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Reactive Oxygen Species in the Biology of Melanoma

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1. Introduction

The incidence of melanoma has been increasing at an alarming rate worldwide. Although melanoma accounts for only 10% of skin cancer, it is responsible for at least 80% of skin cancer deaths. Malignant melanoma remains one of the cancers most resistant to treatment. Moreover, no effective therapy exists to inhibit the metastatic spread of this type of cancer. Research to further understanding of how melanoma cells differ from normal tissues is essential to make the discovery of potential new ways of attack. Increased reactive oxygen species (ROS) levels have been associated with numerous pathological conditions, including cancer. Particularly, melanoma cells constitutively produce high amounts of ROS as compared with their non-tumoural counterpart, melanocytes (Policastro et al, 2009). In relation to this, one promising strategy relates to the development of new therapies taking advantage of the excess of ROS produced by melanomas. In this chapter, we review: our current understanding of the involvement of ROS in cancer and particularly in the biology of melanocytes and melanoma, therapeutic approaches related to intracellular ROS modulation and emerging gene therapy strategies based on intracellular ROS levels of melanoma.

1.1 Role of reactive oxygen species in cancer

In order to further understand the involvement of ROS in the biology of melanoma, we will briefly introduce state-of–the-art advances regarding ROS and cancer. ROS are largely generated as oxidative by-products of normal cellular metabolism which include highly reactive radicals (e.g. superoxide anion, $O_2^{\bullet-}$ and hydroxil radical •OH) and milder oxidants such as hydrogen peroxide (H_2O_2). ROS were traditionally considered as toxic products leading to cellular damage. However, at physiological concentrations, some of these species are involved in the regulation of cellular processes and their levels are tightly controlled by specific antioxidant scavenging systems (Halliwell & Gutteridge, 2007). Antioxidants can be synthesized *in vivo* or taken from the diet. These antioxidant defences contribute to preserve the redox state of cell in homeostatic balance and they comprise enzymes, like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), peroxiredoxins and non-enzymatic

agents such as low molecular weight antioxidants (Fig. 1). Differences in level and composition of antioxidant defences can be found from tissue to tissue and cell type to cell type within a given tissue depending on which ROS are generated, the manner, the time and which of the targets is affected (Halliwell & Gutteridge, 2007).

An imbalance between ROS generation and their removal may induce an oxidative stress or a prooxidant state, which in turn may result in increased proliferation, adaptation, cell injury, apoptosis, senescence, cytotoxicity and cell death. On the other hand, physiological levels of ROS are necessary for proper functioning of cellular processes such as proliferation, whereas scavenging of H_2O_2 inhibits cell proliferation (Stone & Yang, 2006).

ROS have been reported to be involved in over 150 human disorders, e.g. atherosclerosis and cardiovascular diseases, autoimmune and neurodegenerative disorders and cancer (Halliwell & Gutteridge, 2007). ROS may play a role in tumour development, not only as DNA-damaging agents that increase the mutation rate and promote oncogenic transformation (Halliwell, 2007) but also as mediators of signal transduction pathways related to cell proliferation, angiogenesis and migration (Nishikawa, 2008).

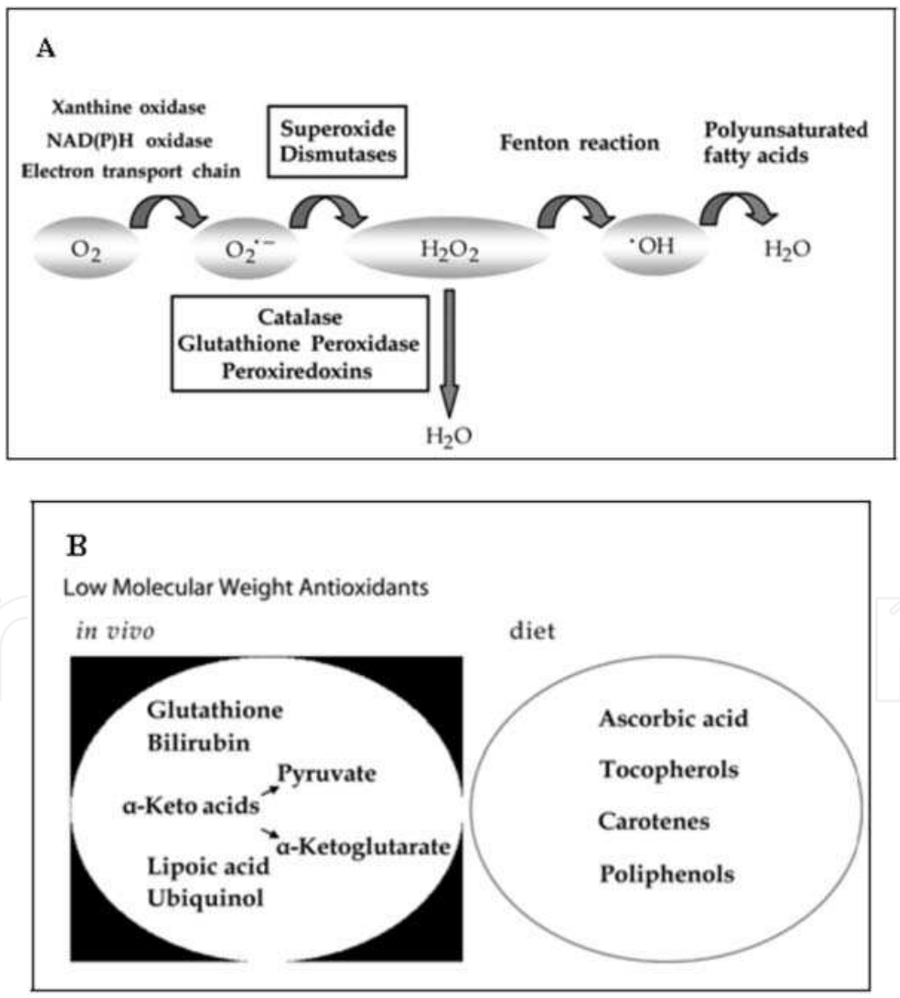

Fig. 1. Antioxidant defence systems. (A) Enzymatic and (B) Non-enzymatic antioxidant defences

One of the major endogenous sources of DNA damage is that produced by ROS (Jackson & Loeb, 2001). It has been estimated that approximately 20,000 bases in DNA are damaged in each human cell per day by ROS (Jackson & Loeb, 2001). Some of the oxidative modified bases constitute blocks to replication, while others are able to misrepair at high frequency, leading to base substitutions (Jackson & Loeb, 2001). Thus, DNA damage induced by ROS in cells exposed to a prooxidant state could result in an overwhelming mutational load that could contribute to carcinogenesis. Indeed, many chemical carcinogens act through free radical metabolites (Guyton & Kensler, 1993), some tumour promoters stimulate the production of free radicals in several cell types and tissues (Cerutti, 1985; Duran & de Rey, 1991), whereas free radical-scavengers protect against cancer development in animal models (Hyoudou et al, 2009) and may be chemoprotective in humans (Cerutti, 1985; Guyton & Kensler, 1993). In addition, $O_2^{\bullet -}$ and H_2O_2 can enhance cell survival and stimulate proliferation. The production of large amounts of H_2O_2 was reported in cancer cells and tissues (breast, colorectal and renal cell carcinoma and melanoma) as compared with its non-tumoural counterpart (Policastro et al, 2004; Policastro et al, 2009; Szatrowski & Nathan, 1991; Toyokuni et al, 1995). This could be attributed in part to deregulation of antioxidant enzymes, which could lead to an increased ROS accumulation. Particularly, an increase in superoxide dismutase and a decrease in catalase activities were found in epithelial tumour cells and in human melanoma cells. In this sense, a correlation between the endogenous levels of H_2O_2 and the degree of malignancy was demonstrated in epithelial tumour cell lines from skin, breast and bladder (Hempel et al, 2009; Policastro el al, 2004). These characteristics further perpetuate a state of oxidative stress in cancer cells. In agreement with this concept, the scavenging of ROS inhibits cell proliferation in tumour cells, as described in cells treated with catalase, glutathione (GSH) or N-acetylcysteine (NAC) (Laurent et al, 2005; Onumah et al, 2009; Policastro et al, 2004).

