REVIEW

Oxidative stress in thyroid carcinomas: biological and clinical significance

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Abstract

At physiological concentrations, reactive oxygen species (ROS), including superoxide anions and H₂O₂, are considered as second messengers that play key roles in cellular functions, such as proliferation, gene expression, host defence and hormone synthesis. However, when they are at supraphysiological levels, ROS are considered potent DNA-damaging agents. Their increase induces oxidative stress, which can initiate and maintain genomic instability. The thyroid gland represents a good model for studying the impact of oxidative stress on genomic instability. Indeed, one particularity of this organ is that follicular thyroid cells synthesise thyroid hormones through a complex mechanism that requires H₂O₂. Because of their detection in thyroid adenomas and in early cell transformation, both oxidative stress and DNA damage are believed to be neoplasia-preceding events in thyroid cells. Oxidative DNA damage is, in addition, detected in the advanced stages of thyroid cancer, suggesting that oxidative lesions of DNA also contribute to the maintenance of genomic instability during the subsequent phases of tumourigenesis. Finally, ionizing radiation and the mutation of oncogenes, such as RAS and BRAF, play a key role in thyroid carcinogenesis through separate and unique mechanisms: they upregulate the expression of two distinct 'professional' ROS-generating systems, the NADPH oxidases DUOX1 and NOX4, which cause DNA damage that may promote chromosomal instability, tumourigenesis and dedifferentiation.

Key Words

- thyroid
- oxidative stress
- genetic instability
- NADPH oxidase
- dedifferentiation

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Introduction

Reactive oxygen species, more commonly called ROS, are derived from oxygen and can be divided into two groups: free radicals, such as the superoxide anion (O₂^{•−}) and hydroxyl anion (OH^{•−}), and non-radical molecules, such as hydrogen peroxide (H₂O₂). Cells produce ROS through a variety of enzymatic systems, including NADPH oxidases, xanthine oxidases, NO synthase, cytochrome P450 reductase and the mitochondrial electron transport chain. Inside cells, a balance exists between the production of ROS and their detoxification by non-enzymatic (ascorbate,

glutathione) and enzymatic (catalase, superoxide dismutase 'SOD', glutathione peroxidase 'GPx' and peroxiredoxins 'PRDX') antioxidant systems that finely control their concentrations and maintain cellular redox homeostasis (Veal *et al.* 2007, Lukosz *et al.* 2010) (Fig. 1). In several diseases, including cancer, the cellular redox balance is disrupted, leading to increases in the intra/extracellular levels of ROS that overwhelm the antioxidant and detoxification proteins producing oxidative stress, which may promote tumour onset and numerous aspects

Figure 1

Cellular mechanisms of ROS generation and elimination. Schematic representation of redox homeostasis of eukaryotic cells. ROS-generating enzymatic systems: eNOS, endothelial nitric oxide synthase; COX, cyclooxygenase; ETC, electron transport chain. Antioxidant enzymatic systems: SOD, superoxide dismutase; GPx, glutathione peroxidases; TRX, thioredoxine reductases; PRDX, peroxiredoxins. ROS: $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; H_2O_2 , hydrogen peroxide; ONOO $^{\cdot}$, peroxynitrite. Extracellular H_2O_2 can diffuse through specific membrane transporters such as aquaporins (AQP).

of progression towards a malignant phenotype, including angiogenesis, proliferation, epithelial-to-mesenchymal transition (EMT), invasion and apoptosis (Holmström & Finkel 2014).

H_2O_2 is an important molecule. Because of its physicochemical properties, it is capable of serving as a messenger that carries a redox signal from the site of its generation to a target site. Among the various oxygen metabolites, H_2O_2 is considered the most suitable for redox signalling because it mediates signal transduction through the modification of specific cysteine residues located within redox-sensitive protein targets (Veal *et al.* 2007, Sies 2017). Additionally, H_2O_2 is a stable, mild oxidant that is electrically neutral; therefore, it can diffuse freely within and between cells, either passively or through specific membrane transporters such as aquaporins, and thus, initiates immediate cellular effects. The spatial distribution of H_2O_2 in cells is not uniform. There are substantial gradients, both between the extracellular to intracellular spaces and between the subcellular spaces (Antunes & Cadenas 2000, Huang & Sikes 2014). The ranges of H_2O_2 concentration during oxidative stress and during cellular responses have been estimated. The intracellular physiological level of H_2O_2 likely ranges from 1 and 10 and up to approximately 100nM (Sies 2017). At these low and moderate concentrations,

H_2O_2 can mimic growth factor stimulation and can activate cell proliferation (Burdon *et al.* 1995). For example, in response to growth factors, a transient increase in the levels of intracellular H_2O_2 , is able to oxidize catalytic cysteines in protein tyrosine phosphatases, which leads to their inactivation and a consequent increase in tyrosine phosphorylation-dependent signalling (Meng *et al.* 2002).

