Thesis for doctoral degree (Ph.D.)
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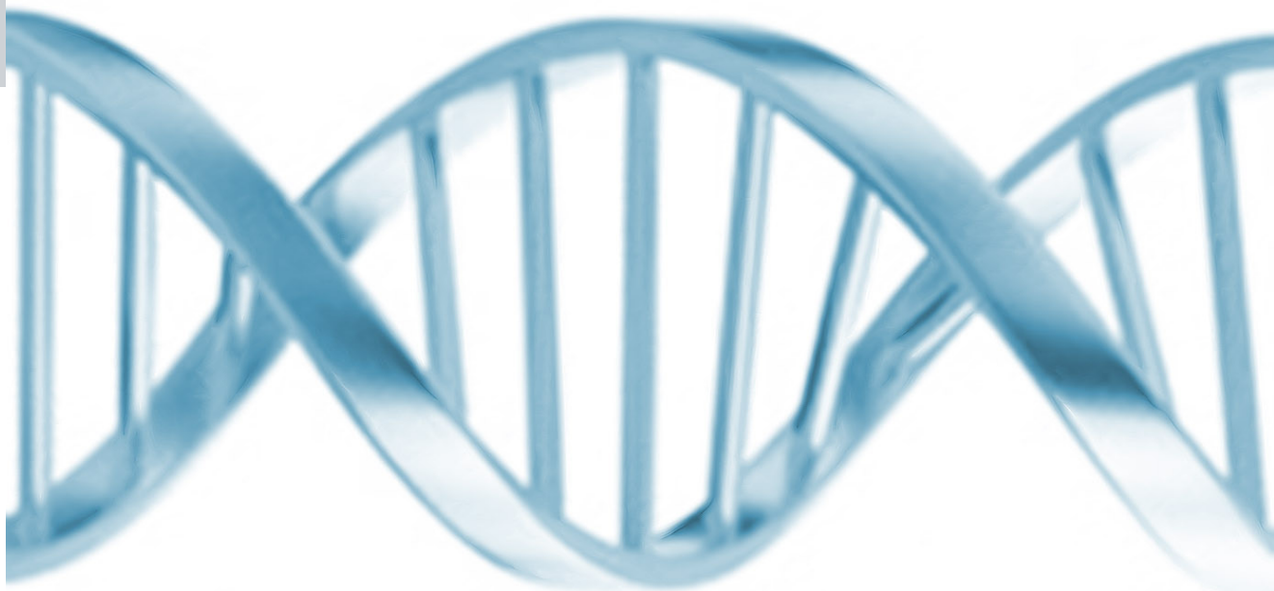
Molecular Regulation and Analysis of Neural Stem and Cancer Cell Characteristics



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TO MY SON

ABSTRACT

Every single cell within an organism is constantly facing the demanding task of preserving the integrity of its genomic material to secure proper function and clearance from disease. To accomplish this cells rely on DNA repair mechanisms that are well-conserved throughout evolution and highly specialized in removing particular types of DNA damage. The ability of rapidly repairing DNA lesions is paramount for cells in both the developing and adult central nervous system (CNS), not only to preserve their differentiation potential but also to ensure that post mitotic cells, which comprise the CNS vast majority, are spared and continue to contribute to the maintenance of CNS homeostasis. Due to their putative enhanced repair capacity, neural stem cells (NSCs) may not only play a critical role in sustaining the pool of neural progenitors over the course of neurogenesis, but also in certain conditions, replenish the loss of terminally differentiated cells due to a variety of damage inducing events.

The work presented in this thesis aimed to further explore the significance of DNA glycosylase activity both in neural stem and cancer cells and to develop a probe for rapid assessment of neuronal characteristics in neurons differentiated from NSCs.

In paper I we propose the DNA glycosylases OGG1 and NEIL3 play roles beyond direct removal of oxidized bases and that they are necessary for typical expression levels of genes conferring normal neural stem cell characteristics and multipotency required for differentiation into the various cell lineages found in the mammalian CNS. Further, we show that NEIL3 deficiency can enhance formation of senescence related heterochromatin foci, suggesting an influence in regulatory functions of this cellular pathway.

In paper II we first demonstrate we can generate C6 glioma cells with stem cell-like characteristics (C6SCs), as it could be assessed by an increased ability of these cells to differentiate into astrocytes and neurons after CNTF and VPA treatment respectively. In addition, we show that RNA knockdown of the DNA glycosylase OGG1 in C6SCs affects differentiation potential and increases the histone modification mark, acetylation of histone 3 in lysine 56 (H3K56ac) associated with increased DNA damage response (DDR). This enhancement of the DDR may confer a certain degree of resistance to cancer therapies that should be carefully taken in account.

Finally, in paper III we introduce a new method for the use of a commercially available voltage sensitive dye (VSD), JPW3027, for accurate characterization of neurons differentiated from NSCs, regarding their ability to generate action potentials (AP). We found that extracellular application of this dye improves labeling of cellular processes, and upon excitation it reports changes in fluorescent translating precise AP kinetics, which are of overall superior quality compared to calcium indicators. Furthermore, JPW3027 proved to possess a lesser degree of toxicity, which represents a great advantage when monitoring cells for extended periods of time after dye loading. Finally, we propose the use of a finite element model of the NSC culture cover slip to optimize electrode positioning relatively to the patched cells this way producing an electrical stimulation that is homogenous to all cells. With this approach we are able to predict isopotential fields where electrodes can be placed minimizing the perturbation of cells away from the field of view.

LIST OF PUBLICATIONS

I. **Reis A**, Hermanson O
The DNA glycosylases OGG1 and NEIL3 influence differentiation potential, proliferation, and senescence-associated signs in neural stem cells. Biochem. Biophys. Res. Commun. 2012, Apr 30 [Epub ahead of print].

II. **Reis A**, Hermanson O
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III. Leão RN*, **Reis A***, Emirandetti A, Lewicka M, Hermanson O, Fisahn A.
A voltage –sensitive dye-based assay for the identification of differentiated neurons derived from embryonic neural stem cell cultures. PLoS One 2010, 5:e13833. *These authors contributed equally to this work.

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LIST OF ABBREVIATIONS

AP	Action potential
ATM	Ataxia telangiestasia mutated
ATR	Ataxia telangiectasia and Rad3 Related
BER	Base excision repair
BMP4	Bone morphogenetic factor 4
CDK	Cyclin-dependent kinase
CNTF	Cilliary neurotrophic factor
CSC	Cancer stem cell
DDR	DNA damage response
DSB	Double strand break
DSBR	Double strand break repair
EGF	Epidermal growth factor
EGFR	EGF receptors
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GSC	Glioma stem cell
MAP2A,B	Microtubule-Associated Protein A,B
MMR	Mismatch repair
MSI	Microsatelite instability
NEIL1-3	Nei endonuclease-like VIII 1-3
NER	Nucleotide excision repair
NSC	Neural stem cell
OGG1	7,8 dihydro-8-oxoguanine DNA glycosylase 1
PARP	Poly (ADP-ribose) polymerase
Rb	Retinoblastoma
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SASP	Senescence associated secretory

	phenotype
SGL	Subgranular layer
SSB	Single strand break
SEZ	Subependymal zone
SGZ	Subgranular zone
SVZ	Subventrical zone
TTX	Tetrodotoxin
VSD	Voltage sensitive dye
VPA	Valproic acid
Wnt3a	Wingless-type MMTV integration site family, member 3A

1 BACKGROUND

1.1 STEM CELLS

Stem cells have the distinct ability to continually renew themselves and differentiate into a wide range of specialized cell types. Upon cell division a stem cell can give origin to either two daughter cells with stem cell properties (symmetric division) or one stem cell plus a differentiated cell type (asymmetric division). Tissue formation during embryonic development and maintenance of proper tissue homeostasis and regeneration throughout adulthood are extremely dependent on the unique features of these cells (Morrison SJ 2006; Rando 2006).

1.1.1 Embryonic stem cells

The cells derived from the epiblast of the inner cell mass (ICM) of an early morula stage embryo (blastocyst) are called embryonic stem cells (ESC). ESC are pluripotent, meaning that during development they have the potential to give rise to cells belonging to all the primary three germ layers: the ectoderm, endoderm and mesoderm. Aside these intrinsic characteristics ESC can also be identified by specific molecular hallmarks such as the presence of certain transcription factors and cell surface marker proteins. For instance, hESC express the transcription factors Oct-4, Nanog, and Sox2, which comprise the regulatory network responsible for repression of genes eliciting differentiation, this way aiding the maintenance of stem cell pluripotency (Reubinoff, Pera et al. 2000).