It is well documented that H_2O_2 is involved in signal transduction pathways (Fig. 2), e.g. increased levels of H_2O_2 induce mitogenic signals, such as those related to epidermal growth factor receptor (EGFR)/Ras/ extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway, and stress-responsive signals, such as those related to Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinase (MAPK) pathways. Moreover, ROS and H_2O_2 were also implied in the modulation of receptor tyrosine kinases (RTK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (Stone & Yang, 2006). These redox-dependent signalling cascades converge on core cell cycle regulators (Burhans & Heintz, 2009). The regulation of cell cycle is highly coordinated by sequential assembly and activation of phase-specific protein kinase complexes (Elledge, 1996; Sherr, 1996), formed by cyclins and cyclin-dependent kinases (CDKs), which are also regulated by the INK4 proteins and the CDK inhibitors (CDKIs). D-type cyclins are expressed throughout the cycle in response to mitogen stimulation (Sherr, 1996). Fluctuations observed in the intracellular redox state during cell cycle progression could link oxidative metabolic processes to cell cycle regulation (Menon et al, 2003; Sarsour et al, 2009). The regulation of cyclin D1 expression was connected to H_2O_2 variations along the cell cycle (Burch & Heintz, 2005). Thus, the removal of endogenous H_2O_2 by overexpression of catalase and glutathione peroxidase induces G0/G1 arrest (Ibanez et al, 2011; Onumah et al, 2009) and decreases cell DNA synthesis (Felty et al, 2005). We demonstrated that the scavenging of H_2O_2 by catalase in tumour cells induced G1/S arrest by modulating the levels of specific regulatory proteins of early to mid G1 (cyclin D1) and G1/S transition ($p27^{KIP1}$). Moreover, we found a modification in the intracellular localization of the inhibitory protein $p27^{KIP1}$. This protein

remained in the nucleus where it inhibits the cyclin E-CDK2 complex after catalase treatment, whereas it showed cytoplasmic localization in proliferating cells. In addition, we demonstrated an increase of $p27^{KIP1}$ in response to H_2O_2 scavenging *in vivo* related to the inhibition of tumour growth after catalase treatment in agreement with *in vitro* results (Ibanez et al, 2011). In view of the aforementioned, the prooxidant state of tumour cells may confer them a proliferative advantage and contribute to the acquisition of a malignant phenotype.

Furthermore, a role of oxidative stress in the regulation of metastasis and tumour progression has been reported. It is becoming clear that a number of steps in the metastatic cascade, such as invasion, intravasation and extravasation, as well as, the angiogenic response are regulated by redox signalling. Particularly, ROS increase the expression and/or activate matrix metalloproteinases (MMPs), adhesion molecules, EGFR, vascular endothelial growth factor (VEGF) and the hypoxia-inducible factor (HIF) transcription factor (Nishikawa, 2008; Toullec et al, 2010). In the process of cancer cells forming metastatic colonies, active MMPs are required to destroy the extracellular matrix and basement membranes for the migration of tumour cells. These cells and their surrounding cells secrete MMPs in latent forms which are activated by ROS (Nishikawa, 2008). The H_2O_2-dependent expression of both MMP-9 and VEGF was demonstrated as removal of H_2O_2 by overexpression of catalase attenuated their expression (Hempel et al, 2009).

In addition, it has been proposed that cancer cells could induce oxidative stress in the tumour microenvironment, resulting in the amplification of oxidative stress in a given tissue area. This would then provide a mutagenic/oxidative field resulting in widespread ROS production and DNA damage. This bystander oxidative stress could favour an increase in the mutation rate of adjacent normal epithelial cells or cancer cells, resulting in more aggressive cancer cells (Martinez-Outschoorn et al, 2010).

2. Reactive oxygen species in melanocytes and melanoma

2.1 ROS in the physiology of melanocytes. Involvement of reactive oxygen species in melanogenesis

The skin represents one of the largest organs of the human body and is a constant target of endogenous and exogenous ROS, counteracted by a powerful defence system, particularly, the biopolymer melanin functions as an intrinsic free radical trap. Also, the UV properties of melanin have a preventive action to protect epidermal proliferating cells (Schallreuter & Wood, 2001)

The pigmentation process takes place in melanocytes, which produce melanin through the process called melanogenesis. The specific organelle involved in this process is the melanosome, which has the full capacity for the biosynthesis and distribution of both eumelanin and pheomelanin.

Tyrosinase, the key enzyme in melanogenesis, contains two copper atoms in its active site, which are primarily coordinated to histidine residues. In its Cu(II) oxidation state, the enzyme is inactive representing met-tyrosinase. Consequently met-tyrosinase has to be activated by the reduction of the two Cu(II) centres to Cu(I) by single electron donors, such as L-DOPA, ascorbic acid or superoxide anion (Schallreuter et al, 2008). Tyrosinase catalyses the hydroxylation of L-tyrosine to L-DOPA and the oxidation of this to dopaquinone. These quinones evolve through a series of steps, both enzymatically and non-enzymatically, to generate several unstable intermediates, which polymerize to melanins (Munoz-Munoz et

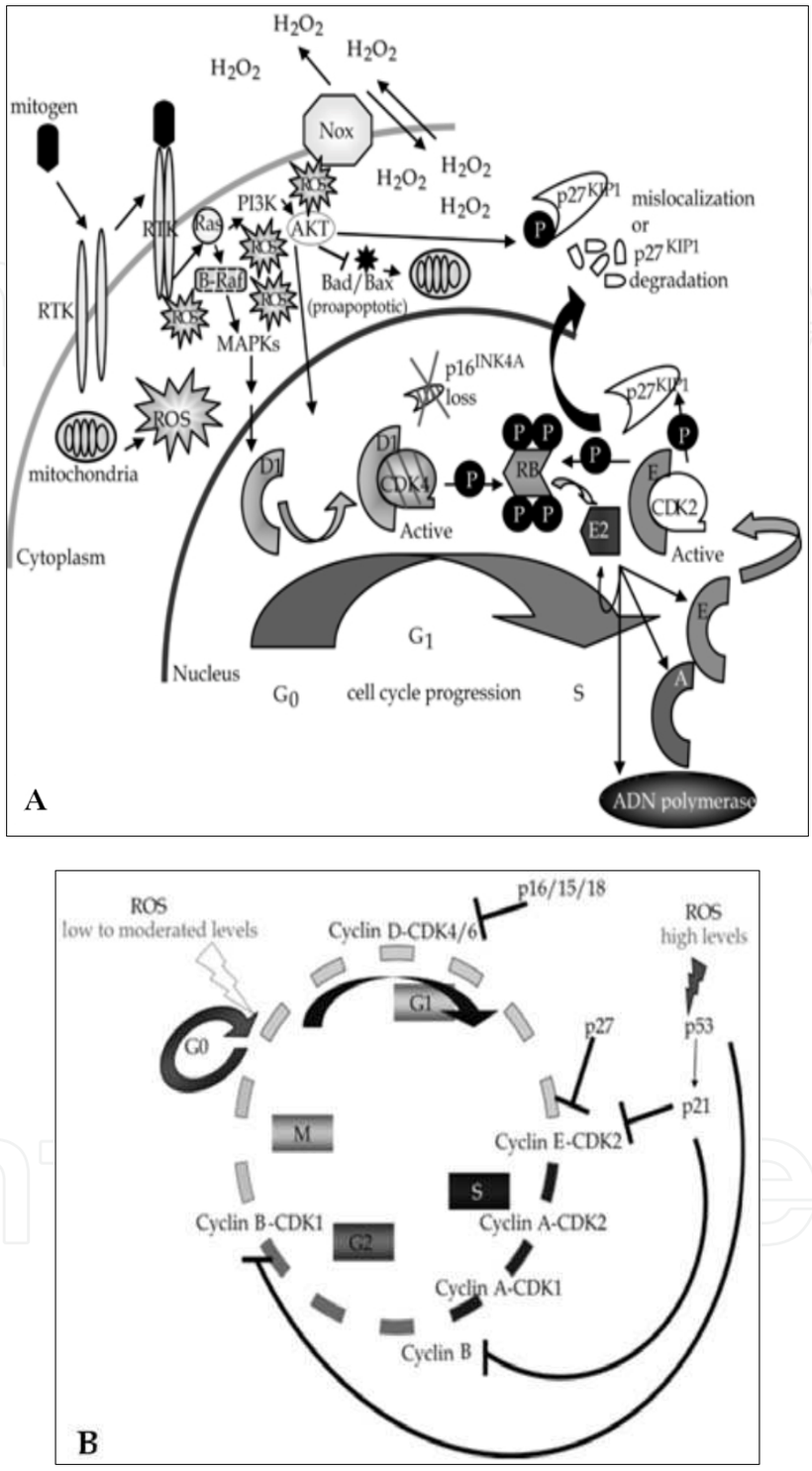

Fig. 2. ROS involvement in cell cycle regulation. (A) Redox-dependent signalling cascades involved in the regulation of G1/S transition in cancer and, in particular, in melanoma. (B) Low to moderate levels of ROS may initiate cell cycle division whereas high levels of ROS may induce cell cycle arrest, apoptosis, terminal differentiation or cytotoxicity

al, 2009). Moreover, L-DOPA is mainly formed from L-tyrosine by tyrosine hydroxylase isoform I (THI). Thus, the availability of L-tyrosine is essential for melanocytes. This aminoacid is provided by facilitated diffusion and by the intracellular enzymatic conversion of the essential aminoacid L-phenylalanine via phenylalanine hydroxylase (PAH) (Schallreuter et al, 2008). The activities of PAH, THI and tyrosinase are controlled by the cofactor 6-tetrahydrobiopterin (6BH4). High levels of ROS, such as O_2•- and H_2O_2, can directly inhibit tyrosinase activity, by leading to structural changes of the enzyme due to the presence of cysteine residues susceptible to be oxidized or by deactivation of the enzyme by the oxidation of a methionine residue to methionine sulfoxide at the active site. Moreover, the oxidation of the cofactor 6BH4 to 6 biopterin may be generated by both H_2O_2 and also by UV-B exposure (Schallreuter et al, 2008; Schallreuter & Wood, 2001). However, low concentrations of H_2O_2, in the micromolar range, are needed to upregulate or activate tyrosinase and other proteins related to melanogenesis. In addition, both tyrosine-related proteins, TRP1 and TRP2, which are located close to tyrosinase in the melanosomal membrane, protect this enzyme from ROS attack. These proteins also contain cysteine rich domains, which are targets of H_2O_2 mediated oxidation. Thus, the levels of ROS and particularly of H_2O_2 are tightly controlled under physiological conditions, maintaining redox homeostasis in the melanosome and regulating the melanogenesis process. Particularly, catalase is an important antioxidant enzyme that regulates this process by H_2O_2 removal.