The endocrine function of the thyroid gland is thyroid hormone synthesis (T_3 and T_4), and during this process, follicular thyroid cells called 'thyrocytes' produce a significant amount of H_2O_2 within the follicular lumen, or colloid, at the apical membrane of thyrocytes (Carvalho & Dupuy 2017) (Fig. 2). Thus, owing to the oxidative nature of thyroid hormone synthesis, the thyroid gland remains one of the most vulnerable organs to the deleterious effects of oxidative stress. Therefore, thyrocytes have the difficult task of tightly regulating the equilibrium between ROS production and scavenging (Kohrle & Gartner 2009).

Figure 2

Hypothetical model of the role of H_2O_2 -generating NADPH oxidases in DNA damage in the thyroid. At the apical membrane of follicular cells, thyroid peroxidase (TPO) uses H_2O_2 locally produced by DUOX2 to catalyse hormone biosynthesis. The role of DUOX1 in the thyroid remains to be established. However, it could compensate for DUOX2 deficiency. In a pathological context, H_2O_2 produced by DUOXs can diffuse through the apical membrane of thyrocyte and could reach the nucleus directly or via redox signalling pathways that might activate the intracellular NADPH oxidase 4 (NOX4), which is detected in the nucleus and in the endoplasmic reticulum. The presence of NOX4 in the perinuclear region might increase the nuclear oxidative stress and promote DNA damage and genomic instability.

In this context, the importance of some selenium (Se)-containing antioxidative enzymes and proteins has been described. These proteins are expressed in thyrocytes, are partially secreted into the colloid lumen and mainly belong to the glutathione peroxidase (GPx) and thioredoxin (Txn) reductase (TxnRd) families (Kohrle & Gartner 2009). Together with GPx, catalase, as well as the peroxiredoxins (PRDX), also protect thyroid cells against H₂O₂ (Kim *et al.* 2000, Nicolussi *et al.* 2017).

After establishing the critical role of oxidative stress in thyroid tumourigenesis, this review will assess how ROS are involved in genetic instability, which contributes to the initiation and development of tumours. This review will also focus on two ROS-generating systems: the NADPH oxidases DUOX1 and NOX4 and their roles in DNA damage, senescence and thyroid dedifferentiation.

Oxidative stress and thyroid carcinomas

Since oxidative stress is associated with the occurrence and development of several cancers, prospective studies have investigated the relationship between the oxidative stress parameters and the serum profiles of thyroid cancer patients and controls in order to identify one or more markers to distinguish thyroid cancer from different types of benign thyroid disease. The oxidative stress parameters were determined by measuring the total antioxidant status (TAS) and the total oxidant status (TOS), and the oxidative stress index (OIS) was calculated as the ratio of TOS to TAS. Analysis of the oxidative status of sera showed that oxidant levels were significantly increased in patients with thyroid cancer compared to controls and that antioxidant levels were lower in patients with thyroid cancer than in healthy controls (Wang *et al.* 2011). An increase in the oxidative stress in the blood of patients affected by thyroid cancer was also detected by electronic paramagnetic resonance measurements, which were significantly altered following a thyroidectomy (Metere *et al.* 2012). In addition, a high concentration of malondialdehyde (MDA), an oxidized lipid-derived aldehyde, was found in the blood of patients affected by thyroid cancer (Akinci *et al.* 2008, Geric *et al.* 2016), and this was associated with a decrease in the antioxidative capacity of whole blood. All these results suggest that there is a relationship between oxidative stress and the presence of thyroid cancer. However, it remains unclear how a tumour of a few cm³ can impact the oxidative status of serum, and whether these changes are risk factors for thyroid cancer or the consequence of its occurrence. The role of oxidative stress in thyroid cancer was characterized by analysing the expression of two

selenium antioxidant molecules, glutathione peroxidase (GPx1) and thioredoxin reductase (TrxR1) in thyroid cancer cells. Decreased expression of both proteins was observed in cancer cells compared to healthy cells and was associated with an increased level of free radicals in the tumour tissue (Metere *et al.* 2018). The expression of catalase was also dramatically decreased in human thyroid tumours compared with normal thyroid tissue (Hasegawa *et al.* 2002, 2003). These findings illustrate the imbalance of the oxidant/antioxidant system in thyroid cancer tissue.

In addition, the expression of peroxiredoxins PRDX1 and PRDX6 was repressed in papillary thyroid carcinomas (Nicolussi *et al.* 2014), but conversely, increased expression of PRDX5 was reported in Hürthle cell carcinoma, a type of papillary thyroid carcinoma (PTC). However, the role of PRDXs in cancer remains controversial (Nicolussi *et al.* 2017).