1.1.2 Neural stem cells

Similar to stem cells, neural stem cells (NSCs) also have self-renewal ability but instead are only able to give rise to cells belonging to the nervous system. NSCs play a critical role during the development of the CNS as they can differentiate into the different cell types (neurons, astrocytes and oligodendrocytes) comprising the CNS. NSCs have been isolated from several regions of the central nervous system (CNS) from both developing and adult mammals (Temple 1989; Reynolds and Weiss 1992). During early development NSCs are restricted to the ventricular zone (VZ) but as neurogenesis progresses a progenitor population is generated through asymetrical division in the VZ that afterwards migrates basally. These intermediate progenitors end up in the subventricular zone (SVZ) where they continue to divide in the same fashion to later differentiate into neurons and glia (Martinez-Cerdeno, Noctor et al. 2006). In the adult CNS, neurogenesis has been consistently observed to persist in two areas, the subependymal zone (SEZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. The SEZ contains GFAP expressing cells and slowly dividing NSCs that can generate neuroblast precursors that ultimately migrate to the olfactory bulb (OB) and differentiate into granule and periglomerular neurons (Goldman and Luskin 1998; Doetsch, Garcia-Verdugo et al. 1999; Lledo, Alonso et al. 2006; Murray and Lledo 2006). During neural repair neurogenesis can take place through endogenous stimulation of NSCs residing in the SVZ. Recent studies suggested participation of these cells in functional recovery following neurological damage by stroke. Presently much of the ongoing related research delves into trying understanding the underlying mechanisms

engaging NSCs in re-establishing damaged neural circuitries in the adult brain as a result of injury or disease (Yamashita T 2006).

1.2 GLIOMAS

Gliomas are primary tumors that usually occur in the frontal or temporal lobes of the brain but that can also grow in other areas namely near the optic nerve, brain stem and the cerebellum. The most common type of gliomas are astrocytomas, however, according to their cell type, they can be further divided into the subgroups oligodendrocytomas and ependymomas. Astrocytomas are graded I-IV relatively to their degree of malignancy with grade IV being the most severe. There are three types of astrocytomas, well-differentiated (low grade or grade I-II), anaplastic astrocytomas (grade III), which grow faster than low grade, and glioblastoma multiforme (GBM) (high grade or grade IV) that grows rapidly and infiltrates the surrounding brain tissue (Louis 2006). Recent technological advances have made it possible to study the genetic and epigenetic alterations these tumors possess in a genome-wide fashion. The majority of gliomas often display mutations in genes controlling three major core-signaling pathways activated during glioma tumorigenesis: the retinoblastoma (Rb) pathway, the p53 pathway and the receptor tyrosine kinases (RTK) pathway.

The Rb pathway exerts tumor suppressor functions by inhibiting the cell cycle through regulation of the E2F family of transcription factors. Rb usually complexes with E2Fs causing blockage of cell cycle progression at G1 phase. Phosphorylation of Rb by cyclin-dependent kinases (CDKs) causes loss of function by releasing Rb from its transcriptional repressive complex this way facilitating S phase progression (Ohtani, Yamakoshi et al. 2004).

The p53 pathway has also a tumor suppression effect by regulating the cell cycle and apoptosis in response to genotoxic and cytotoxic stress. This pathway is mutated in almost all tumors and it is partially regulated by activity of the E3 ubiquitin ligases MDM2 and MDM4 causing transcription inhibition via direct binding or proteosomal degradation of p53. The ARF tumor suppressor is an upstream regulator of the p53 pathway that can inactivate the E3 ligase activity of MDM2, found in the same locus as INK4a members of the cyclin kinase dependent inhibitor family. The $p14^{ARF}$ and $p16^{INK4a}$ genes are both encoded by the CDK2a locus, which plays a pivotal role in the control and linking of both p53 and Rb pathways (Ivanchuk, Mondal et al. 2001; Chen, McKay et al. 2012; To, Pajovic et al. 2012)

The receptor tyrosine kinases (RTK) pathway controls other cellular functions that play an important role in tumorigenesis such as cell proliferation and apoptosis. This pathway is activated through binding of several growth factors, including EGF ands its receptors (EGFR), to phosphatidylinositol-3-OH kinase (PI3K) phosphatidylinositol-3,4,5-triphosphate which in turn phosphorylates and activates protein kinase B (AKT). AKT is known to inhibit the FOXO transcription factors, which are mediators of apoptosis and cell-cycle arrest, by activation of mTOR, leading to cell proliferation and survival. The tumour-suppressor phosphatase with tensin homology (PTEN) negatively regulates PI3K by blocking AKT signaling. In addition RAS proteins can also regulate the activities of PI3K either directly or indirectly through activation of the downstream signaling pathway involving mitogen-activated protein kinase (MAPK). Inhibitors of MAPK have been previously shown to be an efficient cancer therapy in mouse models (Sebolt-Leopold, Dudley et al. 1999; Megan Cully 2006).

Altogether mutations in Rb and p53 pathways, along with deletions or mutations of PTEN and deviant expression or defects in RAS/MAPK signaling affecting the PI3K pathway, cause abnormal cell growth and proliferation perturbing several other cellular activities such as invasion and apoptosis (Mao, Lebrun et al. 2012). A large percentage of high grade astrocytomas and GBM exhibit genetic disturbances in these signaling pathways suggesting that a deeper understanding of these intricate processes will improve the current methods for early diagnosis and treatment of malignant gliomas.

Lastly, current research using newly available genomic data has made possible the identification of previously unknown genetic alterations in brain cancers such as mutation of the enzyme cytosolic isocitrate dehydrogenase 1(IDH1). Arginine 132 mutations to histidine, particularly, have been shown to disrupt its capacity in catalizing the conversion of isocitrate to a-ketoglutarate, a biological compound produced by de-amination of glutamate that plays an important role in glia cell physiology (Ducray, Marie et al. 2009; Hartmann, Meyer et al. 2009). Interestingly these mutations have been found to coexist with mutations in p53 and instead of causing loss of function they rather generate a new ability in IDH1 to catalyse the NADPH-dependent reduction of a-ketoglutarate to R(2)-2-hydroxyglutarate (2HG). Excess accumulation of 2HG represents an increased risk for the development and progression of malignant gliomas (Patrick S. Ward 2009; Ward, Patel et al. 2010; Chowdhury, Yeoh et al. 2011). Identification of patients with mutations in IDH1 may thus provide a good target for adequate early therapies inhibiting 2HG production.

1.2.1 The cancer stem cell model

The cancer stem cell theory proposes there is a small fraction of cells within the tumor with stem cell properties capable of continual self-renewal and differentiation (Tannishtha Reya 2001; Kassem 2008). This view has since fueled a lot of controversy in the field despite being well established that such cells exist in human leukemias (Dick 1997; Wang and Dick 2005). However, convincing proof showing their existence in solid tumors has remained scanty and the debate on whether cancer stem cells (CSCs) are universal to all cancers is still ongoing. Isolation of CSCs from heterogenic solid tumors and inoculation in immunocompromised rat models (NOD/SCID) has been able to phenotypically recapitulate the parental tumors meaning that this CSC subset has the potential to bring about progeny that is similar in composition to the tissue from where it was originally isolated (Singh, Hawkins et al. 2004; Dalerba and Clarke 2007). This has created the notion that CSCs probably arise through neoplastic changes initiated in normal stem cells or downstream progenitors leading to expansion of the stem cell and/or progenitor pool. Thus, accumulation of genetic instability combined with indefinite self-renewal potential in CSCs, and possibly in non-tumorigenic bulk cells, may contribute to tumor progression (Dick 2009). Moreover, many types of CSCs display gene expression patterns resembling the ones observed in ESC as well as they share many of the same markers of "stemness" state detected in normal stem cells including OCT4, NANOG and SOX2, to name a few, suggesting that differences between tumorigenic and non-tumorigenic cancer cells are a consequence of their individual epigenetic profile and that the cellular heterogeneity seen in tumors may be due to epigenetic reprogramming (Vincent and Van Seuningen 2012). Current treatments for GBM are aimed at eliminating the tumor cells of origin to prevent oncogenic transformation and decrease cancer relapse.

1.2.2 Glioma stem cells

Glioma stem cells (GSCs) are thought to be generated either by de-differentiation of lineage-specified progenitors and mature astrocytes (Bachoo, Maher et al. 2002) or transformation of the endogenous NSC population. There is substantial evidence showing that the tendency of gliomas to become more aggressive is associated with progressive de-differentiation. NSCs are found in specific brain regions: the subventricular zone (SVZ) and subgranular layer (SGZ), where together with endothelial and ependymal cells they form a niche that supports their own growth and self renewal (Shen, Goderie et al. 2004; Zhao, Huang et al. 2008). In this scope the concept of a GSC niche is beguiling and has been adapted to CSCs as they also need a particular set of conditions to be able to maintain their "stem cell –like" characteristics. The idea of gliomas originating from astrocytes also presupposes the de-differentiation premise by which cells would be able to regain immature glial and progenitor properties. The likelihood of such event was strengthened when reprogramming of terminally differentiated cells back to pluripotent embryonic stem cells, using a cocktail of transcription factors, was made possible (Takahashi and Yamanaka 2006). Since then many have reported successful generation of gliomas in vitro from early cortical astrocytes using this method. However, there is still insufficient evidence confirming the occurrence of a similar event while using mature astrocytes and in fact recent research has proven this not feasible (Jacques, Swales et al. 2010). There are many difficulties surrounding in vitro culture of mature astrocytes and the use of viral vectors or transgenic mice in such studies has been limited due to the lack of appropriate markers for these cells as the commonly used GFAP can also be expressed by adult NSCs (Brown 1998; Krencik and Zhang 2011).

