, and is highly active in this small and select group of mTECs (Anderson, Venanzi *et al.* 2002; Kyewski and Derbinski 2004; Derbinski, Gäbler *et al.* 2005; Tykocinski, Sinemus *et al.* 2010).

The role of thymic selection and MHC class restriction has multiple benefits, it ensures that the majority of T cells express properly formed TCRs with a vast diversity of epitope recognition, reduces the probability of auto-reactive T cells in peripheral tissue, and maintains a population that is weakly

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reactive to the self-peptide:MHC complex , avoiding what Paul Erlich referred to as *'horror autotoxicus'*(Townsend and Bodmer 1989; Silverstein 2001; Housset and Malissen 2003; Huseby, White *et al.* 2005; Klein, Hinterberger *et al.* 2009).

Having successfully traversed the architecture of the thymus, interacting with a variety of stromal cells in the discrete compartments of the thymic architecture, these fully matured, naive T cells, are attracted by sphingosine 1-phosphate (S1P) at HEVs, and exit the thymus to take their place in the periphery and the secondary lymphoid organs.

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Overview of DNA Repair Mechanisms

by Dave Comstock

for Dr. David Calhoun, Dr. Karen Hubbard, and Dr. Jerry Guyden, The City College of New York

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A discussion human DNA repair mechanisms.

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Intro

Humans DNA is subject to a variety of endogenous biochemical damage within the highly dynamic environment of the cell: tautomeric conversions of nitrogenous bases, hydrolytic excision of bases, base alkylation, deamination, depurination, pyrimidine dimerization, and single- and double-strand DNA breaks (Lindahl, T. and Barnes 2000; Nelson, Lehninger *et al.* 2008; Milanowska, Krwawicz *et al.* 2011).

The adverse effects of DNA damage generally include disrupted DNA metabolism, cell-cycle arrest or cell death, with long-term irreversible mutations contributing to ageing and oncogenesis (Hoeijmakers, Jan H. J. 2001; Vousden and Ryan 2009; Reinhardt and Schumacher 2012). In response to the wide variety and type of damage, numerous, distinct and highly conserved signaling and repair mechanisms have evolved which minimize the consequences of toxic and mutagenic damage (Lindahl, T. and Barnes 2000; Wood, Mitchell *et al.* 2001). DNA is protected from damage by a network of response pathways that detect lesions through a variety of protein sensors (Lagerwerf, Vrouwe *et al.* 2011). This paper will focus on the main DNA damage repair mechanisms, with a bias toward human systems; nucleotide- and base-excision repair, homologous recombination, end joining, and mismatch repair.

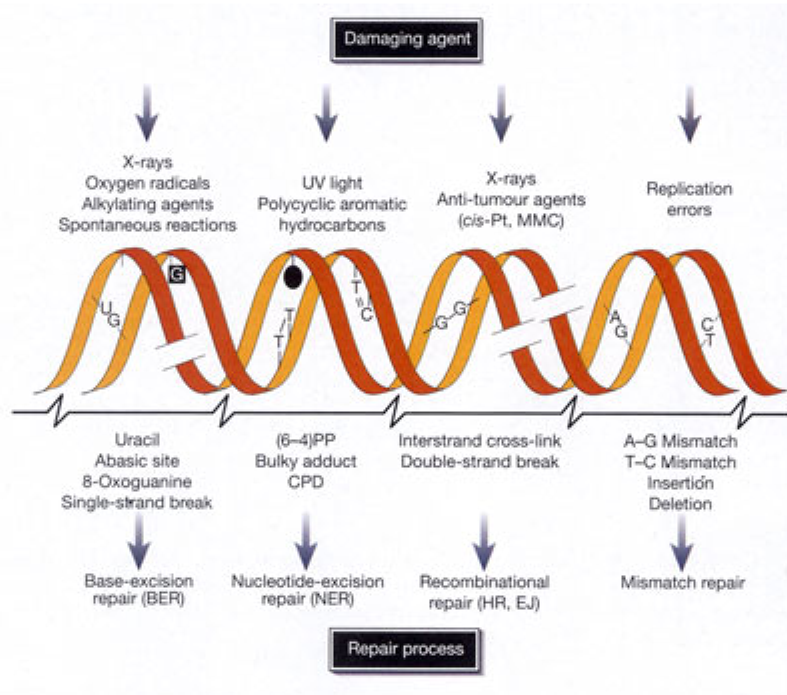

Figure 1 - DNA damage, repair mechanisms and consequences. Common DNA lesions are induced by three main categories of agents: environmental agents, endogenous biochemical reaction byproducts, replication errors, and random spontaneous chemical reactions. The long-term effects of DNA damage include permanent changes to the primary structure of DNA (Hoeijmakers, Jan H. J. 2001).