Oxidative DNA damage in thyroid carcinomas

Oxidative stress is considered to be a cause of DNA damage, which is in turn an initial step of tumourigenesis (Krohn *et al.* 2007). The direct interaction of ROS with DNA can lead to the formation of oxidative DNA base lesions, including abasic sites, single-strand DNA breaks, sugar moiety modifications, deaminated bases and adducted bases (Sedelnikova *et al.* 2010). If unrepaired, these DNA lesions could change some of the genetic information, consequently affecting genome integrity by causing mutagenesis and by inhibiting both replication and transcription. Despite the reported mutagenic and carcinogenic roles of oxidative DNA damage, it is difficult to assess the mutational specificity and the clinical significance of each individual oxidative DNA modification because of the number of these oxidative modifications and their derivative products. Among the four DNA bases, guanine is the preferential target for oxidation because it has the smallest redox potential (Boiteux *et al.* 2017); thus, guanine oxidation is considered to be a useful marker of oxidative DNA damage during carcinogenesis (Kasai 1997). The oxidized form of guanine, 8-oxo-2'-deoxyguanosine (8-oxo-dG), is repaired by the base excision repair (BER) pathway, a multistep process that requires the DNA repair enzyme 8-oxoguanine DNA glycosylase (a domain of the protein N-glycosylase/DNA lyase, encoded by *OGG1*) (Sedelnikova *et al.* 2010). 8-Oxo-dG is frequently studied as a potent mutagenic oxidative base because of its tendency to mispair with an adenine base; thus, GC-to-AT transitions and GC-to-TA transversions are the

most prominent oxidative mutations (Wang *et al.* 1998). In addition, oxidative stress can oxidize the intracellular dGTP (deoxyguanosine triphosphate) pool, which mispairs with adenine during replication and results in a mutation (Scott *et al.* 2014). H_2O_2 itself is almost inert towards DNA; however, the H_2O_2 -derived hydroxyl radicals ($\text{OH}^{\bullet}$) are actively involved in the oxidation of DNA. This is closely linked with the participation of redox-active metals, such as iron, that participate in the Fenton reaction and generate reactive hydroxyl radicals. Although iron regulation ensures that there is no free intracellular iron, under stress conditions, an excess of ROS releases 'free iron' from iron-containing molecules (Valko *et al.* 2006).

In mouse models, the levels of oxidative DNA damage in the thyroid gland were higher than those in any other organ, with high levels of thyroid *OGG1* mRNA expression (Maier *et al.* 2006). Increased nuclear levels of 8-oxo-dG were found in both benign (human follicular adenomas or FTAs) and malignant (follicular (FTC) and PTC) lesions in comparison with matched normal tissue (Young *et al.* 2010, Karger *et al.* 2012); this most likely reflects the deleterious impact of a long-term exposure to chronic oxidative stress that is observed during thyroid malignancy. Interestingly, an investigation of the link between *BER* gene expression and human thyroid malignancy showed a reduction in *OGG1*

expression in FTC compared to FTA (Karger *et al.* 2012). TCGA data analysis highlights a downregulation of the expression level of *OGG1* in papillary thyroid tumours with a high level of *MAPK1* expression, which reflects the activation of the MAPK signalling pathway (Fig. 3A) (C Dupuy, unpublished observations), supporting the potential contribution of oxidative DNA damage to the thyroid dedifferentiation mechanism, which may lead to radioresistance.

However, compared to other tumours, thyroid tumours show a low overall density of somatic mutations, suggesting that DNA repair has an important role in the maintenance of DNA integrity. Distinct signatures that are associated with deficiencies in DNA repair pathways such as HR (homologous recombination), MMR (DNA mismatch repair) and NER (nucleotide excision repair) have been found in various cancers, but no mutational signature has been attributed to impaired BER activity or to oxidative DNA lesions (Helleday *et al.* 2014). This suggests that the signatures might reflect a strong, intermittent process that temporarily overwhelmed the DNA repair capacity or that provoked error-prone repair (Tubbs & Nussenzweig 2017). Both inter- and intra-pathway complementation exists in the repair mechanisms of oxidative base damage (Berquist & Wilson 2012). The majority of driver mutations in the *BRAF* and *NRAS* oncogenes occur at A/T pairs (Hodis *et al.* 2012). It has been suggested that error-prone repair using

Figure 3

POLH, *MSH2* and *MSH6* expression levels are significantly increased in papillary thyroid cancer (PTC) harbouring *BRAF*^{V600E} mutation. A homogeneous cohort of 390 PTCs (170 *BRAF*^{WT} PTCs and 220 *BRAF*^{V600E} PTCs) were included in this analysis. Correlative analysis between *OGG1* gene expression and *MAPK1* gene expression (A). Correlative analysis between *MSH2* mRNA (B) or *MSH6* mRNA (C) or *POLH* mRNA levels (D) and *BRAF*^{V600E} mutation in 390 PTCs. Values are mean $\pm$ s.e. *** $p < 0.0001$. ERK, extracellular signal-regulated kinase.

the translesion DNA polymerase eta (POL η) contributed to these driver mutations, which are expressed in 40–60% (for BRAF) and 6% (for NRAS) of PTCs, respectively. Notably, the MSH2–MSH6 heterodimer, which plays a fundamental role in the recognition of base pair mismatches and the signalling of mismatch repair (MMR), acts in concert with POL η to remove complex DNA lesions induced by oxidative stress in human cells (Zlatanou *et al.* 2011). Moreover, investigations have suggested that oxidative stress caused by inflammation may be partly responsible for mutations of the BRAF gene (Martinez-Cadenas *et al.* 2011). Interestingly, in TCGA data, POLH, MSH2 and MSH6 expression levels are significantly increased in PTCs with a BRAF mutation, suggesting that this complex might be involved in an alternative DNA repair pathway that compensates for the decrease in OGG1 in these tumours (Fig. 3B, C and D) (C Dupuy, unpublished observations). In addition, this complex might be the origin of the oncogenic mutations found in PTCs.