Regarding the alterations of melanogenesis in melanoma, the loss of pigmentation is very common in advanced and in metastatic lesions because of the dysfunction of melanocyte-specific proteins involved in melanogenesis (Watabe et al, 2004). The main regulator of melanogenesis at the transcriptional level is microphtalmia-associated transcription factor (MiTF). This factor controls the expression of all known melanosomal proteins (Levy & Fisher, 2011). However, post-transcriptional processes may also be involved in the pigmentation phenotype of melanoma, particularly the activation of tyrosinase as an important control point for melanin biosynthesis. In this sense, several events have been suggested to be involved in the hypopigmentation of amelanotic melanoma, such as disruption of melanosomal maturation and alterations on the glycosylation and stability of tyrosinase. Moreover, considering the ability of H_2O_2 to inhibit tyrosinase and other proteins involved in the melanogenesis process (Jimenez-Cervantes et al, 2001), the high levels of H_2O_2 associated to a decrease in catalase activity (Picardo et al, 1996; Policastro et al, 2009; Szatrowski & Nathan, 1991) would be relevant in the induction of the amelanotic phenotype. In addition, the down-regulation of melanocyte differentiation markers was preceded by a decrease in microphthalmia transcription factor gene (MITF) expression in H_2O_2-treated melanoma cells. Therefore, it was suggested that oxidative stress may lead to hypopigmentation by mechanisms that include a MiTF-dependent downregulation of the melanogenic enzymes (Jimenez-Cervantes et al, 2001).

2.2 Involvement of ROS in the development of melanoma

Many of the general risk factors for developing melanoma include sun exposure, previous melanoma or non-melanoma skin cancer, family history of melanoma and large numbers of moles (Satyamoorthy & Herlyn, 2002; Wang et al, 2010). The human melanocyte is continuously exposed to reactive biochemical species, but is finely tuned via the intrinsic antioxidant and radical properties of melanin to suppress the build-up of an altered redox phenotype. Meyskens *et al.* propose that this control is lost during the development of

melanoma (Meyskens et al, 2001), moreover they hypothesize that the disruption of melanosomal melanin might be an early event in the etiology of melanoma, leading to increased oxidative stress and mutation (Gidanian et al, 2008).

Sunlight exposure is the major cause of skin cancers, including melanoma. Epidemiological studies implicate ultraviolet radiation (UV) B (290-320 nm) and UVA (320-400 nm) in the development of nonmelanoma skin cancer and melanoma respectively. Although the action of UVB radiation is mediated by specific photoproducts, such as pyrimidine dimers, it is well known that UVA acts mainly through the induction of ROS. The most important source of H_2O_2 is represented by the free radicals generated after UV exposition, though H_2O_2, as a product of melanogenesis itself, also plays an important role since it is able to diffuse outside the melanosome to reach other cellular compartments. Moreover, structural alterations of melanosomal membrane in pigmented melanomas may lead to significant leakage of reactive melanin precursors including free radical species (Borovansky et al, 1991).

As described in the introduction tumour cells produce high amounts of ROS. Regarding melanoma, we demonstrated an increase in the levels of H_2O_2 in human melanoma cells as compared to melanocytes (Policastro, 2009). Figure 3 shows the differences in ROS levels between melanocytes and melanoma. Moreover, an imbalance in the antioxidant system has been described in human melanomas, which can lead to endogenous generation of ROS and to cellular incapability of coping with exogenous peroxidative attacks. In addition, it has been proposed that the functional effect of melanin dysregulation is its evolution to a prooxidant behaviour (Gidanian et al, 2008) and melanin is able to induce DNA damage. Thus, the oxidative stress characteristic of melanoma cells is associated with increased DNA damage and high rates of mutation. In relation to this, we demonstrated high levels of basal DNA damage in melanoma cells (Ibanez et al, 2009) in agreement with other authors (Warters et al, 2005).

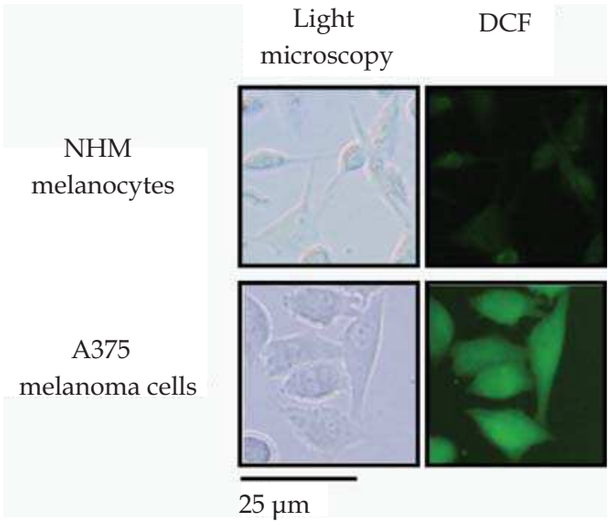

Fig. 3. ROS levels on normal human melanocytes (NHM) and A375 melanoma cells. Representative images of NHM and A375 cells, showing the intracellular ROS levels determined by 2′, 7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) assay, as described by Tarpey *et al*., 2004. DCF: oxidized 2′, 7′-dichloro-fluorescein

2.3 Redox regulation in melanocytes and melanoma

Redox regulation of protein activity is an important mechanism involved in the modulation of signalling and transcription, as part of the large array of posttranslational modifications, including, acetylation, carboxylation, glycosylation, hydroxylation or methylation. Different mechanisms for sensing have been observed in the various redox-regulated proteins. While in many of them, reactive cysteine(s) residues function as redox sensors, others use the metal iron, coordinated in Fe–S clusters as a sensor and, lastly, other use their thiol-coordinated zinc sites as redox switches stimuli (Shlomai, 2010).

2.3.1 Redox regulation of transcription factors

Several master transcription regulators, which control cell growth, development and survival have redox control, many of which are involved in the regulation of signalling transduction pathways in melanocytes and melanoma.

2.3.1.1 Hypoxia inducible factors (HIF)

HIF play a key role in the maintenance of oxygen homeostasis. They are involved in facilitating cell survival and energy supply in reduced oxygen condition. HIF activation affects principally the shift of energy production by increasing glycolysis and decreasing mitochondrial function under hypoxic condition (Denko, 2008). HIF-1, the most studied HIF factor, was initially identified by its response to low oxygen concentration, but it is now apparent that HIF-1 is also crucial in cancer development and progression including angiogenesis and tumour invasion (Harris, 2002). HIF-1 is a heterodimeric complex comprised of an unstable and oxygen regulated subunit (HIF-1α) and a constitutively expressed subunit (HIF-1β), also referred to as the arylhydrocarbon receptor nuclear translocator (ARNT). HIF-1 is constitutively transcribed and translated, however under normoxia it is rapidly degraded by the ubiquitin-proteasome system. Under sustained hypoxia, degradation of the HIF-subunit is inhibited allowing the protein to accumulate, heterodimerize and translocate to the nucleus. Thus, in hypoxia conditions, the functional HIF-1 dimer, binds to a consensus core sequence namely hypoxia response elements (HREs) in the promoter or enhancer of its target genes (Harris, 2002).

A large body of evidence suggests that ROS modulate HIF-1 activity. H_2O_2 and nitric oxide (NO) donors were found to stabilize HIF-1, and genetic and pharmacological interventions modulating ROS generation have been shown to affect the accumulation of HIF-1α (Brune & Zhou, 2007; Kietzmann & Gorlach, 2005). It is well demonstrated that during hypoxia, ROS production is increased at complex III levels in mitochondria, and this condition is necessary for HIF-1α stabilization during hypoxia (Chandel et al, 1998).

A direct role of hypoxia in advanced melanoma is slowly starting to emerge (Bedogni & Powell, 2009). HIF-1 has been shown to play a critical role in uveal melanoma progression and is regarded as one of the critical biomarkers to predict uveal melanoma metastasis (Chang et al, 2008; Victor et al, 2006). The overexpression of HIF-1α and HIF-2α in human melanoma of the skin was reported, and HIF-2α, along with VEGF and vascular density, was associated with poor overall disease survival (Giatromanolaki et al, 2003). Melanoma cells have also shown a marked HIF-1α activity under normoxic conditions. Immunohistochemistry of malignant melanoma showed the focal expression of HIF-1α in cancer tissue independent of regional hypoxia. In this work, it has been demonstrated that constitutive HIF-1α activity under normoxic conditions is associated with the ROS microenvironment around melanoma cells. The inhibition or induction of ROS decreased or

activated, respectively, HIF-1 activity and HIF-1alpha protein expression. In addition, the involvement of nuclear factor kappa B (NF-κB) was demonstrated in the regulation of HIF expression by ROS in melanoma cells (Kuphal et al, 2010).