1.3 **OXIDATIVE STRESS**

Oxidative stress is the result of perturbation in the balance between the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the ability of a biological system to rapidly remove the resulting reactive intermediates and repair eventual damage. ROS usually originate from endogenous sources as by-products of normal cellular metabolism, such as energy generation from mitochondria during cellular aerobic respiration or detoxification activity of the liver enzyme cytochrome P-450 (Robertson, DeCory et al. 2000). Exogenous sources of ROS include exposure to cigarette smoke, carbon emission, ionizing radiation, and bacterial, fungal or viral infections. RNS usually derive from nitric oxide (NO) and superoxide (O_2^-) produced via the enzymatic activity of nitric oxide synthases NOS2 and NADPH oxidases, and has a a direct role in innate immune responses. NOS2 can be secreted by macrophages after induction by cytokines and microbial products, especially interferon gamma (IFN-γ) and lipopolysaccharide (LPS) (Nicole M. Iovine 2008).

ROS are potential damaging agents of cellular molecules including lipids, proteins or DNA. Furthermore, they are also believed to be involved in the development and/or aggravation of several neurodegenerative, lung and heart diseases, inflammation and particularly cancers, because of their direct mutagenic effects on DNA (Gulam Waris 2006; HALLIWELL 2007). These ROS include the less reactive species superoxide radical (O_2^-) and peroxide (H_2O_2). However, metal ion catalyzed reactions (Haber-Weiss and Fenton reactions) converts both (O_2^-) and (H_2O_2) to hydroxyl radical (OH), which reacts strongly with all cellular components including DNA. In normal conditions the availability of iron is very limited due to the fact that iron (Fe) in the cell is bound to proteins like transferrin, lactoferrin, and ferritin, this way minimizing the amount of free cellular Fe available. Caeruloplasmin, and

albumin to a lesser extent, are also proteins with transport functions that bind to copper (Cu). Caeruloplasmin is closely associated to Fe metabolism namely through its Cu-dependent oxidase activity that further oxidates Fe^{2+} (ferrous iron) into Fe^{3+}(ferric iron), which is the form of Fe that can be transported by transferin to the plasma (Zastawny, Altman et al. 1995). Oxidative events producing ROS can release Fe from its natural storage sites and make it work as a catalyst for free radical reactions. To minimize the formation of ROS cells rely on antioxidant protection provided by endogenous enzymatic and non- enzymatic mechanisms (Valko, Leibfritz et al. 2007). Activity of the superoxide oxidase dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase help controlling the exposure to oxidative stress by directly removing ROS. There are three types of SODs in mammals, the soluble or cytoplasmic SOD1, the mitochondrial SOD2 and the extracellular SOD3, each associated with specific metal ions. The different SOD antioxidant capacities are thus a result of their multiple cellular compartment location and metal ion content. SOD catalyzes the dismutation of H_2O_2 to O_2 and GSH-PX catalyzes hydrogen peroxide and GSH to form disulfide GSSG, which prevents free radicals from releasing electrons. GSH reductase (GS), in its turn, reduces GSH to change GSSG back to GSH for it to be re-used in antioxidant reactions (Zhu, Carvey et al. 2007). Peroxides can induce changes in gene expression with consequences ranging from apoptotic to necrotic events. For instance the resulting inflammation and fibrosis seen in liver failure is tightly linked to peroxidation. These toxic effects are ablated through conversion of H_2O_2 to H_2O and O_2.

Endogenous non-enzymatic antioxidants include glutathione (GSH), thioredoxin, vitamin C, vitamin E, carotenoids, natural flavonoids, and melatonin (Mark R McCalla 1999). Some of these antioxidants (Vitamin C, carotenoids and some forms of vitamin E) cannot be synthesized by humans and are obtained essentially through dietary intake. Thioredoxin has important functions in redox signaling while vitamin C and vitamin E aid in a wide range of essential metabolic functions and are crucial scavangers of water soluble and fat soluble ROS respectively (Padayatty, Katz et al. 2003; Azzi 2007). Besides the protective role against U.V light damage in the human retina carotenoids also exhibit antioxidant activity. Natural flavonoids have been shown to have high antioxidant activity in vitro, even surpassing the ones of vitamin C and vitamin E however their protective value in vivo is still unclear especially because they are poorly absorbed in the human organism (La Casa, Villegas et al. 2000; Lotito and Frei 2006). Melatonin is a hormone produced by the pineal gland that is essential for the regulation of the circadian rhythm. Because of its lack of redox cycling, and ease of transport through cell membranes and blood brain barrier, it acts as a powerful antioxidant through direct removal of OH, O_2^-, and NO (Barlowwalden, Reiter et al. 1995; Hardeland 2005; Reiter, Manchester et al. 2010).

1.3.1 The antioxidant paradox

It is generally accepted that in the course of the human life span oxidative stress contributes to aging, neurodegeneration and the development of cancers.
However, there is not any study so far that has convincingly proven that ingestion of supplementary antioxidants is helpful in clearing human disease (Bjelakovic, Nikolova et al. 2007). In fact, the use of antioxidants in rodent models of disease has been far more successful in showing their beneficial effects against disease progression. This lack of evidence challenges the rather simplistic view that free radicals are bad and antioxidants good, raising an issue known as “the antioxidant paradox” (Halliwell 2000). It is still not clear if oxidative stress is relevant in every disease and the fact that antioxidants have more

of a preventive effect is most likely connected to failed therapeutic attempts in patients exhibiting advanced pathological symptoms (Halliwell 2009). Additionally, the used dosages and also the spectrum of the antioxidants used in these preparations are most probably a determining factor for successful interventions. There is a prominent disturbance in the uptake of other nutrients such as α-tocopherol of γ-tocopherol, and β-carotene and other carotenoids associated with administration of high doses of single agents. Similarly, through redox cycling certain antioxidants (e.g. vitamin C) can act as pro-oxidants and contribute to free radical formation. Pro-oxidants can nonetheless have good overall effects by evoking a mild stressful challenge that triggers a rapid response leading to increased levels of endogenous antioxidant defense systems such as reduced glutathione, GSH. It is becoming increasingly more apparent that the best strategy may rely on using mixtures of antioxidants with broad spectrum in optimal doses, to fully take advantage of the protective effects of antioxidants. Ironically such preparations might be just a redundant reproduction of what can already be found in natural food (Halliwell 2012).

1.4 DNA DAMAGE/REPAIR

DNA repair is a crucial cellular routine composed by a plethora of enzymatic activities that protect the fidelity of the genome and aid the maintenance of proper cell function. DNA damage can arise from both exogenous and endogenous sources. The eukaryotic cell has evolved a number of repair strategies to counteract the nocive effects of damaging agents. These repair approaches are organized in orderly fashion in the form of repair pathways that are very specific to the type of damage they are to amend.

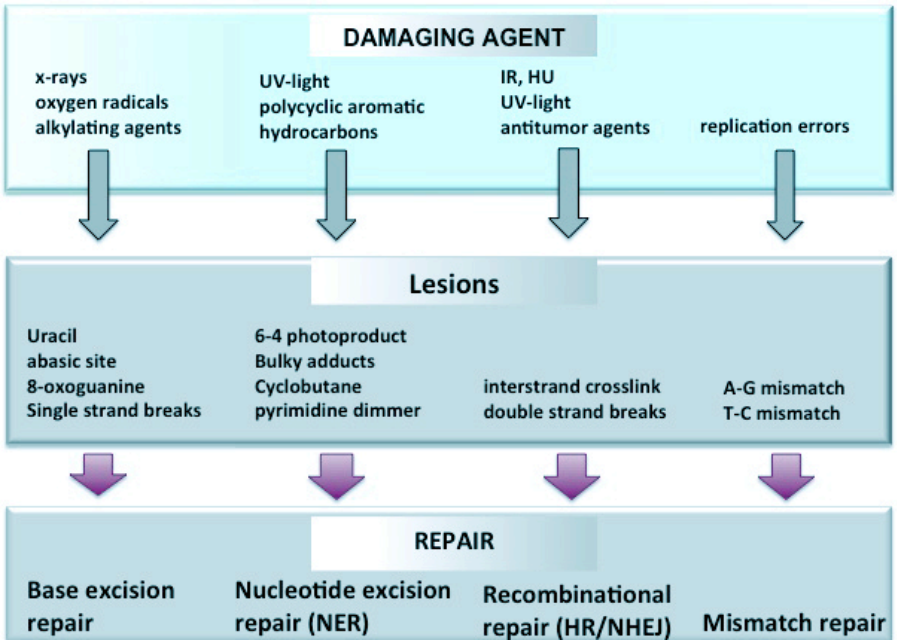

Figure 1.
Resume of DNA repair pathways. Modified from (Hoeijmakers, 2001)