In some circumstances, clustered oxidative DNA lesions, which are difficult to resolve, can lead to the formation of DNA double-strand breaks (DSBs) (Sedelnikova *et al.* 2010). DSBs can promote genetic instability and chromosomal rearrangements (Richardson & Jasin 2000). Interestingly, exposure of human thyroid cells to H₂O₂ induces DSBs (Driessens *et al.* 2009, Ameziane-El-Hassani *et al.* 2010) and, consequently, an oncogenic RET/PTC rearrangement (Ameziane-El-Hassani *et al.* 2010), which is an early event in thyroid follicular cell transformation (Viglietto *et al.* 1995). Upon H₂O₂ exposure, thyroid cells increase the expression of the antioxidant response genes and the inhibition of this response markedly decreases cellular resistance to H₂O₂ and promotes DNA damage (Versteyhe *et al.* 2013); this corroborates that the weakening of the detoxification process might favour the mutagenic effects of H₂O₂.

From an experimental point of view, the phosphorylated form of the histone H2AX (γ -H2AX) is considered a useful marker of both DNA lesions and the DNA damage response or 'DDR' (Valdiglesias *et al.* 2013). Immunohistochemical staining analysis showed that γ -H2AX was increased in PTC compared to normal adjacent tissue and to nodular goitre (Hu *et al.* 2014). Moreover, the nuclear immunostaining of 53BP1, a specific marker of DNA DSBs, reveals its presence in thyroid tumours with intense staining within the nucleus of high-grade thyroid tumours (Nakashima *et al.* 2008); thus, suggesting a strong involvement of DNA DSBs and defects in their repair in the evolution of thyroid cancers.

Thyroid ROS-generating systems: the NADPH oxidases and the mitochondria

In the last decade, the involvement of NADPH oxidases, which produce ROS as their primary and sole function, has become of particular interest in thyroid malignancy. Unlike other enzymes, such as xanthine oxidase, cytochrome P450 and those of the mitochondrial electron transport chain, which are capable of producing ROS, NADPH oxidases produce H₂O₂ and/or O₂^{•-} in a tightly controlled manner and act locally in the redox control of specific signalling pathways (Ameziane-El-Hassani *et al.* 2016). The other enzymes listed above produce ROS as an accidental by-product of their primary catalytic pathways. In this case, the production is neither specific nor transient, which are the two conditions that define signalling. Until the early 2000s, the only known NADPH oxidase was the phagocyte NADPH oxidase, which plays a key role in innate immunity by generating and releasing large amounts of superoxide (O₂^{•-}) into the phagosomes. It is composed of a membranous and heterodimeric flavocytochrome including gp91^{phox}, the catalytic core and p22^{phox}. The discovery of six homologs of gp91^{phox}, which is now called NOX2, over the past 18 years, has given rise to a new interest in this type of enzymes. The six other NOXs that are now identified are NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2.

The thyroid gland expresses three NADPH oxidases: DUOX1, DUOX2 and NOX4. The DUOX enzymes were first identified in the thyroid gland as H₂O₂-generating systems associated with thyroperoxidase (TPO), which is the enzyme that catalyses thyroid hormone biosynthesis at the apical surface of thyrocytes (Dupuy *et al.* 1999, De Deken *et al.* 2000) (Fig. 2).

Each DUOX requires maturation factors (DUOXA1 and DUOXA2) to exit from the endoplasmic reticulum (ER) and to reach the plasma membrane, where they form a stable complex; this is a prerequisite for their H₂O₂-producing activity (Grasberger & Refetoff 2006). Different phosphorylation pathways regulate the enzymes through TSH: DUOX1 is activated by protein kinase A (Gs-PKA pathway); DUOX2 activation occurs through protein kinase C (Gq-phospholipase C pathway) and calcium is the primary activator for both of these enzymes (Rigutto *et al.* 2009).

