Title
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Journal
Archives of Biochemistry and Biophysics, 563

ISSN
00039861

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Publication Date
2014-12-01

DOI
10.1016/j.abb.2014.04.007

Peer reviewed

Archives of Biochemistry and Biophysics 563 (2014) 51–55

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Updates of reactive oxygen species in melanoma etiology and progression

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ARTICLE INFO

Article history:
Received 5 March 2014
and in revised form 14 April 2014
Available online 26 April 2014

Keywords:
Reactive oxygen species
ROS
RNS
Mitochondria
NADPH oxidase
Nox1
Nox4
Melanoma

ABSTRACT

Reactive oxygen species (ROS) play crucial roles in all aspects of melanoma development, however, the source of ROS is not well defined. In this review we summarize recent advancement in this rapidly developing field. The cellular ROS pool in melanocytes can be derived from mitochondria, melanosomes, NADPH oxidase (NOX) family enzymes, and uncoupling of nitric oxide synthase (NOS). Current evidence suggests that Nox1, Nox4 and Nox5 are expressed in melanocytic lineage. While there is no difference in Nox1 expression levels in primary and metastatic melanoma tissues, Nox4 expression is significantly higher in a subset of metastatic melanoma tumors as compared to the primary tumors; suggesting distinct and specific signals and effects for NOX family enzymes in melanoma. Targeting these NOX enzymes using specific NOX inhibitors may be effective for a subset of certain tumors. ROS also play important roles in BRAF inhibitor induced drug resistance; hence identification and blockade of the source of this ROS may be an effective way to enhance efficacy and overcome resistance. Furthermore, ROS from different sources may interact with each other and interact with reactive nitrogen species (RNS) and drive the melanomagenesis process at all stages of disease. Further understanding ROS and RNS in melanoma etiology and progression is necessary for developing new prevention and therapeutic approaches.

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Melanoma is a reactive oxygen species (ROS)[1]-driven tumor based on a copious amount of work done by us and others [1–3]. Searching the PubMed database with "reactive oxygen" and "melanoma" returned 52 publications in 2009 and 103 in 2013; within 4 years the number of publication almost doubled. With the rapid development in the field, we attempt to summarize the tremendous progress in our understanding of the role of ROS in melanoma etiology and progression.

Source of ROS

The term ROS includes chemically reactive molecules such as superoxide anions, peroxides and hydroxyl radicals, which can modify protein and DNA molecules, and permanently or temporally change their cellular behavior. When cells generate excessive ROS, it causes oxidative stress, which has long been recognized as an adverse event for promoting tumorigenesis and progression [4,5]; however, mounting evidence has emerged in recent years indicating that adequate ROS, in particular superoxide and hydrogen peroxide, also serve as signal molecules for cell proliferation, vascular function and wound healing [6–9]. In contrast, extremely low levels of ROS may enable cells to undergo cell cycle arrest [10,11]. However, there has never been a standard measure as to how much ROS is adequate and how much is excessive or insufficient. This deficiency is partially due to the complexity of ROS measurement methods, and partially due to the dynamics of ROS generation and various ROS species in cells.

Cancer cells including melanoma cells exhibit high levels of ROS [12,13]. The source of ROS has not been completely defined. The

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[1] *Abbreviations used:* ROS, reactive oxygen species; NOX, NADPH oxidase; NOS, nitric oxide synthase; Mc1R, melanocortin receptor 1; 8-oxodG, 7,8-dihydro-8-oxyguanine; EMT, epithelial–mesenchymal transition; GEO, gene expression omnibus; Nrarp, notch related ankyrin related protein; 12-LOX, 12-lipoxygenase; UGTs, UDP-glucuronosyltransferases; SODs, superoxide dismutases; RNS, reactive nitrogen species; DPI, diphenyliodonium.

http://dx.doi.org/10.1016/j.abb.2014.04.007
0003-9861/Published by Elsevier Inc.

major source of ROS in cancer cells has traditionally been attributed to mitochondrial uncoupling and dysfunction [14]. However, emerging evidence from specific investigations of melanoma cells indicates that other cellular compartments and enzymes also contribute significantly to ROS generation, including the NADPH oxidase (NOX) family, nitric oxide synthase (NOS) uncoupling, peroxisomes and melanosomes (Fig. 1). In melanoma, the mitochondria may also generate ROS via the electron transport chain, mainly Complex I and Complex III, as well as other sites [15]. How and how much each complex site generates superoxide and how much they contribute to total mitochondrial ROS is not clear. Although melanoma is a ROS-driven tumor [1], mitochondria-generated ROS currently remains as a vague and undeveloped paradigm in melanoma research; most of the studies are indirect or the signal pathways were deduced from other cancer fields. As pointed out in a recent review, mitochondrial DNA mutation is rare in cancer [16], hence, mitochondrial DNA mutation is unlikely a major cause for ROS generation and cancer development in melanoma cells. However it is now recognized that the role of mitochondria in cancer is more linked to defective metabolic regulation [17], consequently it is conceivable that mitochondria-generated ROS may also directly participate in these processes.

