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The transcription factor NRF2 shapes the identity of radio-resistant tumor cells

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Abstract

Tumor recurrence and resistance to therapy remain challenging issues in oncology. Various theories have been tested, including the stem cell hypothesis, but conflicting results were obtained, and are not applicable to all tumors types. We have addressed an overarching theory focused on cell "survivology" in cancer, and analyzed the existing information on the involvement of the cytoprotective transcription factor NRF2 in tumor progression, tumorigenicity and radio-resistance, with special emphasis on cancer stem cells (CSCs). The review is focused on NRF2-mediated responses of tumor cells under the pressure of selective intracellular and microenvironmental forces that shape their genotype and phenotype, deciding which cells survive and which ones die. The cytoprotection provided by NRF2 distinctively to normal and tumor cells, the common and unique mechanisms accounting for the survival and evolution of tumor cells and CSCs in the harsh tumor environment, including the particular NRF2-driven hallmark features of CSCs in the context of radiotherapy, are emphasized. Finally, existing natural compounds and small molecules showing at preclinical and clinical level an inhibitory action on NRF2 are revised. Altogether, the review highlights that NRF2 might be considered as an oncogenic marker with clinical prognostic value for disease evolution and response to radiotherapy-based treatment strategies.

Key words:

NRF2, tumor cells, cancer stem cells, tumorigenicity, radio-resistance.

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Introduction

Despite huge research efforts, cancer remains an important health issue of the modern world (https://ourworldindata.org/cancer). Characterization of individual tumors in the frame of precision medicine for developing personalized and highly targeted therapies still remains a challenge due to the enormous heterogeneity and plasticity within each individual tumor, related both to tumor cells themselves and to the complex influence exerted by tumor stroma (Wingrove et al., 2019). Surprisingly, the apparent simplification ensuing from the distillation of the vast knowledge on the features of particular cancer cells was the working approach that brought huge progress in advancing research (Hanahan and Weinberg, 2000) by pointing major issues that have to be tackled for each individual study. The hallmarks of cancer have been defined as acquired functional capabilities that allow cancer cells to survive, proliferate and disseminate. These particular abilities emerge in different tumor types via distinct mechanisms and at various time points during multistep tumorigenesis (Hanahan and Weinberg, 2011) (**Figure 1**). Aberrant signals coming both from the inside of cells as well as from the tumor stroma and tumor-infiltrating immune cells shape the identity of tumor cells sub-populations (Bhowmick and Moses, 2005).

In the bulk of functionally heterogeneous cancer cells a small subset exhibiting stemness features and exquisite resistance to therapy was identified. This population of cancer cells has the exclusive ability to self-renew and to repopulate the entire tumor through multi-lineage differentiation and asymmetric division, henceforth accounting for tumor progression and relapse. Based on the capacity of these cells to initiate tumors when transplanted into immunocompromised mice (Aguirre-Ghiso, 2007), they have been termed tumor-initiating cells (TICs). Surface markers of TICs have been identified, and proved to be highly dependent on the tumor type. Besides this inconvenient