In addition to the catalytic core, which contains six transmembrane domains and is common among their five counterparts (NOX1–5), the two DUOXs have an N-terminal peroxidase-like ectodomain whose structure, which is controlled by an intramolecular disulphide

bridge, is crucial for both the functioning and targeting of DUOXs (Carre *et al.* 2015, Louzada *et al.* 2018). A functional interaction between DUOX and TPO, which is promoted by H_2O_2 itself, regulates the level of extracellular H_2O_2 (Fortunato *et al.* 2010, Song *et al.* 2010). This association at the plasma membrane might have a role in limiting the diffusion of H_2O_2 into the cell and thereby protecting cells from oxidative damage. Thus, in addition to leading to the sustained proliferation of thyroid cells due to hypothyroidism, the absence of TPO might also promote cancer development. This chemoprotective effect of TPO could explain the severity of the symptoms associated with congenital goitres and the frequent evolution of this condition to nodule and tumour formation in patients who have a defect in TPO. The role of H_2O_2 in thyroid tumourigenesis might be reinforced by the observation that TPO expression and activity are low, or even undetectable, in thyroid cancer tissue. Analysis of TCGA data shows that in the case of PTC, the expression of *DUOX1*, *DUOX2* and their maturation factors is associated with tumour differentiation; lower expression is observed in tumours with the *BRAF*^{V600E} mutation, which are less differentiated than other PTC tumours (Cancer Genome Atlas Research Network 2014). Although the expression of *DUOX1* and *DUOX2* is positively correlated to thyroid carcinoma (Lacroix *et al.* 2001), this finding does not imply that each *DUOX* has the same level of involvement in the early stages of tumour development.

The thyrocytes also express the NADPH oxidase NOX4. The physiological function of NOX4 in the thyroid is unknown. No thyroid dysfunction has yet been described for NOX4-knockout animal models. This NOX, which was first identified in the kidney, is ubiquitously expressed at high levels, which is in contrast to the other NOX proteins. However, the effects of NOX4-derived ROS appear to be cell type dependent, probably owing to the distinct redox-sensitive targets that are affected in each cell type. NOX4 is the only NADPH oxidase with constitutive ROS-generating activity that depends directly on its gene expression. The enzymatic activity strongly depends on the heterodimerization of NOX4 with the membrane protein p22^{phox} but not on cytosolic subunits, such as NOX2. NOX4 is active not only at the plasma membrane but also in different intracellular compartments and organelles, including the ER, mitochondria and nucleus (Ameziane-El-Hassani *et al.* 2016) (Fig. 2). The presence of NOX4 in the perinuclear region might affect the level of oxidative stress in the nucleus and thus contribute to oxidative modifications and damage. NOX4 expression is increased in thyroid cancers and is particularly high in

PTC (Weyemi *et al.* 2010). Moreover, unlike *DUOX*, *NOX4* expression is controlled by TSH at the transcriptional level (Weyemi *et al.* 2010). An association has been found between high TSH levels and an increased risk of the development of malignant thyroid nodules (Haymart *et al.* 2009, Tam *et al.* 2018). In this case, TSH's potential effect on cancer cells would require the maintenance of their receptor's expression. As an ROS-generating system controlled by TSH, NOX4 could be contributing to a mechanism that drives this association.

Despite its ability to generate ROS during respiratory chain function, the causal relationship between mitochondria as a source of oxidative stress and thyroid tumourigenesis does not seem to be well established (Coelho *et al.* 2018). Mitochondrial ROS are produced by the leakage of electrons from complexes I and III of the electron transport chain (ETC) during oxidative phosphorylation, an important process that generates ATP. However, the $O_2^{\bullet-}$ production that occurs through this mechanism does not appear to be a regulated process. Several studies have now demonstrated that NOX4 is localized in the mitochondria and produces ROS under pathological conditions (Block *et al.* 2009). An abnormal accumulation of functionally defective mitochondria was reported in oncocytic/Hürthle thyroid carcinomas (Coelho *et al.* 2018). In this context, the decreased enzymatic activity of complexes I and III of the ETC was correlated with high ROS generation by this organelle (Bonora *et al.* 2006). Interestingly, these results could be related to a recent study that showed that OXPHOS-driven ATP production in the mitochondria controlled NOX4-derived ROS production. Under normal conditions, ATP binds allosterically to NOX4 through Walker A ATP-binding domain, keeping NOX4-derived ROS production low. However, certain cellular events, such as cancer, which switch ATP production to aerobic glycolysis in the cytosol, lead to a reduction of the ATP levels in the mitochondria, thereby relieving this inhibition and leading to NOX4-derived ROS production (Shanmugasundaram *et al.* 2017).