1.4.1 **Repair pathways**

1.4.1.1 Base excision repair (BER)

This repair pathway deals with DNA lesions inflicted by ROS and alkylating agents. Oxidative stress causes base pair modifications that upon accumulation can lead to DNA single strand breaks (Pachkowski BF 2009). The most common oxidative DNA lesion is the 7,8 dihydro 8-oxoguanine (8-oxoG). When left unrepaired this lesion can lead to G:C → T:A transversions which are highly mutagenic. Simple alkylating agents produce N- methyl-purines, which on their turn can affect substrate-enzyme interactions. The most predominant N-alkyl base adducts are the 7-methylguanine and 3-methyladenine and their accumulation can also have severe genotoxic effects to the cell (Larson, Sahm et al. 1985; Beranek 1990). A number of DNA glycosylases have been identified so far, among them OGG1-2, NEIL1-3, NTH1-2, UDG, TDG and MUTYH, each one having well defined substrate specificities however often displaying overlapping repair functions. OGG1 is the main glycosylase in charge of removing 8-oxoG. Its important repair activity in mitochondrial DNA has also been demonstrated by mitochondria-selective overexpression of OGG1 in pulmonary endothelial cells. Enhancement of mitochondrial OGG1 activity protected cells against both oxidative induced mtDNA damage and cell death (Dobson, Grishko et al. 2002). The BER is initiated when a DNA glycosylase recognizes a damaged base and cleaves the N- glycosylic bond between the base and the nucleoside. This produces apurinic/apyrimidinic (AP) site that is subsequently processed by an endonuclease or AP lyase by means of generating a single-strand nick through cleavage of the phosphate backbone adjacent to the AP site. Thereafter, DNA polymerase inserts the correct base and ligase III (short patch repair) or ligase I (long patch repair), in association with PCNA, finalizes repair by sealing the single-strand nick (Almeida and Sobol 2007; Wilson 2007). Several studies have stressed the importance of the interplay between histone modifications and ATP-dependent remodeling complexes in facilitating access to lesions, this The BER is initiated when a DNA glycosylase recognizes a damaged base and cleaves the N- glycosylic bond between the base and the nucleoside. This produces an apurinic/apyrimidinic (AP) site that is subsequently processed by an AP endonuclease or AP lyase by means of generating a single-strand nick through cleavage of the phosphate backbone adjacent to the AP site. Thereafter, DNA polymerase inserts the correct base and ligase III (short patch repair) or ligase I (long patch repair), in association with PCNA, finalizes repair by sealing the single-strand nick (Almeida and Sobol 2007; Wilson 2007). Several studies have stressed the importance of the interplay between histone modifications and ATP-dependent remodeling complexes in facilitating access to lesions, this way linking histone acetylation and DNA lesion repair by BER. The histone acetyl transferase (HAT) CBP/p300 has been shown to interact with DNA glycosylases suggesting a crucial interaction between chromatin remodeling-activity and DNA repair factors (Tini, Benecke et al. 2002; Bhakat, Mokkapati et al. 2006).

1.4.1.2 Nucleotide excision repair (NER)

This excision repair pathway removes DNA helix- distorting bulky lesions inflicted by UV radiation (6-4 photoproducts (6-4 PPs)) and cyclobutane pyrimidine dimers (CPDs). This type of lesion can be repaired either by direct reversal of damage or through excision repair. In yeast, chromatin structure has been shown to play an important role in repair factor accessibility to damage sites. Several studies have demonstrated that repair of both CPDs and 6-4PPs in nucleosome–free regions and in the linker DNA takes place at a higher pace comparing to the nucleosome core particle (Smerdon, Bedoyan et al. 1990; Wellinger and Thoma 1997; S. Tanakaa 2002). Direct reversal repair has been shown to occur in yeast via photoreactivation of photolyases within specific light wave lengths (340-400 nm) (Sancar, Thompson et al. 2000) in a process involving cleavage of the link between adjacent pyramidines, without cutting the phosphate backbone of DNA, and the ATP dependent remodelers SWI/SNF and ISW2 (Aboussekhra and Thoma 1998).

Mutations in genes encoding key players of the NER (eg. XPB, XPD, P8/TTDA, CSA, CBS) are accountable for the onset of xeroderma pigmentosum (XP), XP combined with the progeria Cockayne syndrome (XP/CS), or trichothiodystrophy (TTD) syndromes. These diseases display a broad range of severe symptoms including extreme photosensitivity of the skin, premature aging and retardation (Giglia-Mari, Coin et al. 2004; Oh, Khan et al. 2006; Aguilar-Fuentes, Fregoso et al. 2008).

The NER pathway encompasses two different ways of recognizing damage, global genome repair and transcription coupled repair, that onwards converge into the same excision mechanism (Tornaletti and Hanawalt 1999). In global genome repair recognition of helix distortion is assisted by the complex formed by the DNA binding protein XPC and HR23B, an ortholog of the yeast protein Rad 23 proposed to be involved in XPC ubiquitination and its consequent increase in DNA affinity. The protein CEN2, which is important in centrosome duplication, can also be present as a stabilizer of the XPC-HR23B complex although its interaction *in vitro* has been proven not necessary and it is thought to be rather suggestive of overlapping NER and cell division (Araki, Masutani et al. 2001). Additionally the DBD complex (XPE) enhances recognition of UV induced pyrimidine dimers by increasing the resulting helix distortion and this way making it easier for the XPC-HR23B complex to detect and bind to the sites of damage (Sugasawa 2006). In this context interactions between XPE and the chromatin remodeling complex CBP/p300 are also crucial for efficient removal of DNA damage (Datta, Bagchi et al. 2001; Kulaksiz, Reardon et al. 2005).

Blockage of the RNA polymerase II (RNA pol II) initiates the transcription coupled NER facilitated by the proteins CBS, CSA and XBA, which recognize the lesion and help stabilize the stalled RNA pol II. Following this, either CBS or CSA recruit the human transcription factor IIH complex (TFIIH) to the damaged sites and from this point on the two NER sub-pathways converge (Tsutakawa and Cooper 2000). The members of the TFIIH 10 subunit complex, XPB and XPD, use their ATP dependent helicase activity to unwind the DNA around the lesion and facilitate accessibility. XPA and RPA bind the opposite intact single-stranded DNA, to stabilize the newly formed structure, and recruit the endonucleases XPG and ERCC1/XPF who attain the site of damage and cleave 3′ _ and 5′_ of the lesion, respectively. Thereafter the resulting fragment containing the lesion is excised and the remaining 24–32 base gap filled by repair synthesis performed by DNA polymerase complexes consisting of DNA polymerase d and k, or e (Pol d, k, e) and accessory proteins.

Finally the DNA strand nick is sealed by a DNA ligase (LIG1 or LIG3).

1.4.1.3 Double strand break repair (DSBR)

Double strand breaks (DSBs) are one of the most severe DNA lesions resulting from multiple damaging agents including ionizing radiation, UV light and chemical exposure. DSBs can be induced as response to oncogenic activation and lead to apoptosis or senescence via tumor suppressor p53 activation. It is imperative the presence of DSBs is detected and promptly signaled within the cell because failure in repair, or activation of protective mechanisms can result in defective cells escaping cell cycle checkpoints, evading death and carrying potentially harmful mutations to the progeny (Gorgoulis, Liloglou et al. 2004; Bartkova, Horejsi et al. 2005).

The two major pathways for repair of DSBs are non-homologous end joining (NHEJ) and homologous repair (HR). NHEJ rapidly repairs DSBs without requiring a homologous sequence. NHEJ is required for somatic recombination in B lymphocytes and T-cell receptors, a process responsible for the generation of antibody diversity. However, this repair mechanism is error prone and often causes insertions or deletions at the lesion site. Homologous recombination (HR) is an error free mechanism that uses a sister chromatid as template for repair. The selection of repair pathway for DSBs is dependent on the phase of the cell cycle. During S and G2 phase HR is the preferred repair pathway in mammals whereas NHEJ is generally predominant throughout the cell cycle especially during G1 phase (Takashima et al., 2009).

Detection of DSBs is initiated by MRE11/Rad50/NBS1 (MRN) and the KU70/KU80 complexes (Mordes and Cortez 2008). Further signaling of DSBs is carried through recruitment of the members of the Phosphatidylinositol-3 kinase-related kinases (PIKK) Ataxia-Telangiectasia-Mutated (ATM), Ataxia Telangiectasia and Rad3 Related (ATR) and DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) to the break sites. The ability of these kinases to phosphorylate a number of substrates plays a critical role for the propagation of DSB signaling (Shiloh 2000). The histone H2A variant H2AX, a component of the nucleosome core structure, is extensively phosphorylated by ATM and DNA-PKcs at chromatin regions bearing DSBs (Falck, Coates et al. 2005). Although not strictly necessary phosphorylation of H2AX c-terminal modulates NHEJ and HR pathways and may generate an epigenetic signal that prods the recognition of DDBs by sensor proteins with specific affinity for this phosphorylated epitope (Stucki, Clapperton et al. 2005).

Activated ATM and ATR phosphorylate the cell cycle kinases CHK2 and CHK1. Furthermore, activated ATM, ATR and CHK2 phosphorylate p53, which can transactivate p21 and suppress its inhibitory role on the cyclin-dependent-kinases causing a delay in G1/S transition. Non-repairable lesions can trigger apoptosis namely by p53 transactivation of its pro-apoptotic targets, while kinase activity of CHK1 can lead to either inhibition of CDC2/cyclin B activity, causing G2/M arrest, or CDK2 inactivation and delayed intra-S phase (Mailand, Falck et al. 2000; Smith, Tho et al. 2010).

1.4.1.4 Mismatch Repair (MMR)

MMR is the DNA repair mechanism in charge of removing mismatches arising during replication. Genetic recombination can also introduce mismatches when heteroduplex DNA molecules are formed by association between two homologous but non-identical strands. This occurs when the number of microsatellite-repeat units in the template differs from the ones in the newly synthesized strand causing microsatellite instability (MSI). This phenotype can produce extrahelical non-paired nucleotides known as insertion/deletion loops (IDLs) (Renkonen, Zhang et al. 2003). A significant number of cancers of the colon, endometrium and other organs were found to carry MSI accompanied by IDLs as a result of deficient MMR, leading to a mutation prone phenotype (Jiricny 2006). A variety of other base pair modifications due to DNA damage are also processed by MMR including base pairs containing O^6-methylguanine, 8-oxoG, carcinogen adducts and UV photo products (Aquilina and Bignami 2001; Wu 2008). MMR has been suggested to work as a back up to BER for repair of both mitochondrial and nuclear oxidative damage in yeast (Dzierzbicki, Koprowski et al. 2004).