Early studies indicated that melanocytes and melanoma cells exhibited a unique redox regulation [12,18,19]; hence efforts on seeking a unique ROS source led to discovery of the ROS-generating roles of the melanosome and melanin [20] (Fig. 1). An understanding of the melanosome and melanin-related ROS hypothesis explains how and why melanin is required for melanomagenesis [21]. The red-hair associated pheomelanin has long been assumed to have a pro-oxidant role. Recently, pheomelanin structure has been elucidated and pheomelanin was purified [22,23]. The purified pheomelanin exhibited potent pro-oxidant characteristics in the test tube and in cells when exposed to UV radiation [24,25]. Further investigations should lead to novel mechanistic insights about UV-induced melanomagenesis.

On the other hand, recent studies reveal that NOX family genes also play an essential role in the development of non-melanoma skin cancer and melanoma, which will be discussed in detail below [26,27]. The NOX family of enzymes includes 7 members: Nox1, Nox2 (gp91phox), Nox3, Nox4, Nox5, Duox1 and Duox2; all use NAD(P)H and oxygen as substrates and generate superoxide (except for Nox4, Duox1 and Duox2 which generates hydrogen peroxide). NOX family enzymes have not been well studied in melanoma. Early studies suggested that NADPH oxidases contributed to melanoma cell proliferation [28,29]; although no specific isoforms were identified. Subsequently, Nox4 was discovered to play a critical role in melanoma invasion downstream of the AKT pathway [30]; Nox4 is also a target for MiTF in the B16 melanoma cell line [31]. Our data showed that among the 7 family members, only Nox1 was expressed in normal melanocytes and in all 9 melanoma cell lines examined. Nox4 was expressed in only a subset of metastatic melanoma cell lines [32], consistent with the published results showing that Nox4 is involved in cell invasion [30]. Nox5 was recently found in a melanoma cell line [33], while expression of other NOX family members has not yet been detected in cells of melanocytic cell lineage [32]. Thus, the NOX family of enzymes has emerged as a new and important source of ROS in melanoma.

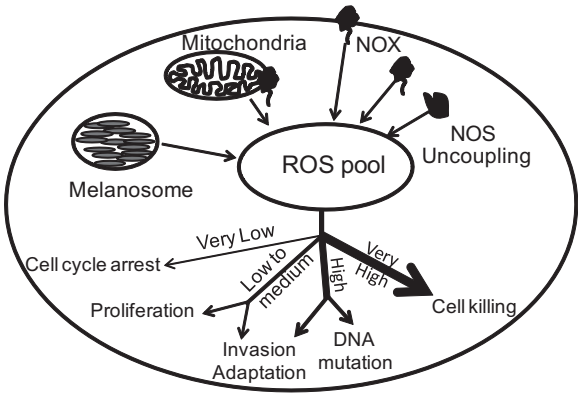

Fig. 1. The source of ROS in melanocytes and their cellular effect. ROS can be generated from melanosomes, mitochondria, NOX family enzymes and/or NOS uncoupling. ROS generated from these different sources may interact with each other and form a cellular ROS pool. When ROS levels are adequate, they serve as proliferation signals; when ROS is raised, they show adverse effect including promoting invasion and DNA oxidative mutations. If ROS level is beyond the cellular antioxidant buffering capacity, they can directly kill cells.

The role of NOX family genes in melanoma etiology

NADPH oxidases are multi-subunit enzymes. Nox1, Nox2, Nox3 and Nox4 share a common membrane subunit CYBA (p22phox, Cytochrome b-245, neutrophil cytochrome b light chain) as part of their holoenzyme, but each prefers different cytoplasmic subunits [34]. Nox1 usually prefers NoxO1 and NoxA1 while Nox2 utilizes a 47-KD NCF1 (phox47) and a 67-KD NCF2 (*neutrophil cytosolic factor* 2, p67phox) [35,36]. Emerging evidence indicates that Nox1, Nox4, Nox5 and NCF1 may impact melanomagenesis and progression.