Risk, causes and dedifferentiation of thyroid cancers: involvement of NADPH oxidases DUOX1 and NOX4

Ionizing radiation (IR) can cause various delayed effects in cells, including genomic instability that leads to the accumulation of gene mutations and chromosomal rearrangements, which are thought to play a pivotal role in radiation-induced carcinogenesis. The persistence of such effects in the progeny of cells has profound

implications for long-term health risks, including the emergence of a secondary malignancy. The thyroid gland is one of the most sensitive organs to the carcinogenic effects of IR. The risk of thyroid tumours is maximal when exposure occurs at a young age, and it increases linearly with the radiation dose (Sinnott *et al.* 2010). More than 90% of these cancers are papillary tumours, which present with an *RET/PTC* chromosomal rearrangement in 70% of cases. The mechanisms by which radiation exposure is internalized and leads to delayed DNA breakage are poorly understood. Hypoxia and antioxidant therapy reduce the delayed X-ray-induced effects, suggesting that radio-induced oxidative stress plays a significant role in determining the susceptibility of irradiated cells to genetic instability (Robbins & Zhao 2004). To analyse how ROS cause persistent instability, we recently investigated the mechanism by which IR induces the generation of ROS several days after irradiation. The results showed that the NADPH oxidase DUOX1 promoted the long-term persistence of oxidative stress after exposure to irradiation (Ameziane-El-Hassani *et al.* 2015) (Fig. 4). Abrogation of DUOX1 expression resulted in a significant decrease in post-irradiation DNA damage in thyroid cells, indicating that DUOX1-dependent H_2O_2 production plays a key role in persistent radio-induced DNA damage. This damage was observed several days after irradiation and was materialized by the presence of γ H2AX and 53BP1 nuclear foci in G1-phase cells. These foci could have formed following a replicative stress that induced chromosomal damage that bypassed mitosis and was then transmitted to daughter cells. Indeed, one of the recognized sources of

replication stress is oxidative DNA damage, which creates physical barriers to the progression of the replication fork. In addition, ROS can also affect, via redox mechanisms, the enzymes involved in the metabolism of nucleotides that are essential for both the synthesis and repair of DNA and/or can inhibit the enzyme systems involved in replication or in the repair of DSBs. There are genomic regions that are prone to replication stress-induced DSBs. These regions, called 'common fragile sites' (CFS), are sensitive readouts for replication stress (Zeman & Cimprich 2014). *RET* and *CCDC6*, the two genes involved in the oncogenic translocation of *RET/PTC1*, are both located in CFS (Gandhi *et al.* 2010). The overexpression of DUOX1 in radio-induced thyroid tumours suggests that this NADPH oxidase may contribute to chronic oxidative stress, thereby promoting genomic instability and tumourigenesis (Ameziane-El-Hassani *et al.* 2015). Replication stress and oxidative stress are intertwined and reinforce each other in the driving of genomic instability (Xu *et al.* 2014). Moreover, a high level of DUOX1 protein was also observed in some sporadic thyroid tumours, suggesting that high levels of DUOX1 might be required for thyroid tumourigenesis. Notably, *DUOX1* was not differentially expressed in sporadic PTCs that occurred in the absence of previous radiation exposure or in radiation-induced PTCs from the Chernobyl Tissue Bank (Detours *et al.* 2007).

PTCs are the most frequent histological type of human thyroid carcinoma and account for approximately 85% of all thyroid carcinomas. PTCs include several tumour types that have mutually exclusive mutations of genes that

Figure 4

Consequences of increased DUOX1 expression after irradiation of thyroid cells. (A) X-ray irradiation of thyroid cells elicits two temporally separated phenomena: first, an oxidative burst mediated by radiolysis of water, and, second, a persistent oxidative stress mediated by DUOX1 upregulation. (B) Irradiation leads to a delayed increased DUOX1 expression through activation of p38 MAPK. This regulation is controlled via IL-13. Radio-induced DUOX1-dependent H_2O_2 production plays a key role in persistent DNA damage whose consequences can be, in particular, induction of genetic instability that promotes the formation of chromosomal rearrangements.

encode effectors that signal through the MAPK pathway (Fagin & Wells 2016). The *BRAF*^{V600E} point mutation, encoding the *BRAF*^{V600E} oncogenic constitutively active protein kinase, accounts for 60% of these mutations; this is followed by *RAS* mutations (15%) and by chromosomal rearrangements that lead to illegitimate expression of the kinase domains of *BRAF* or receptor tyrosine kinases, such as *RET*, *NTRK* and *ALK* (12%). Clinically, *BRAF*^{V600E} is associated with a more extensive disease, a higher rate of recurrence and decreased survival (Xing 2013). *In vivo* and *in vitro* studies have demonstrated the key role of *BRAF*^{V600E} in tumour progression and in the formation of the most undifferentiated stages. It is not fully understood how the expression of mutant *BRAF* contributes to the aggressive character of PTCs, including in particular an alteration of the expression of genes involved in the metabolism of iodine, which is essential for the synthesis of hormones. Iodine uptake is catalysed by the sodium-iodide symporter (*NIS*), a membrane glycoprotein located in the basolateral membrane of thyrocytes. This property has been used for many years in therapeutics that treat thyroid cancer with radioactive iodine-131. However, some patients are initially refractory to this therapy or may become refractory to this therapy because of a loss of *NIS* expression, which occurs fairly early during oncogenesis. An understanding of the mechanisms that underlie this loss of expression is important for the design of a therapeutic strategy that can correct for this anomaly. The inhibition of the mitogen-activated protein kinase (MAPK) pathway is not always efficient, in particular, for the redifferentiation of radioiodine-resistant tumours, which suggests that other compensatory mechanisms contribute to *BRAF*^{V600E} adaptive resistance (Ho *et al.* 2013). Several studies have shown an important role for transforming growth factor B1 (*TGF-β*) in this process. *TGF-β* is overexpressed in thyroid malignancies (Matoba *et al.* 1998, Vasko *et al.* 2007). *BRAF*^{V600E} expression induces the production of *TGF-β*1, which leads to a *TGF-β*-driven autocrine loop that mediates, at least in part, the effects of the *BRAF*^{V600E} oncoprotein; it particularly mediates the decreased expression of *NIS* (Riesco-Eizaguirre *et al.* 2009) and the promotion of cell migration, invasiveness and EMT (Knauf *et al.* 2011). Recently, we showed that *NOX4* is upregulated by *BRAF*^{V600E} via a *TGF-β*-Smad3-dependent pathway in thyroid cancer cells and that *NOX4*-dependent ROS generation has a critical role in the *NIS* repression that is induced by mutant *BRAF* (Azouzi *et al.* 2017) (Fig. 5); this corroborated previous studies suggesting that the *SLC5A5* gene promoter activity that encodes the *NIS* symporter is dependent on the redox state of the cell