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Types of DNA damage

Damage of DNA bases such as deamination, depurination or dimerization, can result from a variety of sources: spontaneous biochemical reactions, errors in DNA synthesis, endogenous by-products of aerobic respiration (reactive oxygen species), exogenous factors such as environmental chemicals found in cigarette smoke or chemotherapeutics, ultraviolet (UV) light and ionizing radiation (De Bont and van Larebeke 2004; Sancar, Lindsey-Boltz *et al.* 2004; Huffman, Sundheim *et al.* 2005; Nelson, Lehninger *et al.* 2008).

DNA purine and pyrimidine bases undergo spontaneous changes in their structure. Base-pair mutations arise through deamination from cytosine to uracil, methylcytosine to thymine, adenine to hypoxanthine, or guanine to xanthine. Ultraviolet light can induce pyrimidine dimerization and the generation of a cyclobutyl ring, or linkage between nucleotides forming kinks and bulges in the structure of DNA which can arrest or prevent transcription and synthesis (Lu, Clark et al. 1983; De Bont and van Larebeke 2004; Huffman, Sundheim et al. 2005; Nelson, Lehninger et al. 2008).

DNA synthesis can result in mismatched pairings that escape polymerase proof-reading mechanisms, stalled replisomes can result in collapsed replication forks, and subsequent single- and double-strand breaks (Nelson, Lehninger *et al.* 2008).

Alkylating agents such as dimethyl sulfate can methylate guanine to produce O^6-methylguanine, which prevent pairing with cytosine, while methylation of bases by S-adenosylmethionine or ethyl methane sulfonate also prevent or alter base-pairing and transcription (Nelson, Lehninger *et al.* 2008; Lagerwerf, Vrouwe *et al.* 2011).

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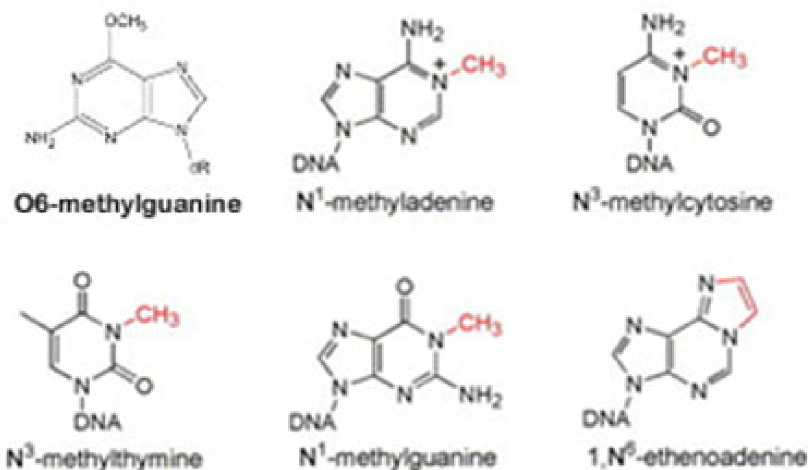

Figure 2 - DNA alkylations known to be repaired by AlkB type proteins (Mishina and He 2006).

Endogenous oxidative damage is the most common type seen in DNA and results from Fenton reactions (Nelson, Lehninger *et al.* 2008). The partial reduction of molecular oxygen during metabolic reactions in the presence of transition metals (iron and copper) yields superoxides ($O_2^{\bullet}$), which are then converted to hydrogen peroxides (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$) referred to as ROS or reactive oxygen species (De Bont and van Larebeke 2004). These products have the potential to cause the oxidation of nitrogenous bases, and deterioration of DNA in the form of single- and double-strand DNA breaks (De Bont and van Larebeke 2004; Cui, Kong *et al.* 2012). The mutagenic potential of oxidative damage is seen in the series of reactions by which ROS oxidize the 5,6-double bond of 5-methylcytosine, then through an intermediate product deaminates the cytosine to form thymine, facilitating a G:C -> T:A transition which is known to block transcription and DNA replication (Lu, Clark *et al.* 1983; De Bont and van Larebeke 2004). One of the most common oxidative DNA adducts is 8-hydroxydeoxyguanosine (8-oxo-dG) (De Bont and van Larebeke 2004; Cui, Kong *et al.* 2012).