As a major melanoma risk factor, skin color is regulated by a series of signaling molecules (e.g., POMC gene products, agouti protein, melanocortin receptors), transcription factors (e.g. MiTF, Sox9/Sox10) and melanin-synthesis enzymes (e.g., tyrosinase, *tyrosinase*-related protein 1, *and tyrosinase*-related protein 2) [37], among which melanocortin receptor 1 (Mc1R) transduces signals from either its agonist α-MSH or antagonist agouti to determine melanin sub-types [38,39]. Consequently, mutations in Mc1R and agouti protein were tightly linked to melanoma risk [38,40–42]. Although emerging evidence indicates that Mc1R can function via a UV-independent melanomagenesis signal pathway [43], it is obvious that Mc1R-mediated skin pigmentation may play a critical role in UV-induced DNA damage response. Abdel-Malek's group showed that α-MSH treatment protected cultured human melanocytes from UV-induced oxidative stress as measured by 7,8-dihydro-8-oxyguanine (8-oxodG) levels, which was mediated by functional Mc1R as the protective effect was not observed in melanocytes carrying loss of function Mc1R alleles [44,45]. A study of immortalized human keratinocytes HACAT cells revealed that expression of wild-type Mc1R, but not the red-hair associated loss of function Mc1R (Mc1R-R151C) gene reduced UVA-induced ROS production [46]. This study further elucidated that UVA-induced the phosphorylation of NoxA1 [46], a subunit for the Nox1 holoenzyme. Phosphorylation of NoxA1 decreases its binding to Nox1 catalytic subunit and Rac1, a key activator for Nox1 [47], hence this phosphorylation decreases Nox1 activity [48]. Thus, Nox1 seems to be responsible for ROS generation downstream of Mc1R. Interestingly, Nox2 (gp91phox) null mice showed a compromised epidermal melanocytes induction after UVB radiation in the eyes, which corresponded to compromised plasma α-MSH induction [49]. These studies suggest that NOX family genes not only regulate UV-induced melanocytes proliferation, but also play a critical role in UV-induced ROS production. Our unpublished data suggests that Nox1 protein is induced by both UVA and UVB in the melanoma cell line SK-Mel28; and more so by UVA (Liu-Smith and Meyskens, unpublished data); consequently, NOX activity assay revealed that UVA also induced higher total NOX activity as compared to UVB (Liu-Smith and Meyskens, unpublished data). These results may suggest that SK-Mel28 cells carry a non-functional MC1R allele. At the same time, this may also suggest that in normal melanocytes from individuals carrying non-functional Mc1R variants the NOX family may play an important role in UV-induced

ROS production, a direct cause for UVA-induced oxidative DNA damage.

Gene knockout in mice did not reveal any tumorigenesis phenotype for NOX family genes, including Nox1, Nox2, Nox4 and CYBA/p22phox [50]. However, knockout NCF1 (*neutrophil cytosolic factor* 1, p47phox), a subunit for Nox2 holoenzyme, reduced xenografted tumor growth [51], suggesting that Nox2 may create a favorable environment for *in vivo* tumor growth. Interestingly, Nox4 knockdown in melanoma cell lines induced G2/M cell cycle arrest [52], suggesting that Nox4 is required for cell proliferation in a subset of aggressive melanomas.

The role of NOX-induced ROS in melanoma progression

Advanced metastatic melanoma is difficult to treat even with the recent therapeutic breakthrough using BRAF and MEK inhibitors and immunotherapy [53–55]; hence understanding the nature of resistant metastatic cells still remains an important research focus. ROS is known to be an important component in driving cancer cell migration and invasion [5]; however, the source of this type of ROS has been rarely described. To date, few reports have investigated mitochondria-derived ROS as a driver of cancer metastasis [56,57]. Recent research on NOX enzymes has established a new playing field for pathway-driven hypotheses focused on the role of NOX-induced ROS in cancer metastasis.