Our current understanding of mammalian MMR is mainly based on data gathered through characterization of Escherichia coli (E. coli) and yeast. In E. coli, mismatches and loops are recognized and bound by the complex MutsS forming a "slidding clamp", triggering an ATP-dependent conformational change that recruits the homodimer MutL to the defective site. Thereafter, also in the presence of ATP, they activate the MutH endonuclease which cleaves the unmethylated strand creating a nick used as a point of entrance for the single-stranded DNA binding protein (Ssb) and DNA helicase II to displace the error bearing strand. The removed single strand is then after hydrolyzed by an exonuclease (either EXO1, EXO7 or EXO10, depending on the direction of the mismatch) and DNA ligase restores continuity to the repaired strand. Finally DNA polymerase III fills in the gap and DNA ligase I completes repair by sealing the remaining nick (Hsieh 2001; Schofield and Hsieh 2003). Five MutS homologues (MSH) have been identified in mammals, where recognition of mismatch is a more complex process. The mismatch-binding factor composed of MSH2 and MSH6 often referred to as MutSα, initiates the repair of base–base mismatches and IDLs of one or two extrahelical nucleotides (Kunkel and Erie 2005). Repair of a larger number of IDL is instead initiated by the heterodimer of MSH2 and MSH3, MutSβ (Drummond, Li et al. 1995). Docking of MutSα recruits MutLα, which forms a ternary structure with the mismatch DNA and MutSα. The ternary structure undergoes an ATP-driven conformational switch, which releases the sliding clamp from the mismatch site. When migrating upstream (5′→3′) sliding clamps run into the DNA polymerase accessory complex RFC, bounded at the 5′ terminus of the strand brake, and replace it with Exo1 that upon activation starts degradation of the strand in a 5′→3′ direction. Replication protein A (RPA) stabilizes the single-strand gap while MutLα inhibits activity of EXO1 upon removal of the mismatch polimerase delta (Pol δ) docks a the 3′ terminus of the original break site bound by PCNA forming a complex that fills the gap and DNA ligase I seals the remaining nick completing the repair process. Sliding clamps moving downstream encounter a PCNA molecule that is bound at the 3′ terminus of the strand break. The recruited EXO1 degrates the region between the original discontinuity and the mismatch whereas RFC that is bound at the 5′ terminus of the discontinuity prevents degradation in the opposite direction (5′→3′). Again, EXO1 activity is inhibited by bound RPA and MutLα and the gap is filled by Pol δ. DNA ligase I seals the nick to finalize repair (Kadyrov, Dzantiev et al. 2006).

1.5 DNA DAMAGE RESPONSE (DDR)

The DDR is a very well orchestrated succession of cellular responses taking place when the cell senses damage to its genome (Rouse and Jackson 2002; Harper and Elledge 2007; Jackson and Bartek 2009). The DDR is at large controlled by DNA damage- sensing kinases ATM, ATR, or DNA-PKcs, which can phosphorylate several proteins including the p53 tumor suppressor. ATM and DNA-Pkcs have important tumor suppressing activities themselves while ATR is required for DNA replication and cell proliferation (Jhappan, Morse et al. 1997; Brown and Baltimore 2003). Depending on the nature and cellular context of the damage DDR signaling can have several outcomes: transient cell cycle arrest coupled with DNA repair, apoptosis, or senescence (Sherman, Bassing et al. 2011). Recent studies now also suggest cell differentiation as a potential outcome in connection with DNA DSBs. Nevertheless the two main features of the DDR are cell-cycle checkpoint activation and DNA repair. Precise control of cell cycle ensures that intact genetic material is passed to daughter cells during cell division. In response to appropriate signals from the surroundings, quiescent cells leave the gap 0 phase (G0) of the cell cycle and enter the gap 1 (G1) phase prior to the DNA replication or synthesis (S) phase, followed by a second gap (G2) phase, and cell division or mitosis (M). Cell-cycle checkpoints are critical steps to maintain the order and fidelity of cell-cycle events in response to replicative stress and DNA strand breaks. Cell cycle arrest comes about in order to stall cell cycle progression and give time for repair to take place this way avoiding replication errors during transcription. DNA repair is the core of the DDR and relies on the election of the appropriate repair mechanism to resolve the harmful DNA lesion. Notable in this context is chromatin remodeling occurring as consequence of histone modifications such as methylation and acetylation of lysine residues (Downs 2008; Amente, Bertoni et al. 2010). These epigenetic changes later facilitate the access of DNA repair factors to sites of damage and instruct the cell to reassume its normal cell cycle progression (Chen and Tyler 2008; Dinant, Houtsmuller et al. 2008; Das, Lucia et al. 2009).

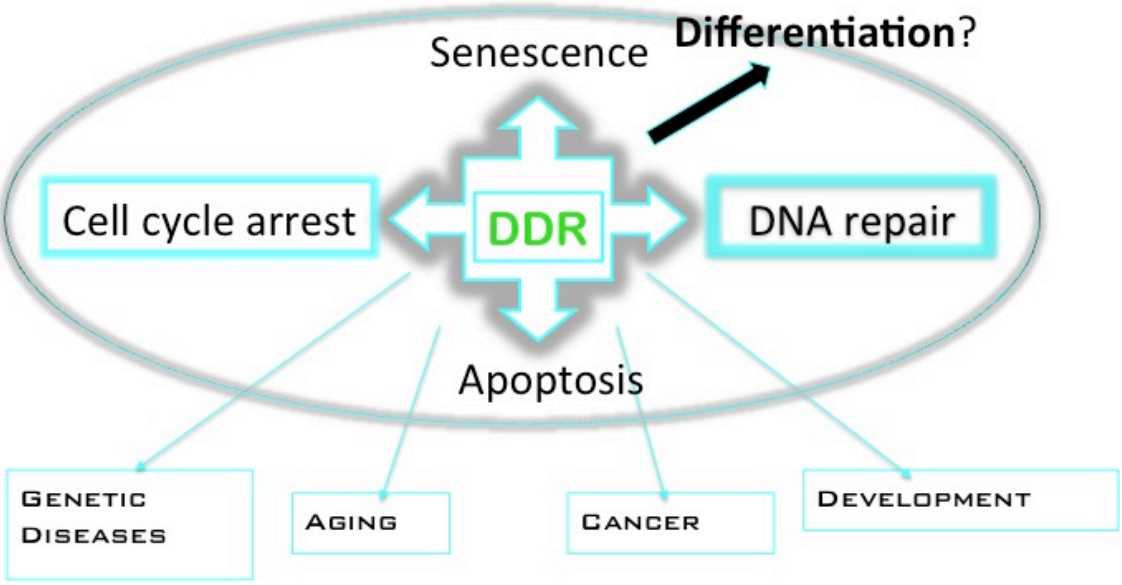

Figure 2.
The DNA damage response (DDR). Recent findings propose differentiation as a fourth outcome of the DDR. Modified from (Harper et al. 2007).

1.6 CHROMATIN REMODELING

In order to fit in the cell nucleus DNA is coiled around nuclear proteins called histones, forming a structure known as chromatin. The further compaction of this structure wrapped around basic histone proteins made up of two copies each of histone H2A, H2B, H3 and H4 along with linker protein H1 form the nucleosome. DNA-associated processes including transcription, replication, recombination, and DNA repair, are all extensively regulated by complex transient changes in chromatin structure. The compact organization of this structure creates an obstacle for important proteins, such as transcription factors, to interact with DNA. To overcome this limitation the chromatin structure needs to be modified to render access to DNA (Groth, Rocha et al. 2007). Chromatin remodeling and compaction can essentially occur via four known processes: (1) active ATP-remodeling activity of the SWI/SNF super family of DNA-dependent ATPases and the ISWI-related protein INO80 (2) incorporation of diverse histone variants by histone chaperones (3) binding of abundant non-core histone proteins, such as the linker histone H1, the family of high mobility group proteins (HMG), or different isoforms of the hetero-chromatin protein 1 (HP1); and (4) covalent and post translation modifications (PTMs) of the core histones such as acetylation, methylation, phosphorylation, and ubiquitylation (He and Lehming 2003; Neves-Costa and Varga-Weisz 2006; Saha, Wittmeyer et al. 2006; Loyola and Almouzni 2007). Some studies have suggested that acetylation of histones may signal nucleosome assembly following repair (Brand, Moggs et al. 2001). Both H3K56 acetylation and deacetylation have been shown to be of great importance in this process thus playing a pivotal role in maintaining genome stability. After completion of repair the histone chaperone ASF1A can engage in an ATM-dependent manner facilitating the recruitment of histone acetyl transferases (HATs) for the reestablishment of the H3K56 acetylation status. In addition, acetylation of H3K56 mediates dephosphorylation of γ-H2AX and its removal from the damage sites, resulting in the recovery from checkpoint arrest and reinstatement of cell-cycle progression (Battu, Ray et al. 2011).