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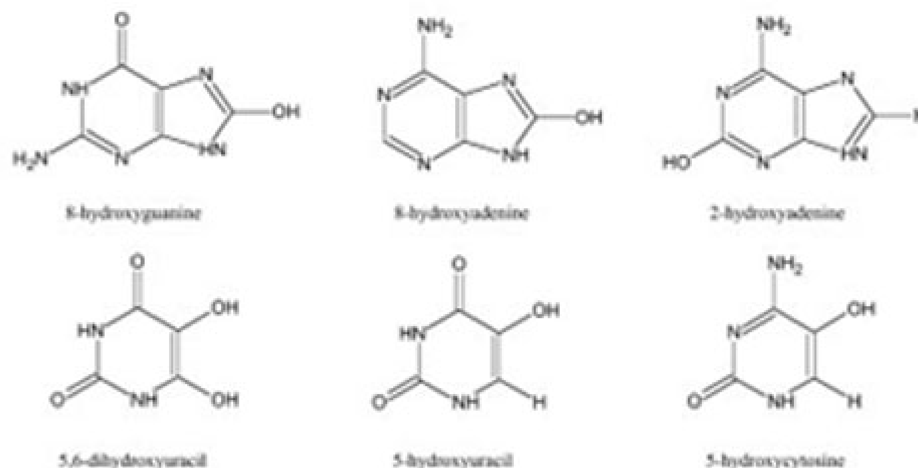

Figure 3 - Common DNA oxidative adducts (De Bont and van Larebeke 2004).

Pathological generation of free radicals occurs through ionizing radiation, the 'oxidative burst' of neutrophilic-driven inflammation, or via the cytochrome p450 system for metabolism of drugs (Lindahl, T. and Barnes 2000; Wood, Mitchell *et al.* 2001; Nelson, Lehninger *et al.* 2008; Jackson and Bartek 2009; Milanowska, Krwawicz *et al.* 2011).

Because of the Chargaff base-pairing specificity and the anti-parallel structure of DNA, each strand can serve as a template for replication or repair of errors during the cell cycle (Nelson, Lehninger *et al.* 2008). This redundancy allows for excision repair of damaged or erroneous DNA by replacing individual bases or a series of nucleotides to restore the integrity of the opposite strand (Lindahl, Tomas, Karran *et al.* 1997; Lindahl, T. and Barnes 2000; Wood, Mitchell *et al.* 2001).

Direct repair

Two human proteins directly reverse base-adduct damage in DNA: the α-ketoglutarate- and Fe(II)-dependent dioxygenase ABH2 and ABH3, of the AlkB protein family, and O^6-alkylguanine-DNA alkyltransferase (AGT) (Milanowska, Krwawicz *et al.* 2011). AGT possesses a helix-turn-helix motif, and participates in direct repair of O^6 -alkylguanine lesions in DNA; the proposed mechanism suggesting that at the C-terminal, the helix swivels about the proceeding loop to bind within the major groove of DNA, which exposes an active-site cysteine, the substrate nucleoside "flips" out and away from the main DNA molecule, and an irreversible transfer of the alkyl lesion occurs (Tano, Shiota *et al.* 1990; Duncan, Trewick *et al.* 2002; Kaina, Christmann *et al.* 2007).

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DNA mismatch repair (MMR) corrects replication errors (mismatches and small insertions and deletions) that escape the proofreading activity of DNA polymerase, resulting in the formation of structural bulges, kinks, or loops (Hoeijmakers, Jan H. J. 2001). In *Escherichia coli*, MMR is initiated by *MutS, MutL,* and *MutH*, while in humans, there are several known *MutS* homologues (Milanowska, Krwawicz *et al.* 2011).

The *E. coli* MMR pathway has been extensively studied and shown to utilize a *MutS-MutL* complex to identify the mismatch and a *MutH* protein to make a strand incision at the nearest hemi-methylated adenine within a palindromic GATC sequence, followed by the action of DNA helicase II and an exonuclease (eg, RecJ or ExoI, ExoVII, or ExoX) to create a gap, which is then filled by DNA pol III and finished by DNA ligase; after repair, the GATC sites are then methylated by D*am* (Iyer, Pluciennik *et al.* 2005; Modrich 2006; Nelson, Lehninger *et al.* 2008). A key feature of this mechanism is the ability to work in a bidirectional manner (Iyer, Pluciennik *et al.* 2005).