Cancer cell metastasis requires a series of regulated movements of the cytoskeleton and cell adhesive molecules. The Rho GTPase family proteins, including Rac1, RhoA and Cdc42, are essential regulators for the cytoskeleton movements and adhesion molecules, hence play pivotal roles in cancer metastasis [58–60]. Rac1 is a crucial activator for NOX enzymes [61], especially for Nox1 [62,63]. Activated Rac1 binds NoxA1 and the C-terminal domain of NOX1 for assembly of the holoenzyme [47,64,65]; ectopic expression of Rac1(Q61L), a constitutively activated Rac1 mutant, enhances the cytosolic activator p67phox (NoxA1 homolog which can also bind to Nox1 and stimulate the enzymatic activity) binding to Nox1 and promotes its localization to plasma membrane, subsequently promoting Nox1 activity and induces ROS [62]. Our finding that Nox1 over-expression promoted melanoma cell invasion provides a new and complementary explanation to Rac1-Nox1 mediated cell motility [32]. Over-expression of Nox1 alone increased ROS levels in melanoma cells, induced MMP2 and MMP9 expression as well as an epithelial–mesenchymal transition (EMT), resulting in an increased invasive phenotype [32]. These results are consistent with the finding that hyaluronic acid induced Rac1 activity, resulting in increased NOX activity and ROS production, leading to increased melanoma motility [66]. Mouse B16F0 non-invasive melanoma cells, when treated with exogenous hydrogen peroxide, also became invasive due to increased Rac1 and p47phox expression [67], further affirming a role for NOX enzymes in ROS-mediated melanoma invasion.

Nox1 protein levels were reported to be associated with colon cancer progression in one report [68], but not in another [69], raising the question whether it can serve as a progression biomarker. Using the microarray data from the Gene Expression Omnibus (GEO) from National Center of Biotechnology Information initially done by Xu et al. [70], we analyzed the expression levels of Nox1 and Nox4 from 31 primary and 52 metastatic human melanoma samples. The Nox1 mRNA levels were not associated with melanoma stages (Liu-Smith and Meyskens, unpublished data). Nox1 mRNA levels are similar in metastatic melanoma samples and early stage non-invasive primary tumors ($p = 0.57$, unpaired t-test). Our published results also showed similar Nox1 protein levels in primary melanoma and metastatic cell lines [32]. Therefore, Nox1 may not be a driving force for melanoma invasion *in vivo*, although it can enhance the invasion capacity *in vitro*. On the contrary, Nox4 seems to be a driving force for melanoma invasion in a subset of tumors. Using the same GEO dataset, we found that Nox4 expression levels were much higher in some metastatic samples as compared to the primary tumors ($p = 0.025$, Liu-Smith and Meyskens, unpublished data). This differential expression of Nox1 and Nox4 in melanomas of different stages is further supported by publications from us and others [30,32,52]. Since Nox4 is required for cell cycle progression in these examples [52], Nox4 may serve as an effective therapeutic target for this subset of melanoma. On the contrary, Nox1 may not be a good biomarker for melanoma progression. Nevertheless, this does not exclude the importance of superoxide and/or hydrogen peroxide in melanoma progression because superoxide/hydrogen peroxide is the ultimate effector, not the NOX enzymes. From this point of view, activation of Rac1 may be considered as a biomarker for melanoma progression, consistent with its newly defined role as a melanoma oncogene [71]. While superoxide and hydrogen peroxide is difficult to detect after fixing procedures in biospecimens, a potential biomarker for superoxide may be Notch related ankyrin related protein (Nrarp) which was found to be down-regulated together with Nox4 in Ang2-heterozygous mice endothelial cells as compared to Ang2 null cells [72]. In this study inhibiting Nox4 by fulvene-5 blocked growth of endothelial tumors in mice [72].

Furthermore, Nox1 was shown to be a downstream effector of 12-Lipoxygenase (12-LOX) signaling in colon cancer proliferation [73]. In this study, knockdown of Nox1 in colon cancer cells prevented 12-LOX mediated proliferation [73]. 12-LOX has also been reported as a biomarker for melanoma progression [74]. In this report, 12-LOX expression was not observed in human melanocytes or nevi, but 12-LOX expression was detected in dysplastic nevi and melanoma. Importantly, 12-LOX expression directly correlated with melanoma progression with melanoma displaying the highest 12-LOX expression [74]. Recent work from our lab demonstrated the opposite expression pattern for the UDP-glucuronosyltransferases (UGTs) in melanoma progression [75]. UGTs are expressed in melanocytes, but not expressed in metastatic melanoma cell lines [75]. This is intriguing since the UGTs can turn off 12-LOX signaling by metabolizing 12-HETE, the main signaling molecule generated by 12-LOX catabolism of arachidonic acid [76]. 12-HETE levels have been shown to correlate with melanoma metastatic potential in mouse models [77]. High concentrations of 12-HETE are found in highly metastatic cell lines compared to cell lines with low metastatic potential [77]. Thus, Nox1 may very well be the critical downstream effector in 12-LOX signaling in melanoma as well.