1.7 CELLULAR SENESCENCE

In 1961 Leonard Hayflick and Paul Moorhead observed that proliferating cells in culture gradually stopped growing after a certain number of cell divisions. This phenomenon was later named the "Hayflick limit" and introduced the concept of replicative senescence (Hayflick and Moorhead 1961).

In general, the maintenance of proper tissue homeostasis represents a great challenge to higher order organisms. Moreover, the rapid cellular turnover of this process requires proliferating cells, which are more prone to acquiring mutations that upon accumulation can trigger carcinogenesis. A common feature to various types of tumors is the presence of highly proliferative cells. From an evolutionary context, cancer has been an obstacle to life span due to its usual fatal consequences. In this scope senescence is believed to be integral part of tumor suppression mechanisms that have evolved to prevent the occurrence of tumorigenesis (Sager 1991; Campisi 2001; Campisi and di Fagagna 2007).

The senescence phenotype is typically characterized by growth arrest in the transition from G1 phase to S phase in the cell cycle along with an upregulation of the senescence -associated β-galactosidase (SA-βgal) activity and expression of the tumor suppressor $p16^{INK4a}$, which otherwise is not usually detected in quiescent and differentiated cells. Assessment of incorporation of BrdU, EdU or H-thymidine and immunodetection of proteins such as

PCNA and Ki-67 have been used as biomarkers for the lack of DNA replication usually associated with senescence. Nonetheless, these methods cannot be used to distinguish between quiescent cells, which can re-enter the cell cycle through various physiological stimuli, and senescent cells, where the growth arrest is irreversible despite the possibility of the cell to remain metabolically active (Goldstein 1990). Markers of senescence- associated heterochromatin foci (SAHFs), such as the protein HP1, and markers of senescence-associated DNA damage foci (SDFs), such as the phosphorylated histone H2AX (γ-H2AX) and 53-binding protein-1 (53BP1), can also spot senescent cells (di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003). However one should not discard that these foci are not entirely exclusive to the senescence state and may also be a reflection of DNA damage and/or telomeric dysfunction.

Understanding the causes and consequences of senescence can help to further clarify its connections to cancer, neurodegeneration related diseases and aging. It is currently postulated that the arrested growth observed in senescent cells is due to both cell autonomous mechanisms via tumour suppressor pathways p53 and $p^{16INK4a}$/pRB, (Hara, Tsurui et al. 1991; Jacqueline J.L. Jacobs 2004) and cell nonautonomous mechanisms, through activity of some of the proteins belonging to the senescence–associated secretory phenotype (SASP) (Acosta, O'Loghlen et al. 2008; Coppe, Patil et al. 2008; Kuilman, Michaloglou et al. 2008; Coppé JP 2010). There are a number of physiological events and stimuli triggering these mechanisms. For instance, DNA polymerases are unable to replicate entire DNA ends and this poses limits in the amount of cell divisions a cell can undergo before falling into replicative senescence. This process is known as the end-replication problem (Harley, Futcher et al. 1990). Several experiments have shown that ectopic expression of the telomerase hTERT in certain human fibroblasts helps to prevent deterioration of telomeres and the onset of senescence. Telomere shortening due to base pair loss in telomeric DNA during S phase introduces chromosomal abnormalities that can trigger the DDR in a similar way to what is seen in response to DSB repair. The DDR is a cellular mechanism that stalls the cell cycle in order to give opportunity for the cell to repair damage. Several of the intervenients in DSB repair such as protein kinases ATM and CHK2, adaptor proteins 53BP1 and MDC1 (mediator of DNA damage checkpoint protein-1) and chromatin modifiers γ-H2AX, are also observed in DNA-damage foci present in senescent caused by telomeric impairment conveying similarities between DSB and telomeric dysfunction (Campisi and di Fagagna 2007).

Damage at non-telomeric sites, especially DSB, can initiate and maintain a continual DDR displaying persistent foci called DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). These foci contain activated protein ATM and Rad3 related (ATR) substrates that can sustain a p53 mediated senescence response (Rodier and Campisi 2011). Senescence can occur without DDR and in this case enforcement of the growth arrest is carried by ectopic expression of the cyclin-dependent kinase (CDK) p21WAF1 and $p^{16INK4a}$ (Rodier and Campisi 2011). In addition certain histone modifications such as acetylation and methylation of lysine residues change chromatin conformation and influence gene expression. Euchromatin is associated with active genes whereas heterochromatin is associated with silent genes. Histone deacetylase inhibition (HDAi) has been shown to induce euchromatin formation and increase senescence through a yet unclear mechanism involving ATM activation, suggesting that HDAi can initiate a p53 dependent DDR (Bakkenist and Kastan 2003).

Tumors usually express genes carrying mutations that have the potential to induce carcinogenesis together with an increased proliferation capacity. Cellular senescence is an essential anti-cancer mechanism that arrests cell growth and diminishes the risk for tumorigenic transformation. However, recent studies warn for unheeded consequences of cell senescence. An argument is raised that paradoxically, the stimuli leading to a senescence response also have the potential to promote or initiate malignant transformation (Rodier and Campisi 2011). In fact, the SASP includes several potent inflammatory cytokines and oxidants produced by immune cells, through NF-κβ activity, that can damage cells and tissues (Freund, Orjalo et al. 2010). This alteration of the tissue environment can cause dysfunction and impairment of stem cell niches. The age–related increase in senescent cells is usually associated with a low-level chronic inflammation phenotype that in certain circumstances can escalate to a persistent acute inflammation, greatly increasing organismal susceptibility to carcinogenesis (Wang, Jurk et al. 2009). This phenotype has been shown to be dampened *in vitro* by the microRNAS mir-146a and mir-146, which help lowering the levels of the SASP factors this way preventing the onset of robust inflammation and occurrence of oncogenic transformation (Bhaumik, Scott et al. 2009). We have clearly only began to fully understand the significance of senescence in human health and further research is much needed to continue to unravel the complexity of this multifaceted mechanism.

1.8 ELECTROPHYSIOLOGY

Electrophysiology is the study of the electrical properties of a given biological sample.
In neuroscience this usually pertains to measurement of the electric activity in neurons that is commonly assessed by recording action potential (AP) propagation. The classical techniques in electrophysiology have in common the use of the electrolyte filled glass micropipette electrode.

1.8.1 Patch-clamp, voltage and current-clamp

The patch-clamp is the gold-standard technique used to report the synaptic and ion channel–mediated changes in membrane potential. Recording of electrical activity utilizes a glass pipette to establish contact with the cell membrane. Next, suction is applied to the pipette interior to seal the cell membrane onto the tip forming a structure known as the "gigaohm seal ". Following this, another suction pulse is applied causing a rupture in the membrane at the point of contact with the pipette tip opening. At this stage it is possible to measure the current passing through the membrane using either the voltage or current clamp. A disadvantage of this technique is that the diffusible substances inside the cell will slowly equilibrate with those contained in the pipette and thus both the internal and external milieu are most likely to be altered by the pipette solution.

In the voltage-clamp one electrode measures the membrane potential while connected to a patch clamp amplifier, which compares membrane potential to the desired pre-set command potential. Whenever the membrane potential differs from the command potential the patch clamp amplifier injects current into the sample membrane through a second electrode. This feedback adjustment causes the membrane potential to equalize the command potential. The current flowing back to the sample across its membrane can be measured at this point.

The current-clamp has a similar set up to the voltage-clamp, however, in this case the membrane potential is not set at any particular value and thus the recorded voltage is either generated by the cell on its own or by external stimulation.

1.8.2 Calcium (Ca^{2+}) signalling

Ca^{2+} is a key signaling ion that can regulate a large number of different intracellular and extracellular processes ranging from muscle contraction to control of synaptic activity and memory formation (Berchtold, Brinkmeier et al. 2000; Greer and Greenberg 2008). Ca^{2+} can enter cells through voltage-gated Ca^{2+} channels and several ligand-gated ion channels, such as glutamate and acetylcholine receptors. Extracellular Ca^{2+} is the main source of Ca^{2+} signal whereas the principal intracellular Ca^{2+} store is the endoplasmic reticulum (ER) from where Ca^{2+} can be released into the cytosol via activation of the inositol 1,4,5-triphosphate receptors (IP_3Rs) or ryanodine receptors raising the cytoplasmic concentration of Ca^{2+} ions dramatically. Several diseases such as Alzheimer's, Huntington's and schizophrenia display abnormal intracellular Ca^{2+} signaling. Ca^{2+} imaging has been a widely used technique to visualize the flux of Ca^{2+} ions within cells through detection of changes in fluorescent signal emitted by a fluorophore. This is usually accomplished by light excitation of a calcium/fluo indicator, which is non-fluorescent unless bounded by Ca^{2+}, or less fluorescent when not bound to Ca^{2+} (Clapham 2007; Greer and Greenberg 2008).