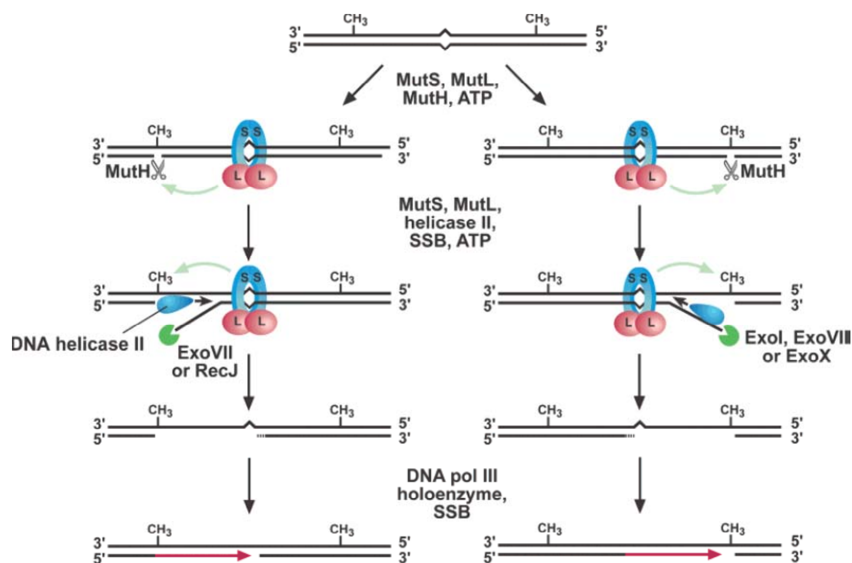

Figure 4 - Model for *E. coli* methyl-directed mismatch repair (Iyer, Pluciennik *et al.* 2005)

Human cells also support mismatch repair, but the human *MutS* and *MutL* homologs (MSH and MLH, respectively) are heterodimers rather than homodimers, and while *E. coli* repair is directed by methylation patterns, it is suggested that human MMR discriminates daughter from template strand using a non-methylation strand-specific nick in the DNA backbone but the mechanism is unknown (Modrich 2006). MSHa has been observed to support repair of all eight base-base mismatch types, including C:C, and insertion/deletion mispairs of up to approximately 10nt in length (Iyer, Pluciennik *et*

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al. 2005). Defects in MMR are associated with genome-wide instability, a predisposition to certain types of cancer, and resistance to certain chemotherapeutic agents (Kanehisa, Goto *et al.* 2012).

Base excision repair

Base excision repair (BER) is one of the major pathways for removing simple lesions encountered in DNA, specifically, nitrogenous base damage, insertion or loss (Parsons, Dianova *et al.* 2005). BER requires coordination between a number of repair and accessory proteins (Parsons, Dianova *et al.* 2005).

The BER process can be summarized as follows: an individual DNA glycosylase recognizes and removes a specific damaged DNA base creating an abasic site, an apurinic/apyriminic endonuclease (APE) then makes an incision adjacent to the abasic site; a phosphodiesterase removes the remaining phosphate-ribose complex at the DNA termini, and a DNA polymerase fills the resulting gap, then finally the strand is sealed by a DNA ligase (Lindahl, Tomas, Karran *et al.* 1997; Hoeijmakers, Jan H. J. 2001; Moon, Garcia-Diaz *et al.* 2007; Dalhus, Laerdahl *et al.* 2009). In some cases, DNA polymerases possess both polymerization and lyase capabilities, eliminating the need for a phosphodiesterase in the pathway (Yamtich and Sweasy 2010).

DNA glycosylases are a family of enzymes involved catalyzing the first step of the base excision repair process; creating an apurinic apyrimidinic site (AP) through a mechanism that "flips" the damaged base out of the double helix, then cleaves the N-glycosidic bond (reviewed in Dalhus, Laerdahl *et al.* 2009). Individual DNA glycosylases typically bend or distort DNA to an angle between 30° and 70°, flattening the minor groove of B-type DNA, which facilitates the excision of a damaged base by rotating the nucleotide around the phosphodiester bonds (Dalhus, Laerdahl *et al.* 2009). Further, DNA glycosylases have extremely tight-fitting substrate recognition pockets that accommodate the damaged bases while blocking normal bases (Moon, Garcia-Diaz *et al.* 2007; Dalhus, Laerdahl *et al.* 2009).