It is evident that Nox4 and Nox1 may function via different upstream signals in invasion, as Nox1 is tightly regulated by Rac1 while Nox4 has no apparent link with Rac1, rather Nox4 is downstream of the AKT pathway. Further, Nox1 participates in EMT process while Nox4 may not. The difference is also reflected by the end product of enzymatic reaction: Nox1 produces superoxide which is not membrane permeable, and which needs to be further detoxified by superoxide dismutases; Nox4 produces hydrogen peroxide which can serve as either a signaling molecule or lead to cellular damaging ROS.

The potential role of ROS in melanoma drug resistance

In experimental therapeutic studies, a number of drugs were reported to induce ROS, which serve as mediators for apoptosis or cell killing. For example, TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis can be sensitized by wortmanin through a ROS-dependent phosphorylation of Bax [78]; The source of wortmanin-induced ROS seems to be Nox4 as its siRNA suppressed Bax phosphorylation and apoptosis [78]. Nox4-generated ROS also played a crucial role in proopiomelanocortin-induced apoptosis [79]. A role of Nox1 in drug response has not yet been explored.

In the clinic a significant breakthrough in melanoma treatment has been the clinical activity of BRAF inhibitor Vemurafenib (Plx4032), which has shown unprecedented efficacy (40–80% response rate) against melanoma as compared to conventional Dacarbazine treatment (~5% response rate) [80,81]. Patients receiving Plx4032 treatment showed a progression-free survival of 5.3 months as compared to 1.6 months for those treated with Dacarbazine [53]. However, after the initial response the tumors uniformly progressed and patients quickly relapsed [54]. A combination therapy of a BRAF inhibitor and Mek1/2 inhibitor produced longer response periods, but unfortunately resistance still developed [55]. The observed Plx4032-induced resistance involved signals from NRAS, CRAF, COT, PDGFRβ, EGFR and IGF-1R [82,83], as well as reactivation of Erk1/2 [84]. In fact, Erk1/2 seems to be at the center of this whole signal cascade because all of the listed upstream signals can be transmitted to re-activate Erk1/2. Interestingly, there have been many studies demonstrating an intrinsic link between ROS and Erk1/2 activation [85]. Hence, it is conceivable that ROS may be a key downstream effector for Plx4032-mediated Erk1/2 reactivation. This hypothesis is supported by recent observation that Plx4032 treatment induced ROS in melanoma cells ([86,87], Liu-Smith and Meyskens, unpublished data). The ROS induction was through PGC1α-induced mitochondria biogenesis [86,87], hence PGC1α may be a good target for developing a combination therapy with a BRAF inhibitor. In addition, considering that ROS can also serve as proliferative signals, this Vemurafenib-induced mitochondria-derived ROS may play a role in the cellular adaptation to the drug treatment; hence anti-oxidants may be useful in combination therapy with Vemurafenib in melanoma.

Emerging evidence show that mitochondria-generated ROS and cytosolic ROS (for example, NOX-generated ROS) interact with each other and may enhance the total cellular ROS production [88]; or some NOX isoforms may locate in mitochondria and become a source of mitochondrial ROS in addition to the ROS generated by electron transport complexes [89].

Redox balance in melanoma: co-evolution of ROS and cellular antioxidants

Numerous studies have shown that cancer cells including melanoma cells exhibit elevated ROS levels [12,90], which led to testing of a wide range of antioxidants as potential chemotherapy drugs. However, melanoma cells also carry high levels of ROS-metabolizing enzymes including superoxide dismutases (SODs), catalase, and a series of redox-related transcription factors such as the AP-1 family, APE/Ref-1 gene and NFκB [91–95]. The two major antioxidant systems, glutathione (GSH) and thioredoxin (TRX) systems, are also up-regulated in melanoma cells as compared to normal human melanocytes [96–101]. Thus, high ROS levels and a more robust detoxifying antioxidant system seem to co-evolve and form a new balance in cancer cells in order to maintain ROS levels in the non-toxic ranges. One consequence of this new balance is that a more robust antioxidant enzyme system exists in melanoma cells, which renders tumor cells extremely adaptive to challenges like ROS-inducing chemotherapy drugs [90,102]. We and others have discovered that APE/Ref-1 and Hif-1α genes are transcriptional targets for melanocytes master transcriptional factor MiTF [91,103], suggesting that this redox balance may be through a currently undefined melanocyte lineage specific mechanism.