2.3.1.2 Nuclear factor kappa B (NF-κB)

NF-κB is a conserved inducible transcription factor that plays a key role in the control of a diverse range of genes involved in inmunological response, cell proliferation, apoptosis, tissue remodelling, cellular responses to stress and oncogenesis (Ghosh & Hayden, 2008). In mammalian cells, the NF-κB family comprises five related proteins: p50, p52, RelA (also known as p65), c-Rel, and RelB which can homo- and heterodimerize through the rel homology domain (RHD). Only RelA, c-Rel, and RelB contain a transcriptional activation domain, while p50 and p52 can only activate transcription through heterodimerization with RelA, c-Rel, or RelB. NF-κB dimers are generally found inactive in the cytoplasm bound to inhibitory proteins IκB (IκB-α and IκB-β). In response to a wide array of stimuli, IκB proteins are phosphorylated and NF-κB dimeric complex is released and its nuclear localization signal is unmasked. NF-κB translocates to the nucleus, where it can 'turn on' the expression of specific genes that have DNA-binding sites for NF-κB (Perkins, 2007). ROS have long been known to activate NF-κB; however, the role of redox in NF-κB regulation is apparently different in cytoplasm and in the nucleus (Kabe et al, 2005). Whereas in the cytoplasm ROS have been described in multiple reports to enhance signal transduction pathways, leading to NF-κB activation and translocation into the nucleus, it was shown to significantly inhibit the capacity of NF-κB to bind the DNA in the nucleus, a capacity that is consequently restored through the action of reducing enzymes (Kabe et al, 2005).

In melanoma, NF-κB is constitutively active unlike to normal melanocytes and is increased under conditions of oxidative stress (Meyskens et al, 1999). In this study, the basal DNA-binding activity of NF-κB in metastatic melanoma cells was found to increase 4-fold as compared with normal melanocytes. This level of binding was paralleled by a 1.5- to 4-fold increase in the expression of p50, p65 (RelA), and IκB-α. In contrast, the expression of c-Rel was markedly decreased (60%) in melanoma cells as compared with normal melanocytes. Following oxidative stress produced by enzyme-generated H_2O_2, free H_2O_2, or incubation with buthionine sulfoximine, NF-κB binding activity increased 1.5- to 2.5-fold in melanoma cells, but only slightly in normal melanocytes (Meyskens et al, 1999). Shattuck-Brandt and Richmond reported an endogenous activation of NF-κB in melanoma due to posttranslational modification of the inhibitory element IκB, as a result of oxidative processes, that enhances its degradation (Shattuck-Brandt & Richmond, 1997). In addition, melanoma cell lines that exhibit high or low activity of the membrane-bound gamma-glutamyl transpeptidase (GGT), a basal source of H_2O_2 in the extracellular space, were associated either with higher or lower levels of NF-κB respectively (Maellaro et al, 2000). Moreover, stimulation or inhibition of GGT activity in higher GGT activity cells resulted in progressive activation or inactivation of NF-κB, respectively. A role of NAD(P)H oxidase and NAD(P)H:quinone oxidoreductase in oxidant signalling of melanoma cell growth has also been demonstrated by activating the redox-regulated NF-κB transcription factor (Brar et al, 2003).

2.3.1.3 Activating protein 1 (AP-1)

AP-1 is a dimeric transcription factor comprising proteins from several families whose common denominator is the possession of basic leucine zipper (bZIP) domains that are

essential for dimerization and DNA binding. The Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) subfamilies are the major AP-1 proteins. Activating transcription factor (ATF) proteins ATF2, LRF1/ATF3, B-ATF, JDP1 and JDP2 are also components of AP-1. The last group of AP-1 proteins, and the least studied, is the Maf subfamily, which includes c-Maf, MafB, MafA, MafG/F/K and Nrl (Eychene et al, 2008). Different combinations of these proteins in the AP-1 complex bind to their specific DNA consensus sequences, determining which genes are regulated (Chinenov & Kerppola, 2001). AP-1 family is involved in the regulation of central cellular processes, including cell proliferation, survival, growth, differentiation, apoptosis, migration, and transformation. AP-1 proteins can function as anti-oncogenic factors, by inducing apoptosis, or as oncogenic factors, by signalling cell survival (Eferl & Wagner, 2003; Hess et al, 2004; Shaulian & Karin, 2002; Verde et al, 2007). Multiple different stimuli induce AP-1, including cytokine, growth factors, neurotransmitters, hormones, bacterial and viral infections, stress signals, and oncogenic stimuli. Its activity can be regulated at the levels of transcription of the genes encoding AP-1 subunits, mRNA stability and translation, protein turnover, and posttranslational modification (Vesely et al, 2009). AP-1 transcriptional activity is controlled by ROS and reactive nitrogen species (RNS) at several levels of its regulation. This includes the regulation of expression of the genes encoding AP-1 subunits, the interactions of the AP-1 proteins with their DNA-binding sites, their interactions with co-activators, as well as at the level of chromatin remodelling, through the modulation of histone acetyltransferase and histone deacetylase (Shlomai, 2010).

Abnormal redox regulation of AP-1 has been reported in melanoma (Yang & Meyskens, 2005). Treatment of human metastatic melanoma cells with resveratrol, a potent ROS scavenger, produces some morphological changes, such as reduced anchorage-independent growth and decreased AP-1 binding and transcriptional activities. These changes in AP-1 activities were related to different AP-1 composition, where c-Jun/JunD/Fra1 turned to JunD/Fra1/Fra2, with increased levels of JunD, Fra1, and Fra2 expression in the nucleus. Furthermore, the overexpression of Fra2 in human melanoma cells reduced significantly both AP-1 transcriptional activity and 12-*O*-tetradecanoylphorbol-induced transcriptional transactivation. Addition of H_2O_2 partially reversed the inhibition of colony proliferation. Although H_2O_2 restored participation of c-Jun in AP-1 complexes, H_2O_2 addition did not affect the induction of Fra1 and Fra2 by resveratrol nor the morphological changes. The authors hypothesized that alterations in AP-1 composition and reduction of intracellular oxidative status by resveratrol might contribute to the recruitment of normal AP-1 transcription signalling in melanoma and subsequently induce cellular biological features consistent with a more normal phenotype.

2.3.1.4 Forkhead box O (FoxO) family of transcription factors

FoxO transcription factors regulate development, proliferation, survival and longevity. Members of the mammalian FoxO family, which include FoxO1, FoxO3, FoxO4 and FoxO6, are relatively expressed in an ubiquitous way. However, FoxO proteins are not equally expressed in all tissues, thus individual FoxO proteins may have specificity in regards to cellular function (Maiese et al, 2009). Moreover, target gene regulation appears to be controlled in a cell-type-specific manner due to association of FoxO isoforms with specific cofactors. Many of the cellular processes modulated by FoxO are themselves deregulated in tumorigenesis, and deletion of FOXO genes has demonstrated that these transcription factors function as tumour suppressors (van der Vos & Coffer, 2011). FOXOs were originally

identified as downstream components of insulin/insulin-like growth factor signalling through phosphoinositide 3-kinase (PI3K) and AKT (Brunet et al, 1999; Kops et al, 1999). In mice, FoxOs act as functionally redundant tumour suppressors (Paik et al, 2009), and in cell systems, FoxOs can either mediate apoptosis or quiescence in response to growth factor deprivation (Medema et al, 2000). The DNA binding domain (DBD) is highly conserved between FoxO proteins. FoxOs can function both as transcriptional activators and repressors, depending on the associated cofactors that they recruit upon DNA binding. Growth factors, cytokines, and hormones negatively regulate FoxO transcriptional activity through inhibitory phosphorylation predominantly mediated by AKT.
Regulation of FoxOs by ROS occurs through numerous posttranslational modifications (van der Horst & Burgering, 2007). Besides, FoxO activation increases resistance to oxidative stress through transcription of antioxidant enzymes as MnSOD and catalase as a result of a negative feedback loop (Kops et al, 2002).
It has been described that FoxO4 is involved in oncogene-induced senescence (OIS) through redox mechanisms. Oncogenic BRAF signalling through mitogen-activated protein kinase/extracellular signal-regulated kinase kinase results in increased ROS levels and JNK-mediated activation of FoxO4 via its phosphorylation, which induces $p21^{CIP1}$-mediated cell senescence (de Keizer et al, 2010). In this sense, the ectopic introduction of FoxO4 in endogenous oncogenic BRAF-expressing melanoma cells induces a growth arrest through cellular senescence (de Keizer et al, 2010). OIS represents a barrier for tumour formation, and consequently, the melanoma cells have to bypass this barrier, suggesting that FoxO inactivation is one of the requirements for senescence bypass and melanoma growth (de Keizer et al, 2010).