APE1 is a human class II endonuclease that creates a nick in the phosphate-ribose backbone adjacent to an AP site. Across organisms, there are several types of AP endonucleases, classified base on incision dynamics: AP endonucleases class I and II cut the DNA backbone at the phosphate groups 5´ to the abasic site leaving a 5´-phosphate and a 3´-OH and termini (Myles and Sancar 1989; Lindahl, Tomas 1993; Marenstein, Wilson Iii *et al.* 2004).

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DNA polymerase β (Pol β) is a small enzyme, approximately 39kDa (335 amino acids), capable of synthesizing DNA in a template-directed manner, using the free 3'-OH as a primer, binding preferentially to small gaps of 2-6 nucleotides (Yamtich and Sweasy 2010).

After APE1 makes a backbone incision, it recruits PolB to the site, then dissociates from the abasic site (Sokhansanj, Rodrigue *et al.* 2002; Lagerwerf, Vrouwe *et al.* 2011). Pol β binds to the lesion and uses the undamaged strand as a template for resynthesizing the sequence, and then recruits a DNA ligase III/XRCC1 heterodimer complex to reform the phosphodiester bond and repair the DNA backbone (Myles and Sancar 1989; Parsons, Dianova et al. 2005; Nelson, Lehninger et al. 2008). Most damaged bases are repaired by this single-nucleotide patch pathway, but there is an alternative pathway in which the PolB is replaced by Pol Delta, which displaces a flap consisting of nucleotide residues, and synthesizes a longer patch sequence (Matsumoto and Kim 1995; Kim, Biade *et al.* 1998). The flap is recognized and and cleaved by the flap endonuclease FEN1, and the strand is ligated by DNA Ligase 1 (Kim, Biade *et al.* 1998).

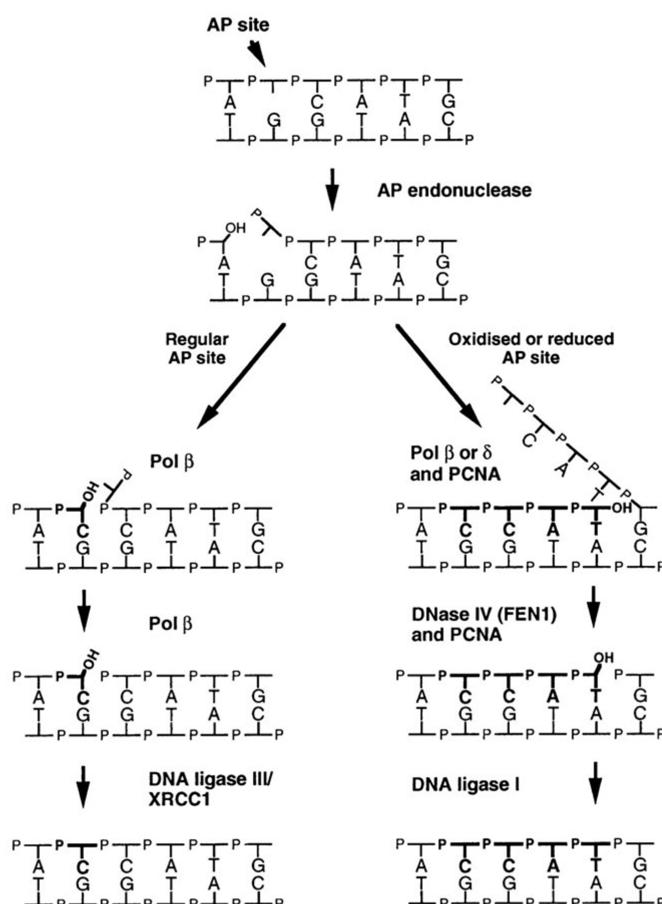

Figure 5 Short and Long-patch BER Repair. Base excision repair is the primary pathway for repairing nucleotide lesions caused by ROS and alkylating agents, and consists of short patch (left), long-patch (right) (Klungland and Lindahl 1997; Kanehisa and Goto 2000).

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Nucleotide excision repair (NER) is characterized by specific proteins that recognize the damage, cleave the DNA strand on each side of the lesion, and excise a fragment of nucleotides in which the lesion exists; DNA polymerase and DNA ligase then restore the strand sequence and phosphopentose backbone to its initial state (Huffman, Sundheim *et al.* 2005).