RNS (reactive nitrogen species) and ROS: a perspective relationship in melanoma

Reactive nitrogen species are a series of active molecules generated from nitric oxide and superoxide, and participate in essentially all aspects of cancer development. Nitric oxide is synthesized by nitric oxide synthase (NOS) which consists of three isoforms: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2 and endothelial NOS (eNOS, NOS3). iNOS has been well characterized in melanoma: its expression promotes cell proliferation; is associated with poor patient survival; and increases resistance to cisplatin [104–109]. Although an early study demonstrated nNOS expression in melanocytes and melanoma cells [110], these investigations were not followed up until we recently discovered that nNOS played a role in melanoma invasion and proliferation [111,112]. The eNOS uncoupling is also an important source of superoxide in melanoma which drives the malignant transformation [113]. There have been large amount of data from cardiovascular research showing interactions between NOX and NOS pathways [114]. It is conceivable that the NOS product NO interacts with NOX product O_2^- and generates *peroxynitrite* ONOO–, an extremely reactive molecule which can nitrosylate key signal molecules such as PTEN in melanoma, hence impacting melanoma development.

NOX inhibitors and their potential as melanoma drugs

Recent studies of NOX enzymes in cancer development identified these enzymes as novel targets for cancer therapy [115–117]. Nox1 enzymatic activities can be inhibited by Diphenyliodonium (DPI, a classic NOX inhibitor), VAS2870 (a NOX inhibitor in clinical trial for cardiovascular conditions), apocynin (a natural compound found in the anti-inflammatory herb *Picrorhiza kurroa* and a potent Nox1 inhibitor), GKT137831, ML171, triphenylmethanes, Gentian violet, GKT136901 [118–124]. All these compounds inhibit NOX activity in low μM concentration range [119,125,126]. Our results showed that DPI, VAS2870 and apocynin are able to kill melanoma cells *in vitro*, with DPI as the most potent compound and apocynin the least (Liu-Smith and Meyskens, unpublished data). However due to the non-specificity of DPI and high toxicity [127], its clinical use has been limited. VAS2870 was developed as a pan-NOX inhibitor [128], but off-targets were discovered soon after [129]. ML171 showed selectivity for Nox1, as its IC50 for Nox1 is about 20-fold lower than that for Nox2 compared to Nox4 [120]. GKT137831 inhibits both Nox1 and Nox4 [121], and effectively provided renoprotection against diabetic nephropathy in mice [130,131]. Triphenylmethanes inhibited Nox1, Nox2 and Nox4 and showed efficacy in reducing hemangiomas growth in mice model [122]. Gentian violet antagonized Nox1-induced p53 inactivation and induced cancer cell death [123]. GKT136901, similar to GKT137831, inhibits Nox1, Nox2 and Nox4, and showed renoprotective effect in diabetic mouse model [132–134]. To date validation of most of these NOX inhibitors as targeted therapy in cancer is still needed preclinically *in vivo* before moving them to testing in clinical trials, but the proof of principle of the use of reactive oxygen inhibitors in melanoma is demonstrated by the use of gentian violet and imiquimod in advanced melanoma of the scalp [135].

Summary

We summarize recent discoveries on the source of ROS in melanocytes and melanoma cells, which mainly include three types: mitochondria ROS, melanin/melanosome ROS and NOX-generated ROS. These sources of ROS may interact with each other and promote oxidative stress in cells and play causative roles in UV-dependent or UV-independent melanomagenesis and progression, as well as drug resistance. As the majority of single anti-oxidant therapy trials failed to show significant effect, we reason that the elevated ROS levels co-evolve with anti-oxidant levels in cancer cells, thus largely masking the exogenous anti-oxidant effects. Hence identification of specific ROS inhibitors, such as nNOS or

NOX inhibitors, may prove efficacious in combination. Possibly, dual therapy with a specific ROS inhibitor and an effective chemotherapy drug such as Vemurafenib may achieve a better outcome for patients.

Acknowledgments

This work is supported by Chao Family Cancer Center Seed Grant (CCSG, P30 CA 62230, to F.L.M. and F.L.S.), the Alan Hubbell Education Grant (CSUF and UCI-CFCCC Partnership for Cancer Health Disparities Research P20 CA174188 to Hubbell and subgrant to F.L.S.), NCI K07 (CA160756 to F.L.S.) and Waltmar Foundation (to F.L.M. and F.L.S.). The authors thank Dr. Sun Yang for critical scientific discussions.

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