1.8.3 Voltage sensitive dyes

Voltage sensitive dyes (VSDs) are part of optical electrophysiological techniques with a fluorescent component developed to overcome the limitations of classical techniques. Using this method the membrane potential can be measured optically at the level of an individual cell or groups of neurons allowing to follow the dynamics of neuronal population activity with high spatial and temporal resolution. This is contrasting to classical techniques that only allow observation of electrical activity at a single point within a volume of tissue and may therefore lead to generalization of a single observed phenomenon (Zochowski, Wachowiak et al. 2000). Nevertheless, the loading of neurons with fluorescent dyes can yet be a demanding task. One of the shortcomings is to not be able to identify which neurons are being labeled when a dye is applied extracellularly in bulk loading. Also when dyes are applied intracellularly there is an obvious limitation on the number of cells that can be observed (as one cannot load a large number of neurons by direct dye injection). The temporal and spatial resolutions of voltage-sensitive dye recordings can also be limited by the imaging system and the signal-to-noise ratio, hence, the improvement of these parameters remain a major challenge to fully explore the potential of this approach (Zhou, Yan et al. 2007).

2 AIMS OF THE THESIS

Paper I. Elucidate the contribution of the DNA glycosylases OGG1 and NEIL3 repair activity in maintenance of neural stem cell characteristics and ability to differentiate into the diverse cell types found in the CNS.

Paper II. Unveil functional roles for the DNA glycosylase OGG1 in a C6 glioma cell line to help determine some of the factors involved in resistance to anticancer treatment, hoping to develop strategies for modulating repair capacity to either overcome resistance or meliorate sensitivity to cancer therapies.

Paper III Develop an alternative method for quick assessment and identification of stem cell-derived neuronal populations capable of eliciting action potentials using voltage sensitive dyes.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Embryonic neural stem cells provide an excellent experimental system to study the molecular mechanism behind the extraordinarily complex process of the CNS development, and furthermore, they represent one of the most promising avenues towards finding suitable therapies for alleviating and most hopefully curing the devastating symptoms accompanying neurodegenerative diseases and brain tumor formation. Treatment of NSCs with the histone deacetylase (HDAC) inhibitor valproic acid (VPA) has shown to rapidly induce increase expression of genes enhancing neuronal differentiation such as brain derived neurotrophic factor (BDNF) (Castelo-Branco 2012, submitted). Previous studies performed by our lab had found that the expression of DNA glycosylases could be affected by VPA treatment suggesting that the balance of DNA damage/repair possibly impact survival and differentiation of neural stem cells. In an attempt to elucidate roles for the DNA glycosylases in NSCs we have tested several differentiation protocols and made the intriguing observation that NSCs treated with VPA tend to accumulate 8-oxoG in the cell nucleus at a higher frequency comparing to control and FGF2 conditions. This has prompt us to perform gene expression studies of NSCs treated with VPA comparing to FGF2 conditions as well as cilliary neurotrophic factor (CNTF) (data not shown), known inducers of astrocytic and smooth muscle differentiation, respectively, in order to clarify putative roles for DNA glycolylase repair activity in NCS. Despite these efforts none of the DNA glycoylases neither DNA repair related protein XRCC1 nor the nuclear protein MBD4 showed any significant changes related to the different treatments. To further investigate the relevance of the DNA glycosylases in NSC differentiation we used siRNA against NEIL3, recently suggested to be implicated in early stages of differentiation (Hildrestrand, Neurauter et al. 2009), and OGG1, which is by default the main DNA glycosylase in charge of removing 8-oxoG and previously demonstrated to be closely linked to activity of the demethylase LSD1 (Perillo, Ombra et al. 2008). Knockdown of OGG1 and NEIL3 in –FGF2 conditions resulted in decrease of both neuronal and astrocytic differentiation of NSCs, shown by a downregulation of the expression of both neuronal (DCX) and astrocytic (GFAP) markers compared to control siRNA. These findings pointed to a new function for the DNA glycosylases as regulators of neural stem cell differentiation and to improve our understanding of this phenomenon we have looked at the expression of important multipotency markers of NSCs under the same conditions. Confirming our hypothesis results from these experiments revealed a downregulation of the expression of the gene Musashi-1, after 24h of OGG1 and NEIL3 knockdown. However, no significant changes were seen in the expression pattern of the genes Sox2 and Prominin-1. Conversely to the knockdown experiments in –FGF2 conditions, we have determined that treatment with the HDAC inhibitor VPA and bone morphogenic protein4 (BMP4), a potent inducer of neuronal differentiation of NSCs plated at high density (Andersson, Sodersten et al. 2009), counter acted the knockdown effects of siOGG1 and siNEIL3 as downregulation of both DCX and GFAP could not be detected (data not shown). The same lack of effect was obtained when treating the cells with CNTF (data not shown).

Because of the general roles for DNA glycosylases in maintaining the integrity of DNA, we investigated putative effects of OGG1 and NEIL3 RNA knockdown on cell viability. To our

surprise, knockdown of either OGG1 or NEIL3 at 24h did not produce any significant changes in survival rate of NSCs (Live-positive cells: siControl: 78.4%, siOGG1: 77.7%, siNEIL3 75.7%) suggesting that decrease in cell differentiation potential did not directly affect cell death rate neither was a consequence of an increased cell death. Furthermore, analysis of NSC proliferation revealed that knockdown of OGG1 and NEIL3 resulted in significant decreased incorporation of the cell proliferation detector 5-ethynyl-2'deoxyuridine (EdU) (17.2% and 13.8%, respectively compared to control 24.0%) coherent with the notion that repair impairment can arrest cell cycle progression. To further explore possible outcomes of oxidative stress combined with DNA glycosylase deficiency we therefore looked at cell senescence using specific antibodies against heterochromatin protein 1 (HP1-γ), a protein associated with heterochromatin foci formation commonly used as a marker of senescence. These experiments unveiled a significant predominant labeling of HP1-γ in siNEIL3 (82.4%) samples comparing to siOGG1 (64.4%) and control siRNA (65.5%) samples. Embryonic NCSs seldom show signs of classical senescence, except in certain conditions, however, the tumor suppressor p^{16INK4} can be induced by telomeric and DNA damage subsequently activating the pRb pathway causing formation of senescence-associated heterochromatin foci (SAHF), which can silence critical pro-proliferative genes (Narita, Nunez et al. 2003). Considering the results of the cell death and proliferation assays its is reasonable to speculate a similar senescence mechanism triggered by NEIL3 knockdown.

In short, our results supply additional proof suggesting both OGG1 and NEIl3 are regulators of multipotency in embryonic cortical progenitor cells. NEIL3 deficiency especially, affected negatively the expression of pro-neuronal (DCX) and pro-astrocytic (GFAP) genes and notably both OGG1 and NEIL3 targeting siRNA downregulated the expression of the stem cell multipotency gene Musashi-1. We have however not seen any direct correlation between induced cell differentiation and regulation of DNA glycosylase gene expression. The observed increase of 8-oxoG in the cell nucleus associated with VPA treatment could be coupled to increased demethylase activity of LSD1 as response to HDAC inhibitory effects of VPA. Although this effect needs to be further investigated in future studies, it could perhaps be linked to the concept of cell differentiation occurring as a protective event in response to both extrinsic and intrinsic oxidative stress. Activation–induced Cytidine Deaminase (AID) is an example of "self-induced" damage mechanism in order to drive productive transcription (Muramatsu, Kinoshita et al. 2000). Lastly, our results suggest that NEIL3 deficiency may influence an increase in senescence-associated signs.

3.2 **PAPER II**

Originally derived from rat brain tumors induced by *N*-nitrosomethylurea (Benda, Lightbod.J et al. 1968) the C6 glioma cell line are widely used as an experimental model system for studying glioblastoma, the most common and aggressive form of malignant primary brain tumor in humans. The percentage of cancer stem cells (CSCs) in a tumor remains a controversial issue but nonetheless current anti-cancer therapies such as radiation aim to induce cell death and avoid tumor relapse, which is believed to be strongly associated with these CSCs. However, DNA repair factors can counter act the damaging effects of this approach and hence represent an obstacle for successful elimination of tumors. With this in mind many have endorsed therapies directed to DNA repair enzymes as a means to increase DNA damage and decrease cell viability in aggressive tumors such as gliomas. Nonetheless, this approach may instead create a new problem especially in differentiation therapies aiming to more easily identify the CSCs in the tumor. To deepen our understanding of this phenomenon we therefore conducted this study.

Using the C6 glioma cell line we have successfully implemented a cell culture system similar to the one used for NSC culture to generate C6 glioma-derived stem-like cells (C6SCs). Imunocytochemistry revealed these C6 glioma cells stained positively for nestin at higher proportions when treated with the mitogen FGF2 indicating a more neural stem cell-like phenotype. Moreover, these cells became responsive to VPA and CNTF treatment and displayed both characteristic morphology and marker expression for neuronal (TuJ1) and astrocytic (GFAP) differentiation that otherwise could not be seen in control cells. Surprisingly, gene expression analysis has revealed that C6 glioma cells lack some of the DNA glycosylases that can be found in NSCs (data not shown) suggesting that the BER back up system is probably disturbed in these cells. However OGG1 expression was comparable to the one seen in NSCs and therefore we developed siRNA to efficiently knockdown OGG1.
Notably, we found that increase in GFAP expression induced by CNTF was substantially lower in C6SCs that had received siRNA against OGG1 compared to cells cultures that had received control siRNA suggesting that differentiation potential of CSCs are at least to an extent depending on OGG1 expression similar to our previous observations in NSCs (Reis et al., 2012).