NER recognizes a wide array of DNA lesions, and is the primary mechanism for repairing DNA lesions such as pyrimidine dimers, intrastrand cross-links and bulky adducts that form large helical distortions (Myles and Sancar 1989; Hoeijmakers, Jan H. J. 2001). Most of these lesions occur through exogenous sources, such as UV radiation , or exposure to aflatoxin, benzopyrene and other chemotherapeutic agents such as cisplatin (Lindahl, Tomas and Wood 1999). These distortions can disrupt both DNA synthesis and RNA transcription (Lindahl, Tomas and Wood 1999; Hoeijmakers, Jan H. J. 2001; Huffman, Sundheim *et al.* 2005).

NER in *E. coli* is among the most extensively studied systems, and while the complexity of NER is greater in eukaryotes, similar principles operate in bacterial models (Hoeijmakers, J.H.J., van Duin *et al.* 1986; Kanehisa and Goto 2000; Huffman, Sundheim *et al.* 2005; Hanawalt 2007). Xeroderma pigmentosa is a human genetic repair disorder resulting from mutations in one of seven genes: XPA, XPB, XPC, XPD, XPE, XPF, and XPG, that produces hypersensitivity to UV radiation, and a 1000-fold risk increase for skin cancer (Hoeijmakers, Jan H. J. 2001; Kanehisa, Goto *et al.* 2012); Cockayne syndrome, typified by premature ageing, dwarfism, and dysmyelination, results from mutations in either of the CSA or CSB genes (Kanehisa and Goto 2000; Hoeijmakers, Jan H. J. 2001). Mammalian NER involves 9 major proteins, named according to the disease associated with their deficiency(Kanehisa, Goto *et al.* 2012).

NER consists of two related subpathways known as global genomic repair (GG-NER) and transcription-coupled NER (TC-NER), and although the pathways differ in damage recognition, many of the proteins involved in the repair of damage are shared between them (Lindahl, Tomas and Wood 1999).

GG-NER is considered to be transcription-independent, removing lesions from non-transcribed regions of genome in addition to non-transcribed strands of transcribed regions, while the repair of damage in transcription-active genes, typically resulting from UV exposure, is known as transcription-coupled NER (TC-NER)(Hoeijmakers, Jan H. J. 2001).

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Global Genome NER

After recognition of the damage by the XPC:HR23B complex, XPA and Replicative protein A (RPA) initiate formation of the multisubunit repair complex - TFIIH creates a bubble, unwinding the damaged section of DNA, XPG then hydrolyzes a phosphodiester bond on the 3'- side of the lesion, and an ERCC1:XPF complex cleaves the phosphodiester bond on the 5'-side of the lesion, generating a fragment 27 to 30nt in length (Lindahl, Tomas, Karran *et al.* 1997; Lindahl, Tomas and Wood 1999; Wood, Mitchell *et al.* 2001). The cleavage sites correlate to the unwound DNA at the junctions of single- and double-strand DNA (Wood, Mitchell *et al.* 2001). After creation of a gap in the nucleotide sequence, DNA polymerase ε (Pol ε) synthesize the new base pairings, and DNA ligase seals the phosphodiester bonds (Hoeijmakers, Jan H. J. 2001; Nelson, Lehninger *et al.* 2008). Damage recognition, incision of the DNA strand on both sides of the lesion, and excision of the damaged sequence, are followed by polymerization and ligation to restore the DNA to its original state .