2.3.1.5 p53 tumour suppressor protein

The tumour suppresor protein p53 (encoded by TP53 gene) is an important transcription factor that is induced in response to many forms of cellular stress. This protein has been named ''the guardian of the genome'' because it plays a central role in coordinating the cellular responses to a broad range and levels of cellular stress factors, leading to apoptosis, cell cycle arrest, senescence, DNA repair, changes in cell metabolism, or autophagy (Green & Kroemer, 2009; Helton & Chen, 2007). There are hundreds of p53 DNA-specific binding sequences located close to promoters of target genes in the human genome.
The role of redox signalling in the regulation of p53 has been well demonstrated. The p53 DNA-binding activity depends on redox regulation by the oxidation of several reactive cysteine residues located within its DNA binding domain (Hainaut & Mann, 2001). ROS can also regulate the function of p53 through indirect oxidative modifications of its interacting partners such as Mdm2 (Shlomai, 2010). Moreover, it was demonstrated that p53 regulates energy metabolism and its resulting ROS generation by modulating the transcription of p53 target genes that control mitochondrial respiration, glycolysis, and the pentose phosphate shunt (Bensaad & Vousden, 2007).
Approximately half of human cancers were found to have inactivating mutations of TP53. Although most melanomas have wild type TP53, loss of function of p53 is rather common. This protein is kept inactive and at low levels predominantly bound to Mdm2, targeting it for proteasomal degradation (Box & Terzian, 2008).

2.3.1.6 Microphthalmia-associated transcription factor (MiTF)

MITF is the master control gene for melanocyte lineage survival. The mutation of MITF gene causes a microphthalmia phenotype in mice, deafness and lack of pigmentation

(Steingrimsson et al, 2004). In humans, mutations of MITF gene cause Waardenburg syndrome type IIA in which patients exhibit deafness and pigment disturbances because of a lack of melanocytes (Tassabehji et al, 1994). The role of MiTF in differentiation and development is well established, but evidence of its role in tumorigenesis remains contradictory. In human melanocytes transfected with mutant BRAF, overexpression of MiTF leads to transformation (Garraway et al, 2005). As the MITF gene is amplified in some melanomas, it has been suggested that MITF can function as a melanoma oncogene (Levy et al, 2006). In another report, however, MiTF was downregulated in Braf-transformed murine melanocytes and BRAF-expressing human melanocytes; reexpression of MiTF in these cells inhibited cell proliferation (Wellbrock & Marais, 2005). These contradictions have been elegantly reconciled in a recent review that has pointed out that an optimal amount of MiTF is required for maintaining proliferation and differentiation, whereas MiTF levels that are too low or too high may cause cell-cycle arrest and apoptosis (Gray-Schopfer et al, 2007).
It has been demonstrated that MiTF regulates cellular response to ROS by upregulating APE-1 at the basal level as well as enhancing its stimulation by ROS (Liu et al, 2009). APE-1 has two essential well characterized functions: a DNA endonuclease activity, which has a key role in the base excision DNA repair pathway and a role as redox sensor that enhances the DNA binding activities of a large number of transcription factors, including p53, HIF-1α, AP-1, and NF-κB upon an increase of intracellular ROS. Depletion of APE-1 by siRNA leads to apoptosis in human cells due to accumulation of damaged DNA, especially when ROS increase (Fung & Demple, 2005).

2.3.2 Redox regulation in the melanoma signalling network

Melanocyte and melanoblast proliferation, survival, differentiation and migration are all critical elements of normal developmental physiology. The signalling circuitry that regulates these events is often indelibly altered at the genetic level by the potential melanoma cell (Fig. 4).
At least four major signalling abnormalities have been described in melanoma. They include the Ras signalling network, both through RAF/ERK MAPK and PI3K/AKT cascades; the $p16^{INK4A}$/CDK4 network; the Bcl-2/p53 network and the melanocyte developmental pathways that involve receptor tyrosine kinase (KIT), microphtalmia transcription factor (MITF) and β-Catenin. The abnormalities in these signalling cascades are involved in the different steps of promotion and progression of melanoma, from the radial growth, the resistance to apoptosis and radio- and chemotherapy up to the vertical growth and the invasion and metastasis processes (Hocker et al, 2008).

2.3.2.1 The Ras signalling network

The Ras signalling network regulates cell growth, cell proliferation, survival and invasion through two distinct cascades, the Ras/MAPK and the Ras/PI3K signalling streams.
One of the first genes shown to be specifically mutated in melanoma was NRAS, a gene encoding a member of the Ras family of small GTP-binding proteins (Berger & Garraway, 2009). These proteins lie at the top of the Ras/Raf/MEK/ERK MAPK pathway. This activates a large number of growth-promoting genes in response to growth factors and cytokines. NRAS is mutated in 15-30% of melanomas (Demunter et al, 2001; van Elsas et al, 1996). These mutations impair GTPase catalytic activity of Ras, resulting in a constitutively GTP-bound and active state. It has been suggested that the spatial distribution of NRAS-mutated tumours on the skin and their proclivity for dipyrimidine mutations have a possible correlation with UV exposure (Berger & Garraway, 2009).

As noted above, Ras works in the MAPK pathway (Fig. 2A and 4). This pathway is hyperactivated in up to 90% of human melanomas (Cohen et al, 2002). This emphasizes the contribution of NRAS mutations to the development of melanoma but also indicates the involvement of other genes in the pathway. The BRAF gene was observed to be mutated in 80% of short-term melanoma cell cultures and 66% of uncultured melanomas thereby making BRAF the single most commonly mutated gene in melanoma (Hocker et al, 2008). The product of BRAF gene is a serine-threonine kinase that induces MEK to phosphorylate ERK, which enhances cell growth and proliferation (Berger & Garraway, 2009). Notably, up to 90% of the BRAF mutations in melanoma involved a single substitution of valine to glutamic acid in the kinase domain (V600E) (Berger & Garraway, 2009; Davies et al, 2002). The oncogenic potential lies in the ability of the V600E mutant to lead to a constitutively active BRAF product (Berger & Garraway, 2009; Davies et al, 2002; Shinozaki et al, 2004; Wan et al, 2004). Unlike the mutation patterns observed in NRAS, the T>A transversion at the 1799 position associated with the V600E mutation is not suggestive of UV-induced DNA damage, although there is an apparent association between this mutation and intermittent exposure to sun (Maldonado et al, 2003). Thus, mutations in both NRAS and BRAF occur in a mutually exclusive manner suggesting some degree of functional redundancy between these genetic events (Hocker et al, 2008).

Once activated, Ras induces the membrane translocation and activation of PI3K, which leads to activation of a prominent downstream oncogenic effector, AKT (Fig. 2A and 4). Downstream effectors of AKT promote proliferation, survival and invasion (Hocker et al, 2008). Downstream components of the PI3K pathway have been shown to be altered in up to 50-60% of melanomas (Stahl et al, 2004; Zhou et al, 2000) The expression of PI3K and AKT have shown to gradually increase during the progression from benign nevi to early melanoma, and finally, to metastasic disease (Hocker et al, 2008; Stahl et al, 2004). Interestingly, the AKT3 isoform is highly expressed in neural crest-derived cells such as melanocytes, and AKT3 has been found undergo chromosomal copy gains and/or overexpression in up to 60% of melanomas (Stahl et al, 2004). Moreover, activating point mutations in AKT3 have also been described in melanoma (Davies et al, 2008).

A key downstream component of the PI3K pathway, PTEN (also named PTEN/MMAC1), is frequently altered in melanoma and is thought to be one of the main mediators of PI3K pathway activation and melanoma tumorigenesis (Fig. 4). Deletions or loss-of-function mutations in PTEN were observed in 40% of melanoma cell lines (Guldberg et al, 1997; Wu et al, 2003). Inactivating mutations in PTEN and activating mutations in NRAS were proved to exist in reciprocal fashion. This suggests that the loss of PTEN and the activation of NRAS product may functionally overlap (Hocker et al, 2008). The protein PTEN is involved in the termination of PI3K signalling, upregulation of cell cycle arrest and proapoptotic proteins, and downregulation of antiapoptotic proteins of the Bcl-2 family. The expression of PTEN is lost or decreased in 15-50% of melanomas even in the absence of demonstrable mutations (Zhou et al, 2000). Remarkably, ROS participate as second messengers both in RAF/MAPK and PI3K/AKT cascades stimulating cell proliferation (Fig. 2A and 4). ROS often go along with aberrant Ras signalling, either caused by oncogenic forms of the protein or by strong upstream growth factor signalling through the Ras/Raf/MAPK pathway (Finkel, 2006; Kopnin et al, 2007). Moreover, Ras can be activated by UV radiation, ROS, metals and mitogenic stimuli (Valko et al, 2006). It has been reported that the serine/threonine kinases of the MAPK family can be regulated by oxidants. In this sense, $O_2^{\bullet -}$ and H_2O_2 can activate the MAPK cascade at the level of MEK and ERK1/2 (Valko et al, 2006). Disregulation of

MAPK function was found in human skin cancers (Valko et al, 2006) and this pathway is invariably activated in early-, intermediate-, and last-stage melanomas (Verhaegen et al, 2006). Moreover interplay among the MAPK pathway, ROS and antiapoptotic factors in the control of melanoma viability was demonstrated. Verhaegen *et al* reported a tumour cell-selective role of the MAPK pathway upstream of the mitochondria, controlling ROS production. This function was critical to prevent the activation of proapoptotic functions of p53 in melanoma cells, but it was dispensable for normal melanocytes (Verhaegen et al, 2006).
In addition to PTEN and BRAF mutations, proto-oncogene pathway activation can occur in melanoma as a result of ROS potentiating AKT signalling by inhibition of PTEN phosphatase activity (Fruehauf & Trapp, 2008). Moreover, as described in 2.3.1.2 section, the NF-κB transcription factor is constitutively active in melanoma (Meyskens et al, 1999). Furthermore, it is well known that this transcription factor is usually activated under prooxidant conditions (Stone & Yang, 2006). Govindarajan *et al.* found that $O_2^{\bullet -}$ can be induced by AKT in melanoma, and AKT can prevent cells from superoxide mediated cell death. Thus AKT may serve as a molecular switch that enhances aggressiveness through tumour angiogenesis and the generation of $O_2^{\bullet -}$. The payoff for the tumour is generating ROS-NF-κB signalling, which allows increased angiogenesis and resistance to chemotherapy (Govindarajan et al, 2007).