(Puppin *et al.* 2004). The reversible nature of the reduced expression of the *SLC5A5* gene by *NOX4* suggests that an epigenetic mechanism may be active that may involve a redox-sensitive control mechanism for the recruitment of silencing complexes that contain DNA methyl transferase and histone deacetylases (HDACs) to the promoter region.

Figure 5

Oncogenic BRAF-dependent regulation of *NOX4*. (A) Mutant *RAS* and *BRAF* activate mitogen-activated protein kinase (MAPK) signalling at different degree with high MAPK signalling and *NOX4* expression in tumours driven by *BRAF* compared to that driven by *RAS*. This was inversely correlated with cell differentiation. (B) Proposed model for *BRAF*^{V600E} mediated *NIS* repression in thyroid cancer cells. *BRAF*^{V600E} increases *TGF-β* synthesis and secretion via the MAPK signalling pathway. *NOX4* is upregulated by *BRAF*^{V600E} in a *TGF-β*-Smad3-dependent pathway and *NOX4*-derived ROS participate to *NIS* repression. The mechanism could involve redox-sensitive epigenetic modifications.

The link between *BRAF*^{V600E} and *NOX4* was confirmed by a comparative analysis of *NOX4* expression in human (TCGA) and mouse thyroid cancers. The level of *NOX4* expression in human and murine *BRAF*^{V600E}-mutated thyroid tumours is inversely correlated with thyroid differentiation; this suggests that genes other than NIS that are involved in thyroid differentiation might be silenced by a mechanism controlled by *NOX4*-derived ROS (Azouzi *et al.* 2017). However, further investigations are needed to determine whether this increase in *NOX4* expression is a cause or a consequence of the thyroid dedifferentiation process. Moreover, tumour-associated mutant p53 proteins enhance *NOX4* expression in both TGF- β -dependent and TGF- β -independent processes in human breast and lung epithelial cells (Boudreau *et al.* 2014). Inactivation of p53 has been considered a hallmark of advanced thyroid tumours (Fagin *et al.* 1993). *TP53* mutations are highly prevalent in ATCs, which have a higher mutation burden. This might be related to an increase in *NOX4*-induced DNA damage via ROS production, in addition to the loss of the canonical function of ATM kinase in some of these tumours (Landa *et al.* 2016) as ATM is required for successful DNA repair (Cremona & Behrens 2014).

In addition, regardless of its level of expression, NIS must be sufficiently expressed at the plasma membrane to mediate effective RAI uptake. Several studies have demonstrated that NIS is mis-localised to the cytoplasm in thyroid cancers (Dohan *et al.* 2001, Smith *et al.* 2013). At the present time, it is unknown whether ROS affect NIS trafficking. However, we recently observed that *NOX4* inactivation increased the cell surface expression of NIS in two *BRAF*-mutant thyroid cell lines, suggesting that the *NOX4*-derived ROS might also impact the signalling pathways involved in NIS trafficking (Azouzi *et al.* 2017).