Acetylation of histone 3 in lysine 56 (H3K56ac) is a histone modification proven to be relevant for DNA repair and longevity of several organisms. This epigenetic mark has been recently shown to occur in mammals (Yuan, Pu et al. 2009) and it is currently an accepted hallmark of initiation of the DDR, a cellular process that has recently received much attention because of its newly suggested implications in regulation of cell differentiation (Das, Lucia et al. 2009; Sherman, Bassing et al. 2011). To weight the relevance of OGG1 in the context of the DDR we therefore tried to identify epigenetic changes related to defective repair namely by elucidating the H3K56ac levels after several different treatments using immunocytochemistry. Unchallenged C6SCs that had received siRNA against OGG1 showed no alterations in H3K56ac levels compared to control (ECFP siRNA). However when treated with VPA there was a clear increase in the H3K56ac levels in cells receiving siRNA against OGG1 compared to vehicle-treated cultures. To clarify whether this increase in DNA damage response was specific we compared to C6SCs nucleofected with siRNA against another DNA glycosylase, NEIL3. We have previously characterized this siRNA in NSCS but C6SCs express very low levels of NEIL3 compared to NSCs. While a slight increase in H3K56ac levels could be detected in C6SCs that had received siRNA against Neil3 after VPA treatment

compared to vehicle, this increase was comparable to control cultures and much less pronounced than the H3K56 levels in VPA treated cells that had received siRNA against OGG1.

Activation of Poly (ADP-ribose) polymerase (PARP) due to accumulation of SSBs connected with defective BER has been shown to trigger apoptotic pathways (Oka, Ohno et al. 2008), and thus can alter cell survival rates. We did not however observe any significant changes in cell viability as consequence of DNA glycosylase knockdown (data not shown) perhaps suggestive of a robust DDR through a mechanism avoiding cell death. We conclude that knocking out OGG1 RNA interferes with the differentiation potential of C6SCs associated to an increased DDR. These results should be taken in account when using cancer therapies targeting OGG1 because they may produce unwanted side effects.

3.3 PAPER III

To fully understand NSC biology and appreciate the contribution of DNA repair and chromatin modifications to their differentiation potential and function, it is important to be able to identify and determine the precise electrophysiological properties of the cells being analyzed. The field of Neuroscience has long been using molecular biology techniques and electrophysiology in order to identify and characterize different cell populations of the CNS. However, these techniques face several limitations, for instance electrode-based recordings can describe well the behavior of either a single or a few neurons (intracellular recordings or patch-clamp), but are not effective when studying large population responses or recordings from neuronal populations with very little detail on the single cell level (extracellular recordings). Similarly, immuno-labeling techniques can be misleading because they can identify neurons that, despite expressing canonical neural markers, do not behave like bona fide neurons in terms of ability to generate action potentials (AP) (Quian Quiroga and Panzeri 2009; Schnell, Dijk et al. 2012). More recently, fluorescent imaging has added a powerful tool to the field allowing the development of a number of fluorescent dyes that enable the detection of changes in many different ions (e.g. Ca^{2+}, K^{+}, Na^{+}, Cl^{-}), neurotransmitters (e.g. serotonin), synaptic vesicles and voltage. Technological innovations, such as ultra-fast cameras, together with the improvement of probes of cell voltage have permitted the development of voltage sensitive dyes (VSDs) making it possible for scientists to visualize the propagation of AP. This has made it feasible to study large populations of neurons and network behavior by observing changes in fluorescence in a way that would be impossible with purely electrical recordings (Baker, Kosmidis et al. 2005).

To explore the possibility of using this method we first tested the ability of VSDs to label cellular membranes and for this purpose we used embryonic multipotent NSC cultures derived from mid-gestation rat embryonic cortex and expanded as monolayers in FGF2. The NSCs were differentiated with a protocol based on treatment with VPA, which generates increased number of cells with neuronal-like morphology and marker expression a vast majority of which, however, were electrically inactive. We first looked at passive membrane properties of cells loaded with JPW3027 and observed they were not significantly different from control cells. Next, coverslips containing embryonic NSC cultures differentiated with VPA (7, 14 and 21 days) were labeled with JPW3027, RH795 and the Ca^{2+} indicator Fluo-4. Quantification of specific versus non-specific cell labeling revealed higher percentage of bright pixels (N_{ROI}/N_{im}) in JPW3027 (90.6±3.3%) comparing to RH795 (65.5±2.9%) and Fluo 4 (42.3±3.8%), showing that extracellular loading of JPW3027 produces specific labeling of cell body and processes. In

addition, to evaluate dye toxicity, we estimate cell viability upon dye loading after 10 days of VPA differentiation. Results from these experiments showed that cell viability was equal to 93.7±1.1% for coverslips loaded with the vehicle, 59.2±8.5% for JPW3027, 43.1±4.7% for RH795 and 38.3±1.8% for Fura-2 (we used Fura-2 instead of Fluo-4 as the latter's excitation/emission wavelengths coincides with the wavelengths of our live/dead assay). Because Ca^{2+} imaging assays fail to efficiently mirror fast AP dynamics we assessed the possibility of using VSD to analyze AP dynamics. Using dynamic clamp, we simulated a fast and a slow current in cells that did display any native I_{Na} (differentiated for 14 or 21 days with VPA, loaded with JPW3027). A 100 pA current step was used to trigger an artificial AP. The half-width (HW) of the fluorescence signal (after fitting a single exponential to the re-polarization trace) and the current clamp recording produced by the artificial AP showed a strong relationship ($r^2 = 0.98$).

In order to improve the signal-to-noise ratio others have averaged several image acquisition sweeps only. Using an EM-CCD camera we were able to separate spikes in the fluorescence signal from the background noise based on single sweeps, applying a very low excitation light while at the same time minimizing cell damage. Together these results confirmed that VSDs are capable to resolve changes in membrane potential with a linear variation in fluorescence in the sub-millisecond range and therefore can be used to distinguish fast versus slow AP kinetics. Further they offer the advantage of being less harmful to the cells as it could be observed particularly in cultures loaded with JPW3027.

In coverslips containing cells differentiated over two and three weeks using combined treatment with BMP4 and Wnt3a almost no visible difference in morphology was observed among the generated neuronal-like cells nor in the detection of the late dendro-somatic neuronal marker MAP2A,B. In coverslips containing NSCs differentiated by co-treatment of BMP4 and Wnt3a for two weeks we found no active neurons after recording from 15 cells using the patch clamp technique. Only 3 out of 20 cells showed some form of immature APs after current injection in cultures differentiated for three weeks. Moreover, whereas morphological characterization could not distinguish from mature and immature neurons obtained through either BMP4 and Wnt3a or VPA treatment, we found that membrane mean resting potential and input resistance of cultured cells expressing MAP2A/B did not resemble the ones of functional neurons. However cells labeled with JPW3027, that produced $\Delta F/F_0$ peaks, also showed APs when patched (12 out of 12 cells, 3-week BMP4 and Wnt3a-treatment culture) while the majority of cells in which no fluorescence peak was observed showed no AP during current-clamp recordings (9 out of 12 cells). Interestingly, even in cultures differentiated for three weeks, all AP-capable cells displayed immature APs that could be suppressed with 1 μM TTX (data not shown). With these results we concluded that JPW3027 could reliably detect electrically active neurons derived from stem cell cultures and distinguish such cells from morphologically indistinguishable non-active cells. We further evaluated the ability of detecting firing cells using bipolar stimulation in primary hippocampal cultures (containing both neurons and glia) labeled with JPW3027. Initially the bipolar stimulation electrodes were placed equidistantly from the neuron while using a 200 W metal-halide lamp to excite the dyes and applying a 1 V, 5 ms stimulus pulse, which produced a mean $\Delta F/F_0$ of 3.6±0.4%, however, displaying considerably higher noise and bleaching compared to LED excitation (data not shown). To analyze the effect of stimulation electrode positioning on the emission of the JPW3027 we used non-neuronal (glial) cells in order to avoid interference of the non-linear electrical stimulation-to-fluorescent emission relationship exhibited by neurons with the experimental aim. The anode was placed at a −2.5 mm position in relation of a glial cell (astrocyte; set at position 0) while the cathode was placed at the opposite direction in

different positions (2.5, 5 and 10 mm). Moving the cathode to the positions 2.5, 5 and 10 mm caused an increase in fluorescence peaks producing mean $\Delta F/F_0$ of 0.5±0.7%, 1.9±0.4% and 3.1±0.7%, respectively.

To simplify the detection of firing-capable cells within stem cell cultures, stimulating electrodes can be positioned around a cell group to produce AP-triggering depolarization of the membrane. For time lapse experiments however, the stimulus has to be weak enough to not cause cell damage but at the same time strong enough to produce an AP in every cell in the area of interest. In a monolayer, the change in potential across the membrane produced by an extracellular stimulus can be well approximated by the finite element method (calculates the electric potential across the electrodes) and the cable equation (calculates the current across the membrane produced by the extracellular variation in electric potential). Lastly we show we are capable of reproducing the effects of a changing cathode position on membrane potential using our finite element model. We were able to optimize the positioning of the stimulation anode and cathode to produce changes in extracellular potential that do not disturb cells away from the field of view. In summary, our method offers several advantages comparing to classical methods and current procedures for application of VSDs, the most striking being better overall cell labeling, simple and less time consuming dye loading, less cell toxicity and better linear relationship between changes in fluorescent and AP.

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