2.3.2.2 The $p16^{INK4A}$/CDK4/Cyclin D1 network

Although NRAS and BRAF play a key role in melanocytes proliferation, activating mutations in those genes are alone insufficient to lead to malignant transformation. Consequently the requirement for cooperation of other pathways is highlighted. In order for malignant transformation to occur, the mechanisms that mediate senescence must be overridden. Oncogene-induced senescence is thought to involve activation of tumour suppressors such as CDKN2A, RB and TP53 (Mooi & Peeper, 2006). Increased susceptibility to melanoma was observed in patients with germline mutations in these genes. The CDKN2A gene encodes two proteins: $p16^{INK4A}$ and $p14^{ARF}$, and both of them are involved in melanoma growth and survival (Sharpless et al, 2003). The $p16^{INK4A}$ protein inhibits the phosphorylation of the retinoblastoma protein by CDK4/6. On the other hand, $p14^{ARF}$ inhibits the p53 antagonist Mdm2 and it is suggested that $p14^{ARF}$ also possessed tumour-suppressive effects that are independent of p53 (Ha et al, 2007). Thus CDKN2A appears to play a major role in preventing cancer formation by mediating a senescence-like state upon oncogenic stress (Hocker et al, 2008; Serrano et al, 1997) Melanoma cell lines often exhibited loss of both CDKN2A copies (Fig. 4). Those that retain at least one copy of CDKN2A frequently carried nonsense, missense or frameshift mutations of the remaining allele (Hocker et al, 2008). This loss of CDKN2A is required in addition to NRAS mutations in order to progress to frank melanoma (Hocker et al, 2008).
Moreover, both somatic and germline mutations in CDK4 have been detected in melanoma (Hocker et al, 2008). The mutations in CDK4 fall in the $p16^{INK4A}$ binding region (Lys22 or Arg24) and prevent $p16^{INK4A}$ from binding to and inhibiting CDK4 protein (Hocker et al, 2008). Thus, these alterations in CDK4 mimic $p16^{INK4A}$ loss.
Cyclin D (encoded by CCND1) is another fundamental component of the CDK4/6 complex. Oncogenic Ras positively impacts the cell cycle by upregulating cyclin D1 through MAPK signalling, drawing another link between Ras network and $p16^{INK4A}$/CDK4 network (Fig. 4). A less common melanoma subtype, acral lentiginous melanoma, frequently exhibits amplification of the chromosomal region that encompasses the CCND1 locus (Bastian et al,

2000; Sauter et al, 2002). Additionally, CCDN1 amplifications were noted to occur more often on melanomas that take place on skin with chronic sun-induced damage (Curtin et al, 2005). The tumour-suppressor $p16^{INK4A}$ was recently suggested as an endogenous regulator of carcinogenic intracellular oxidative stress and the increased susceptibility of melanocytes to elevated ROS in the context of the characteristic oncogenic $p16^{INK4A}$ depletion was reported (Jenkins et al, 2010). Considering the importance of cell cycle regulation tightly bound to Ras/MAPK and PI3K/AKT signalling cascades and to the $p16^{INK4A}$/CDK4 network, we studied the effects of ROS modulation on cell cycle and we demonstrated that the high levels of intracellular ROS of melanoma cells are associated with increased levels of cyclin D1 and mislocalization of the negative regulatory protein of Cyclin/CDKs complexes, $p27^{KIP1}$ (unpublished results), favouring the increased proliferation rate of melanoma cells (Fig. 2A). Notably, cyclin D1 expression is directly controlled by Ras/MAPK pathway and ROS (Burhans & Heintz, 2009).

2.3.2.3 The Bcl-2/p53 apoptotic pathways

It has been shown that alterations within apoptotic pathways, as well as aberrant modulation by upstream signalling networks, such as MAPK and PI3K, contribute to the inherent chemoresistance observed in melanoma (Helmbach et al, 2001; Hocker et al, 2008).
The protein p53 functions to initiate DNA repair and/or apoptosis when exposed to cellular stresses, such as UV radiation or high levels of ROS (Fig. 2B). The overall mutational rate in melanoma of this gene is near to 13% (Hocker & Tsao, 2007). As described in Section 2.3.1.5, direct mutation of TP53 is rare in sporadic melanoma, whereas p53 is frequently inactivated (Sekulic et al, 2008). Induction and activation of p53 play an essential role in halting cellular growth and repairing damaged DNA under conditions of substantial cellular stress. An important mediator of the cell cycle-inhibitory function of p53 is the cell cycle inhibitor $p21^{CIP1}$, which is induced by p53 and inhibits the cyclin E-CDK2 complex. This leads to decreased phosphorylation of Rb protein with the consequent cell cycle arrest at the G1/S transition point. Thus, melanoma cells can increase their proliferation rate taking advantage of their high levels of ROS and the defective function of p53 (Fig. 4).
The Bcl-2 network is one of the most crucial regulators of melanoma cell apoptosis (Soengas & Lowe, 2003). The Bcl-2 family is formed by both antiapoptotic and proapoptotic members. Bcl-2 protein has a physiologic role in the skin: in response to UV radiation, keratinocytes secrete NGF, which binds to melanocyte receptors and leads to increased Bcl-2 expression and resistance to apoptosis (Zhai et al, 1996). Aberrations in various signaling pathways contribute to elevated Bcl-2 levels in melanoma. Particularly, oncogenic NRAS and MITF favour the enhanced expression of this antiapoptotic protein in melanoma (Hocker et al, 2008). Alterations in others antiapoptotic members of the Bcl-2 family, such as Mcl-1, may play a role in decreasing the dependence on Bcl-2 for cell survival. Indeed, Mcl-1 has emerged as potentially critical melanoma survival gene and is highly expressed in primary as well as advanced melanoma (Tang et al, 1998). Various growth factor receptors on the melanoma cell surface signal through AKT to increase the levels of the Bcl-2 family proteins that can block apoptosis and this could be mediated by ROS (Fruehauf & Trapp, 2008)

2.3.2.4 Melanocyte developmental pathways alterations

The genetic factors involved in the coordinated balance of proliferation, migration and survival required for melanocyte homing to the cutaneous surface may be usurped by melanoma cells during tumorigenesis.

KIT is an essential gene for melanocyte survival and development, which encodes a receptor tyrosine kinase (RTK) for the stem cell factor (SCF) ligand and functions as an upstream activator of the MAPK signalling pathway (Berger & Garraway, 2009). The role of KIT in melanoma tumorigenesis is rather controversial (Hocker et al, 2008). The fact that KIT alterations are associated with certain less common types of melanoma, would explain the relatively low frequency of mutations of this gene in unselected cases (Hocker et al, 2008). However, in uveal melanoma, c-Kit expression is present in nearly 80% of cases (Berger & Garraway, 2009; Sekulic et al, 2008). It is generally accepted that ROS generated by these ligand/receptor-initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death (Valko et al, 2006). Indeed, ROS act as second messengers in the MAPK pathway activated by KIT and they can mediate uveal melanoma cell proliferation through this signalling.

The MITF gene has emerged as a master regulator of melanocyte development (Hocker et al, 2008). As described in section 2.3.1.6, its product is a transcription factor involved in multiple processes, such as the regulation of pigment cell phenotype and melanocyte proliferation and survival (Widlund & Fisher, 2003). Interestingly, MITF amplifications are coincident with mutations in BRAF (Berger & Garraway, 2009). Unlike oncogenic NRAS and BRAF, which acquire new and tumour-specific cellular functions through nucleotide mutations, MITF becomes oncogenic via deregulation, affecting survival mechanisms that are also present in the normal melanocyte lineage (Berger & Garraway, 2009).

MiTF is a downstream target of β-catenin, a critical regulator of melanoma cell growth (Widlund et al, 2002) which, in turn, is an important downstream mediator of Wnt pathway signalling (Hocker et al, 2008). Thus, melanomas often show constitutive activation of the Wnt signalling as revealed by nuclear accumulation of β-catenin (Rimm et al, 1999). Wnts are secreted proteins with important developmental functions, particularly in neural crest cells such as melanocytes (Sekulic et al, 2008). The Wnt signalling pathway is also regulated by ROS (Fig. 4). A thioredoxin-related protein, nucleoredoxin (NRX), governs ROS-stimulated Wnt signalling in a temporal manner (Funato & Miki, 2010). NRX usually interacts with Dishevelled (Dvl), an essential adaptor protein for Wnt signalling, and blocks the activation of the Wnt pathway (Funato & Miki, 2010). Oxidative stress causes dissociation of NRX from Dvl, which enables Dvl to activate the downstream Wnt signalling pathway (Funato & Miki, 2010).