Thyroid tumour-associated oncogenes (*BRAF*^{V600E}, H-RAS^{V12}, RET/PTC1 and RET/PTC3) have been shown to trigger permanent cell cycle arrest, known as oncogene-induced senescence (OIS), in human thyroid cells (Vizioli *et al.* 2011, 2014, Weyemi *et al.* 2012, Bellelli *et al.* 2018). Severe or irreparable DNA damage is involved in the proliferative arrest and induces a DNA damage response that precedes senescence. Initially, senescence acts as a barrier to tumourigenesis, and a bypass of senescence at later stages might enable cellular transformation and tumour progression. Immunohistochemical analysis of human thyroid tumours has revealed high expression levels of senescence markers during the early stages of thyroid tumourigenesis that are lost during the later stages, which provides an indirect demonstration that

senescence evasion is a mandatory step during thyroid tumourigenesis (Vizioli *et al.* 2011). Conditional expression of H-RAS^{V12} in human thyroid cells positively regulated *NOX4*-derived H₂O₂, which induced DNA damage that led to replicative stress and subsequently to senescence (Weyemi *et al.* 2012). Analysis of human PTCs (TCGA) showed that the *NOX4* expression level was correlated with ERK activation (Azouzi *et al.* 2017). Thus, the level of *NOX4* expression in thyroid tumours may depend on the level of MAPK activation, and the expression level may thus contribute to the establishment of senescence. However, senescence-associated ROS may also promote the emergence of pretumoural cells through mutagenicity of ROS and thus may explain why some tumours, such as *BRAF*^{V600E}-mutated thyroid tumours, overexpress *NOX4*.

Conclusion and perspectives

Oxidative stress is a risk factor associated with thyroid tumourigenesis. Until recently, the sources of ROS were not defined. The identification of the NADPH oxidases DUOX1 and *NOX4* as sources of ROS in thyroid tumourigenesis has opened the way for research concerning their respective roles in the mechanisms that lead to genetic instability and to dedifferentiation. The dysregulation of their expression and activity can threaten DNA stability and, therefore, influence cell fate. This dysregulation may have far-reaching health implications. DUOX1 promotes persistent DNA damage after irradiation, which is also a risk factor for thyroid cancer. Further studies are now needed to determine how DUOX1 is involved in radiocarcinogenesis and, in particular, what is its potential role in DNA break formation at chromosomal fragile sites, which are hotspots for chromosomal translocations such as RET/PTC. *NOX4* also seems to have a critical function in thyroid tumourigenesis, particularly in PTC harbouring the *BRAF*^{V600E} mutation, which are associated with cell dedifferentiation and radioiodine refractoriness. The mechanisms that may connect *NOX4* to the dedifferentiation process need to be studied in depth, but *NOX4* may become a new potential therapeutic target due to its role in NIS repression.

The absence or loss of radioactive iodine (RAI) uptake in thyroid cancer cells is a major challenge for the treatment of patients with thyroid cancer. In cases with distant metastases, RAI can cure one-third of the patients, but in other cases, it is not concentrated by the tumours or the tumours are resistant to RAI (Durante *et al.* 2006). An approach for the treatment of RAI-refractory patients is to re-enhance RAI uptake or to redifferentiate

tumours. Restoring RAI uptake is indeed the first step of redifferentiation. It is mandatory, but to be clinically effective, it must be followed with anti-tumour effect. Recently, drugs targeting the MAPK pathway have been studied since the activation of the MAPK pathway is known to be associated with cell dedifferentiation and with a decrease in RAI uptake. In human *BRAF*-mutant PTCs, there is a low expression level of NIS and a low tumour differentiation score (Riesco-Eizaguirre *et al.* 2006, Romei *et al.* 2008, Fagin & Wells 2016). In an engineered mouse model bearing the *BRAF*^{V600E} mutation, thyroid cancer is insensitive to RAI treatment, and the treatment of these mice with small-molecule inhibitors of either MEK or mutant *BRAF*^{V600E} reduces their proliferative index and partially restores thyroid-specific gene expression (Chakravarty *et al.* 2011). Based on these studies, MEK inhibitors and *BRAF* inhibitors have been developed for *BRAF*- and *RAS*-mutated TC patients. Pilot clinical studies in RAI-refractory patients showed an increase in the RAI avidity and tumour response following RAI treatment, when it was preceded by treatment with a MEK inhibitor (selumetinib) or a *BRAF* inhibitor (dabrafenib or vemurafenib) in patients with a *RAS* or *BRAF*^{V600E} mutation (Ho *et al.* 2013, Rothenberg *et al.* 2015, Dunn *et al.* 2018). Unfortunately, not all patients respond to these treatments, and further inhibition of the MAPK pathway with combination treatment is being investigated. Indeed, a strong inhibition of ERK signalling with potent anti-MEK drugs was shown to be essential to maximize *BRAF*^{V600E}-thyroid cancer redifferentiation, providing a rationale for the combination of anti-*BRAF* and anti-MEK drugs in patients (Nagarajah *et al.* 2016). Clinical trials are underway with such a combination. Based on the understanding of oxidative stress in thyroid carcinoma that has been developed previously, future treatments with drugs targeting NOX4, as a single agent or in combination, have to be tested. Finally, the dedifferentiation of thyroid tumours is associated with an increase in the mutation burden, which might reflect the error-prone repair of oxidative DNA lesions. Tumour mutation burden has been demonstrated to be useful biomarker for immune checkpoint blockage across some cancer types. However, the mutation burden in thyroid cancer remains low compared to what is seen in lung cancer or melanoma. We have few data regarding response to immune checkpoint inhibitors at the moment.

Declaration of interest

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