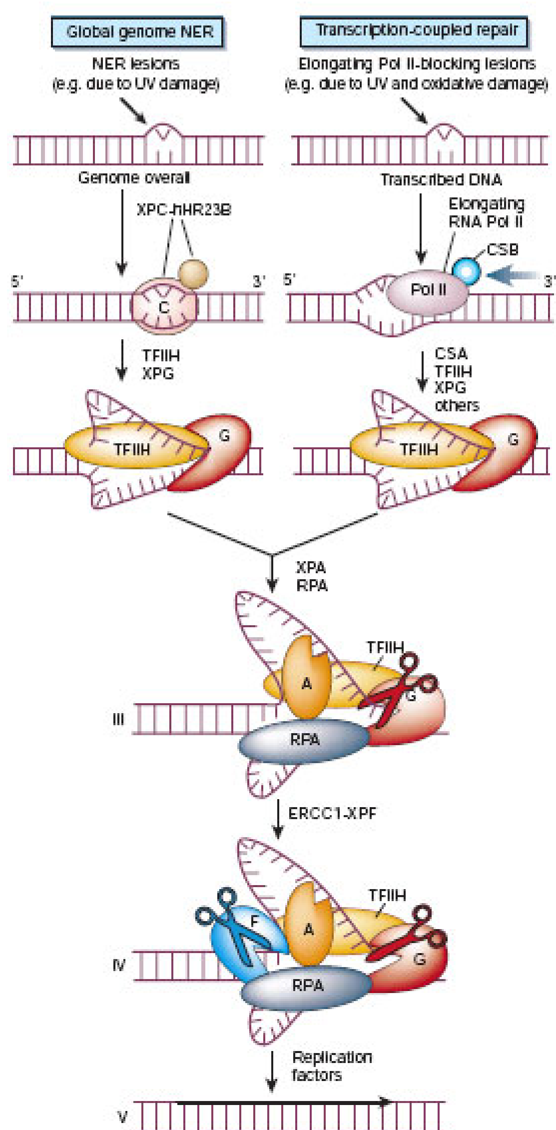

Figure 6 - Nucleotide Excision Repair. Global Genome (left) and transcription-coupled (right) models. (Hoeijmakers, Jan H. J. 2001)

Transcription-Coupled NER

During active gene expression, DNA helix-distorting lesions can block transcription by stalling the RNA polymerase II (Pol II) and preventing elongation (Araújo and Wood 1999; Svejstrup 2002; Lagerwerf, Vrouwe *et al.* 2011) In response to stalled RNA pol II, various repair proteins are recruited to the site, including CSA, CSB, TFIIH, XPG and the XPF:ERCC1 complex elongation (Araújo and Wood 1999; Svejstrup 2002; Lagerwerf, Vrouwe *et al.* 2011) . It is presumed that CSB uncouples the polymerase and removes it from the lesion site, the TC-NER repair complex consisting of CSA, CSB, TFIIH, ERCC1:XPF,

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XPG, TFIIS then forms, and DNA pol δ and/or ε synthesizes the repair elongation (Araújo and Wood 1999; Svejstrup 2002; Lagerwerf, Vrouwe *et al.* 2011).

Recombinational Repair

Eukaryotic chromosomes are subject to rounds of replication, segregation, condensation and decondensation throughout the cell cycle, and different types of lesions are more likely to occur in specific phases (Branzei and Foiani 2008). While BER and NER (both GG and TC), operate throughout the cycle, MMR is primarily active during S-phase, correcting mismatches, insertions and deletions generated during DNA synthesis (Iyer, Pluciennik et al. 2005).

Simultaneous double-strand breaks (DSBs) of the phosphate backbone of the two complementary strands are one of the most serious DNA lesions. Failure or incomplete repair may result in genetic mutation, cell-cycle arrest, apoptosis or genetic instability leading to cancer (reviewed in Symington and Gautier 2011). DSBs can form as a result of ionizing radiation through the induction of ROS, or free-radical production by cytotoxic drugs such as doxorubicin or etoposide; actinomycin and cisplatin, chemotherapeutics that induce DNA cross-links, can also generate DSBs (Ohnishi, Mori et al. 2009). These drugs interfere with topoisomerases that, when covalently bound at the replication fork, terminates leading strand synthesis and creates a DSB (Ohnishi, Mori et al. 2009).

Double-stranded breaks of DNA are known to be repaired by a number of pathways: homologous recombination (HR), non-homologous end-joining (NHEJ), alternative NHEJ (alt-NHEJ), and single-strand annealing (SSA) (Ciccia, A and Elledge 2010). Coincidentally, there are also four independent sensing mechanisms for detecting DSBs: poly adenosine-ribose polymerase (PARP), Ku70/80, the MRN protein complex (Mre11, Rad50 and Nbs1), and with auxiliary processing RPA (Ciccia, A and Elledge 2010).

NHEJ

The quickest response to a double strand break, comes from the Ku70/Ku80 heterodimer, which exhibits a strong affinity for DNA, and forms a ring around DSBs within seconds, and recruits DNA protein kinase to the site (Walker, Corpina *et al.* 2001; Ciccia, A and Elledge 2010). Once bound, it activates the DNA protein kinase catalytic subunit (DNA-PKcs), and the recruitment of ARTEMIS which initiates NHEJ, followed by ligation by the XRCC4/Ligase4 complex (Ciccia, A and Elledge 2010).