Gene transcription and post-translational modifications of MiTF are strongly influenced by multiple upstream pathways, some of which are modulated by ROS, such as RTK, MAPK and PI3K pathways (Fig. 4). Therefore, some authors linked the involvement of ROS in these signalling pathways to the progression of melanoma by the term reactive oxygen-driven tumour (Fried & Arbiser, 2008). In this sense, the overexpression of APE-1, a transcriptional target for MiTF, protected melanoma cells from H_2O_2-induced cell death (Yang et al, 2005). Interestingly, Liu *et al.* demonstrated that MiTF regulates cellular ROS response by upregulating APE-1. Thus, MiTF-positive melanoma cells are more resistant to H_2O_2-induced cell death as compared to MiTF-negative cells. MiTF accumulation in advanced melanoma may increase APE-1 levels and other survival factors such as Bcl-2, which in turn lead to better survival outcome for tumour cells (Liu et al, 2009). This is associated to the fact that high MiTF levels were correlated with poor outcome of patients with melanoma (Koyanagi et al, 2006).

As we can see so far, the melanoma network is highly interconnected with a great deal of functional redundancy built into it. The signalling pathways active in melanoma cells are

interconnected through multiple feedback loops, and ROS are involved in the regulation of many of them (Fig. 4).

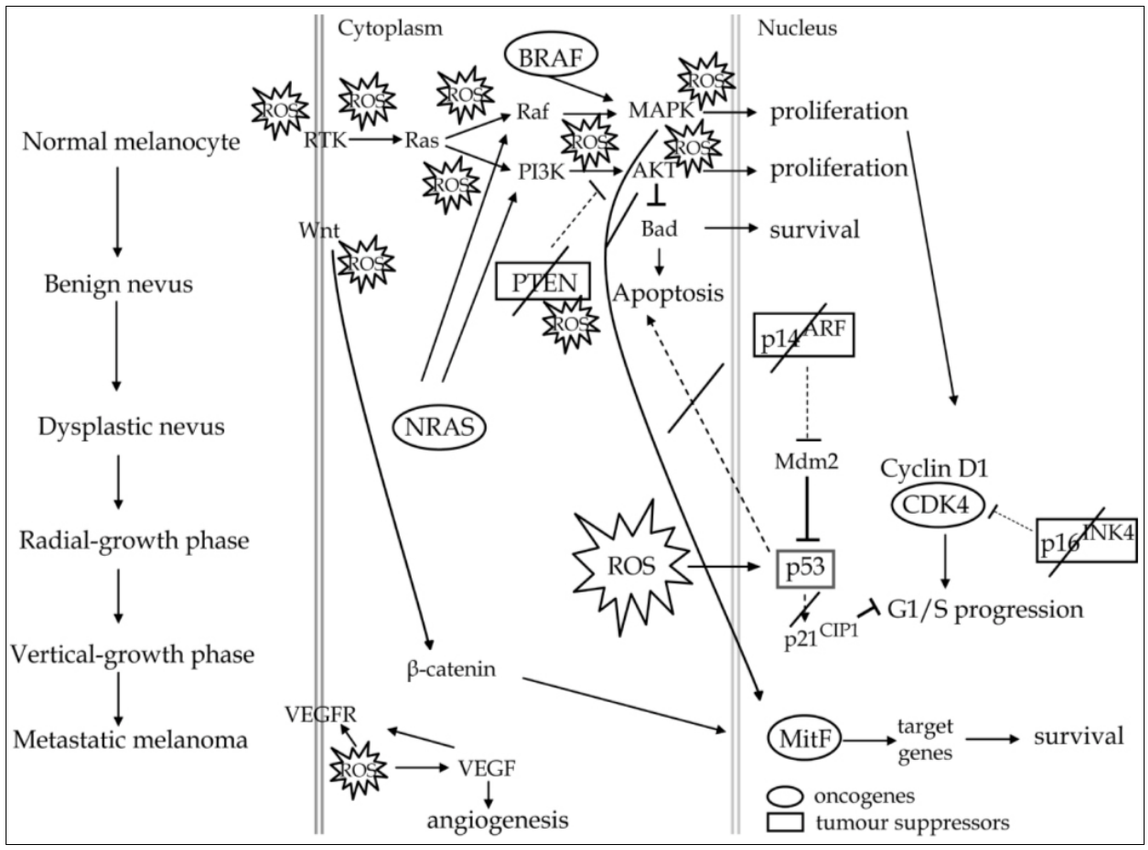

Fig. 4. ROS and the altered signalling pathways involved in the different steps of promotion and progression of melanoma.

2.4 ROS in invasion and metastasis of melanoma

Regarding the involvement of ROS in metastatic melanomas, several proteins related to different steps of migration, invasion and metastasis have been found to be induced by transcription factors or activated by signalling pathways described as mediated by ROS. For example, connexin 43 (Cx-43), which participates in tumour cell diapedesis and attachment to endothelial cells (Villares et al, 2009) along with a chondroitin sulfate proteoglycan of the extracellular matrix that favours the detachment of cells and the metastatic dissemination (Domenzain-Reyna et al, 2009), i.e. versican, are overexpressed in melanomas and are regulated by the redox-responsive transcription factor AP-1. Another mechanism that relates high ROS levels of melanoma cells to metastatic potential would be the cytoplasmic mislocalization of $p27^{KIP1}$ (unpublished results), since reduced nuclear levels of this protein increases proliferation and cytoplasmic localization would drive tumour cell invasion by other functions of this protein not related to cell cycle regulation (Wander et al, 2010).

NEDD9 has been supported as a melanoma gene important for invasion and metastasis (Kim et al, 2006). The overexpression of this gene induces malignant features related to metastatic potential in primary melanocytes and in nonmetastatic melanoma cells. This

increased invasiveness is conferred by a functional and physical interaction between NEDD9 and focal adhesion kinase (FAK) at the cell periphery (Berger & Garraway, 2009). Hef1 (encoded by NEDD9) positively regulates the Src-FAK-Crk "migratory switch". Hef1 overexpression also induces the activation of ERK, p38, and JNK kinases through interactions with intermediary signalling effectors. As previously explained, these proteins that interact with Hef1 can be modulated by ROS (O'Neill et al, 2007).
Angiogenesis signalling in melanoma is mediated by growth factor receptors, including the VEGF, platelet-derived growth factor (PDGF), and basic-fibroblast growth factor (b-FGF) pathways. VEGF activation of VEGF receptors (VEGFR) is likely to play an important role in melanoma growth, survival, and invasiveness through autocrine loop formation (Graells et al, 2004; Lacal et al, 2005). As described above, ROS can modulate these pathways involved in angiogenesis (Fig. 4). In this sense, it has been well described that the expression of VEGF is induced by ROS and in particular it is most strongly activated by H_2O_2 (Valko et al, 2006).

3. Therapeutics approaches for melanoma based on reactive oxygen species levels

It is widely accepted that melanoma is resistant to all therapeutic modalities once the metastatic process started. Current treatment protocol for early-stage melanomas consists in surgical tumour excision, while in advanced stages, the adjuvant treatment including chemotherapy, unspecific immunotherapy and interferon offers poor results regarding free disease terms rate of survival. Thus, none of the currently available FDA approved therapies clearly alter the natural history of the disease for the population as a whole.
On the other hand, advanced personalized therapeutic procedures like golden nanospheres and gene therapy are recently being studied and represent an alternative for future treatment of melanoma. Despite all new knowledge and technological support the advanced stage melanoma management still remains an unsolved problem.

3.1 Use of ROS as therapeutic agents

The cytotoxic nature of ROS has been widely used to kill tumour cells in many cancer treatments. Various anti-cancer agents in clinical use today—including anthracyclines, cisplatin, bleomycin and ionizing radiation—generate ROS within the cells (Nishikawa, 2008). However, around a decade ago, the concept of taking advantage of the increased oxidative stress of cancer cells was proposed as a strategy to induce preferential cancer cell death based on the different redox states in normal and malignant cells. Different approaches exploit the fact that increased ROS may induce cell death by apoptosis.
Arsenic trioxide (ATO) is studied as an anticancer agent whose use originated in traditional Chinese medicine. In several tumour types, it is an established cell growth inhibitor and apoptosis inducer (Baysan et al, 2007; Han et al, 2008). In patients with acute promyelocytic leukemia, for example, low concentrations of ATO (less than or equal to micromolar concentrations) induce longlasting remission without significant myelosuppressive side effects. The ability of ATO to induce apoptosis in leukemic cells depends on the activity of the enzymes that regulate cellular H_2O_2 content (Jing et al, 1999). Regarding melanoma and ATO, in several melanoma cell lines a study demonstrates that a combination of treatments of melanoma cells with ATO and the antibiotic thiostrepton results in significant growth inhibitory effects, reversed when the cells are also treated with the free radical scavenger NAC, indicating an increase in intracellular ROS as part of the mechanism. Thiostrepton is

commercially available as part of a topical antibiotic mixture used to treat bacterial infections in animals. It has also been used to effectively treat murine bacterial systemic infections (Bowling et al, 2008). However, to date, it has not been approved for human use.