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Alternate NHEJ

Binding of DNA DSBs by PARP promotes alt-NHEJ by attracting the MRN complex, in competition with Ku70/Ku80, to the DNA end-breaks (Ciccia, A and Elledge 2010). In response to DNA breaks, MRN activates ATM which in turn phosphorylates CtIP in a BRCA-dependent manner (Chinnadurai 2006; Ciccia, A and Elledge 2010). The XRCC1/Ligase1 complex then repairs the phosphodiester bonds to restore the chromosome (Ciccia, A and Elledge 2010).

Homologous Repair

When NHEJ fails, the combination of MRN, CtIP and BRCA1 promotes deletion of nucleotides from the 5' ends of the break, in a process called resection (reviewed in Ciccia, A and Elledge 2010; Kousholt, Fugger *et al.* 2012). Replication protein A (RPA) coats the single-strand 3' prime ends, along with RAD51 binding at these sites, and (depending on which phase of the cell cycle the break occurs) if the DSB occurs in either S-phase or G2 phase of the cycle, with the assistance of BRCA2, then promotes strand invasion as part of a homology search for a sister chromatid (Ciccia, A and Elledge 2010; Kousholt, Fugger *et al.* 2012). Successful strand invasion results in formation of D-loops and Holliday junctions and synthesis by DNA polymerases is engaged (Ciccia, Alberto and Elledge 2010). After synthesis completes, crossover events are then resolved by either the BLM/TopoIII complex, Gen1, or Mus81/EME1 complexes (Ciccia, A and Elledge 2010).

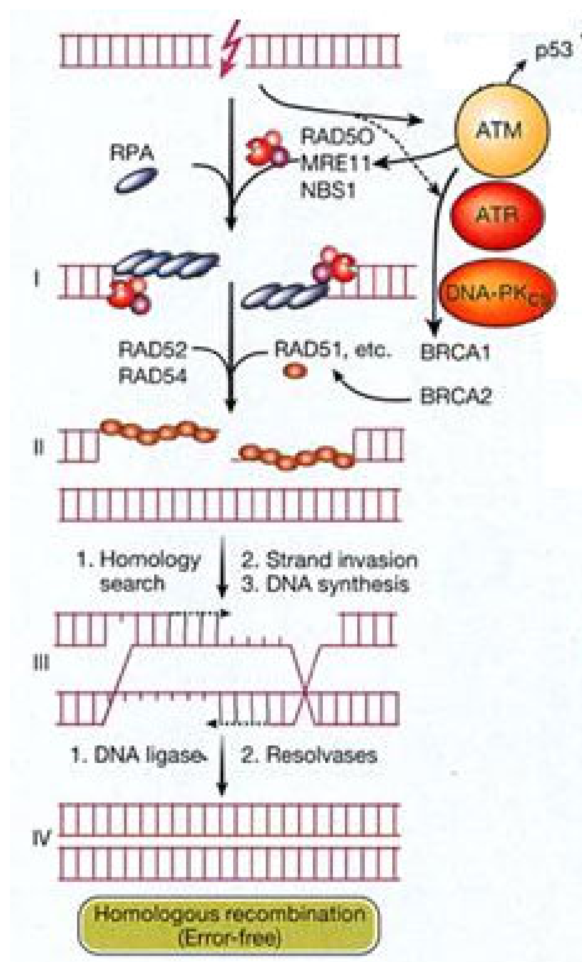

Figure 7 - modified from (Hoeijmakers, Jan H. J. 2001).

Interstrand Crosslink Repair

Breaks in DNA that occur as a result of stalled replisomes at a replication fork are particularly dangerous, threatening mitotic catastrophe, complex chromosomal rearrangements, and cell death (Ciccia, A and Elledge 2010). The interstrand crosslink repair pathway consists of 13 genes associated with Fanconi

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anemia, a genetic disease that predisposes the patient to cancer, and bone-marrow failure (Garcia-Higuera, Taniguchi *et al.* 2001).

Summary

DNA repair pathways include base excision repair, nucleotide excision repair, DNA strand break repair, direct reversal of DNA damage, and the bypass during replication of DNA lesions. These pathways often involve similar, or the same, proteins and enzymes, with the mechanism by which particular damage is recognized being a primary difference between them. Nuclear DNA repair proteins protect genomic integrity of cells, and many systems have evolved over time, to address the specific damage occurring through DNA synthesis, cellular metabolism and exposure to exogenous chemicals and radiation. Defects in these repair mechanisms may result in aging or disease, cancer in particular, and may provide insight to treatment and pharmaceutical targets.

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