Other drugs are studied alone or in combination with ATO to induce apoptosis. Disulfiram (DSF), an agent used to treat alcoholism for the past few decades, has been shown to induce apoptosis in thymocytes. It has been demonstrated that redox regulations in melanoma cells are aberrant and metal chelators (such as dithiocarbamate) alter the redox status and induce apoptosis in melanoma cells. Cen *et al.* have explored the effect of disulfiram (DSF), a member of the dithiocarbamate family, on apoptosis in melanoma cells, founding that DSF caused a 3 to 5-fold increase in apoptosis in all three melanoma cell strains being tested at a very low dose (25-50 ng/ml). The same dose of DSF did not have significant apoptotic effect on melanocytes (Cen et al, 2002). Disulfiram in combination with ATO were selected for a study based on their ability to alter GSH redox balance. They were chosen because ATO has been reported to be synergistic against various solid tumour cell lines *in vitro* and *in vivo* when given with buthionine sulfoximine (BSO), another agent that acts as DSF, both modulating GSH metabolism (Wu et al, 2004); and the effect of Disulfiram (DSF), has recently been explored on apoptosis in melanoma cells.

Another compound that showed therapeutic activity in malignant melanoma is Elesclomol (STA_4783), that was able to prolong the progression-free survival time in a Phase II clinical trial (Kirshner et al, 2008). The mechanism underlying proapoptotic activity of elesclomol is through the induction of ROS and oxidative stress. Elesclomol rapidly induced ROS in cancer cells, generating a transcription profile characteristic of an oxidative stress response. Moreover, pretreatment of cells with antioxidants blocked all *in vitro* activities associated with elesclomol, including ROS production and the induction of apoptosis. Unfortunately, the Phase III clinical trials of elesclomol were suspended in 2008 due to safety concerns.

Finally, Taxol (paclitaxel) is a chemotherapeutic agent that has shown promise in the treatment of previously unresponsive breast, ovary, and non-small cell lung carcinoma (Crown & O'Leary, 2000). It interferes with mitosis during the G2/M phase of the cell cycle. Besides, it was demonstrated that taxol mediated activation of MAPK signalling pathways is linked to the activation of the cell death machinery in human melanoma cell lines. The same study demonstrates that the downregulation of a protein UCP2 and subsequent induction of ROS are essential parts in this mechanism (Selimovic et al, 2008).

ROS are capable of exerting different effects according to their nature, localization and levels (Davies, 2000). Although classically they are thought of as cytotoxic and mutagenic or as inducers of oxidative stress, evidence show that ROS play a role in signal transduction. ROS can be implicated in stimulation or inhibition of cell proliferation, apoptosis, and cell senescence (Chen, 2000; Sundaresan et al, 1995). In BHK-21 and human prostate cells, a low concentration of exogenously added H_2O_2 caused a modest increase in proliferation, whereas higher levels resulted in slowed growth, cell cycle arrest, and/or apoptosis (Burdon, 1996; Wartenberg et al, 1999).

Furthermore, considering the relationship between ROS and metastasis, reviewed in the literatures, it indicates that ROS accelerate adhesion (Orr et al, 2000), invasion (Orr et al, 2000), migration (Wu, 2006; Wu et al, 2008) and angiogenesis (Ushio-Fukai & Nakamura, 2008). Therefore, ROS scavengers, such as catalase and superoxide dismutase, have been examined to inhibit tumour metastasis, although their effects are not very marked. Targeted delivery of these enzymes to the sites where ROS are generated is necessary to obtain sufficient inhibition of tumour metastasis.

Thus, an increase in ROS levels is not the only alternative for a possible cancer therapy. Scavenging of H_2O_2 in several tumour cells inhibits cell proliferation (Arnold et al, 2001). The inhibition of lung metastatic tumour growth of melanoma cells has been reported to occur in a mouse model by sustained delivery of catalase (Hyoudou et al, 2009). In that sense, a recent study of our group demonstrates that H_2O_2 scavenging impedes nuclear exportation of $p27^{KIP1}$ allowing cell cycle arrest through the accumulation of this protein in the nucleus (unpublished data).

Interleukin (IL)-2 has been shown to have clinical activity in metastatic melanoma. Nevertheless, *in vitro* studies have demonstrated that ROS generated by monocyte/macrophage populations may inhibit cytotoxic activity within the tumour, leading to the hypothesis that cytokine-based immunotherapy may be more effective if the generation of ROS were inhibited (Hansson et al, 1999; Kono et al, 1996). Clinical evidence in support of this hypothesis came from phase II studies that combine histamine, a potent inhibitor of ROS formation, with cytokine-based therapies for metastatic melanoma, acute myelogenous leukemia, and renal cell carcinoma (Hellstrand et al, 1997; Hellstrand et al, 1994).

Manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells. It is worth noting that redox alterations in cancer cells are very complex because of the multiple factors involved in the redox regulation and stress response, and that the simple addition of ROS-generating agents may not always lead to a preferential killing of cancer cells. This explains the importance of understanding the complex redox alterations in cancer cells.

3.2 Gene therapy

Gene Therapy is a relatively new paradigm for treatment of human diseases and is becoming a rationale area for the development of new therapeutic agents for cancer treatment. One major strategy of gene therapy to provide effective and specific activation of medical products inside the tumour mass implicates the control of the therapeutic agent activity by a Tumour Specific Promoter (TSP). TSPs corresponding to genes differentially activated in cancer cells have been used to control viral replication inside the tumour or the antitumour activity of therapeutic genes in melanoma (Cao et al, 1999; Diaz et al, 1998; Schoensiegel et al, 2004). Selection of a TSP is central for the design of a well defined medical product for gene transfer. Since solid tumours are highly heterogeneous it is more tempting to drive gene expression by designing promoters that include transcriptional regulatory elements responsive to the tumour environment conditions (Laurent et al, 2005).

Recently, we described a novel approach of gene therapy, which takes advantage of the prooxidant state of melanoma cells. (Policastro et al, 2009). This therapeutic strategy is based on a ROS-responsive chimeric promoter, containing a ROS-response motif located in the VEGF gene promoter and a second ROS-response motif obtained from the EGR1 gene promoter. This chimeric promoter was based on a ROS-response motif located in the VEGF gene promoter placed and a second ROS-response motif obtained from the EGR1 gene promoter. The activity of the chimeric promoter (named E6(40)VE) was largely dependent on variations in intracellular ROS levels and showed a high inducible response to exogenous H_2O_2. Transient expression of the Herpes Simplex virus thymidine kinase therapeutic gene (TK) driven by the chimeric promoter, followed by gancyclovir administration, inhibited human melanoma cells growth *in vitro* and *in vivo*. Moreover, electrotransfer of the TK gene followed by GCV administration exerted a potent therapeutic effect on melanoma

established tumours. This response was improved when combined with chemotherapeutic drugs such as doxorubicin, which act by themselves by increasing ROS levels (Policastro et al, 2009).

4. Conclusion

Throughout this review, we summarized the updated knowledge about the involvement of ROS in the biology of melanoma, analyzing the alterations in both, specific pathways of melanocytes development and general ROS-regulated signalling pathways common to different types of tumours. Moreover, we provided a glimpse of the state-of-the-art advances in relation to therapeutic approaches for melanoma treatment in view of the high levels of ROS characteristic of melanoma cells as a differential feature that may be exploited to develop selective treatments with low toxicity for normal tissues.

Further studies on the biology of melanoma and ROS regulation in the context of recent new advances in genomics, proteomics and bioinformatics will allow a better comprehension of the complex signalling network in response to the high oxidative stress of melanoma. This approach will help to characterize individual patient tumour specimens identifying vulnerable targets and preventing deleterious damage in normal tissues.

5. Acknowledgements

The authors gratefully acknowledge Andrea C. Cruz for proofreading our work and advising us on the use of English language.

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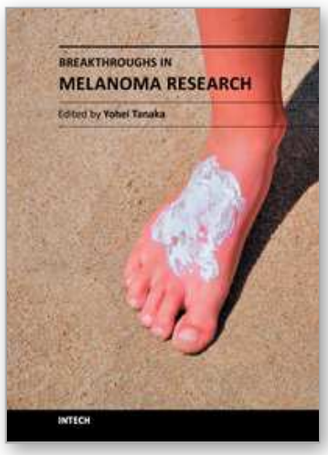

Breakthroughs in Melanoma Research
Edited by Dr Yohei Tanaka

ISBN 978-953-307-291-3
Hard cover, 628 pages
Publisher InTech
Published online 30, June, 2011
Published in print edition June, 2011

Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Irene L. Ibañez, Cintia Notcovich, Lucĺa L. Policastro and Hebe Durán (2011). Reactive Oxygen Species in the Biology of Melanoma, Breakthroughs in Melanoma Research, Dr Yohei Tanaka (Ed.), ISBN: 978-953-307-291-3, InTech, Available from: http://www.intechopen.com/books/breakthroughs-in-melanoma-research/reactive-oxygen-species-in-the-biology-of-melanoma

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