Abbreviations

ABC, ATP-binding cassette;
ALDH, aldehyde dehydrogenase;
ARE, antioxidant response elements;
ATM, ataxia–telangiectasia mutated;
ATR, ataxia telangiectasia and Rad3-related protein;
ATRA, all-trans retinoic acid;
BMI, polycomb suppressor protein complex I;
CBP, cAMP-responsive element-binding protein (CREB) binding protein;
CDDO-Me, bardoxolone methyl;
Chk, checkpoint kinase;
CP, clobetasol propionate;
CSC, cancer stem cell;
CUL3-RBX1, Cullin 3-Ring Box Protein 1;
EMT, epithelial-to-mesenchymal transition;
ERK1/2, extracellular regulated serine-threonine kinases;
FLASH-RT, FLASH radiotherapy;
FOXO, forkhead box proteins O;
GCL, glutamate–cysteine ligase;
GCLC, glutamate–cysteine ligase catalytic subunit;
GCLM, glutamate–cysteine ligase modifying subunit;
GEO, Gene Expression Omnibus;
GSH, glutathione;
GSK-3, glycogen synthase kinase 3;
Gy, Grey;
HIF-1, hypoxia-inducible factor 1;
HO-1, hemoxygenase 1;
HRD1, endoplasmic reticulum-E3 ubiquitin ligase;
HT, hadron therapy;
IGF, insulin-like growth factor;
IRE1, inositol requiring enzyme;
JNK, c-Jun NH2-terminal kinase;
KEAP1, Kelch-like ECH associated protein 1;
MAPK, mitogen activated protein kinase;
miRNA, microRNA;
NFE2L2, the gene encoding NRF2;
NRF2, nuclear factor erythroid 2-related factor 2;
PDT, photodynamic therapy;
Ph-RT, photon radiotherapy;
PINK1, PTEN-induced kinase 1;
PPARγ, nuclear peroxisome-proliferator activator receptor γ;
PS, photosensitizer;
ROS, reactive oxygen species;
RT, radiotherapy;
RXRα, retinoid X receptor alpha;
SOD, superoxide dismutase;
STING, stimulator of interferon genes;
TCGA, The Cancer Genome Atlas;
TIC, tumor-initiating cell;
β-TrCP, β-transducin repeat-containing E3 ubiquitin protein ligase;
xCT, glutamate–cystine antiporter.

such markers greatly helped to isolate positive TICs from the bulk of tumor cells using multi-parametric flow cytometry (Greve et al., 2012), hence allowing for in-depth molecular and functional studies. Yet, removal of cells out of their natural environment, especially in the case of solid tumors, can significantly alter their phenotype and functions, considering that they transiently adapt to the microenvironment (Dirkse et al., 2019).

It has been found that many of the identified TIC markers and signalling mechanisms were also specific for normal stem cells, and therefore TICs were alternatively termed cancer stem cells (CSCs). The cancer stem cell theory considers that the heterogeneous population of tumor cells may arise from a common stem cell ancestor, and tumor is seen as an "abnormal organ" (Reya et al., 2001; Gisselsson, 2011). This theory is in good agreement with the quiescence, self-renewal ability and pluripotency of TICs (Aguirre-Ghiso, 2007). Nevertheless, the finding regarding the differentiation and de-differentiation shifts between CSCs and regular tumor cells (Huang et al., 2015) questions the stem cell origin of TICs. For instance, it has been shown that a surprisingly high fraction (25%) of phenotypically diverse melanoma cells is capable of tumorigenesis when cells were injected into highly immunocompromised NOD/SCID IL2Rγnull mice, far above the low percentage of CSCs (0.05-1%) generally identified using CSC markers (Quintana et al., 2010). The connection between genomic instability, selective forces, clonal variation, survival of the newly generated clones and the competition among diverse clones in different microenvironments should be further investigated for better answering the question whether cancer stem cells really exist (Gisselsson, 2011). The cancer stem cell theory and the existing highly variable data have been recently re-evaluated using the hierarchy-of-hypotheses approach (Bartram and Jeschke, 2019), in which an overarching hypothesis is divided into sub-hypotheses to create a hierarchical structure into which every empirical study in question is then sorted. The study brought arguments for the urgent need for: (a) clarity the terminology regarding CSC biology, (b) communication of negative results or data pointing in the wrong direction, and (c) the development of specific assays with well-defined endpoints for increasing the reproducibility of results and henceforth their analysis.

It is also hypothesized that CSCs do exist but describe only a transient state of cancer cells that are able to transiently switch their phenotype (Gupta et al., 2011). A new hypothesis (Teng et al., 2018), based on the "cell survivology" concept, suggests that cells with CSC features might arise from the de-differentiation of regular cancer cells, driven by microenvironmental stress, in the attempt to survive at the expense of the rest of cancer and host cells. This feature does not match the paradigm of stem cells which are using proliferation, migration and differentiation for constantly building homeostasis with surrounding cells through

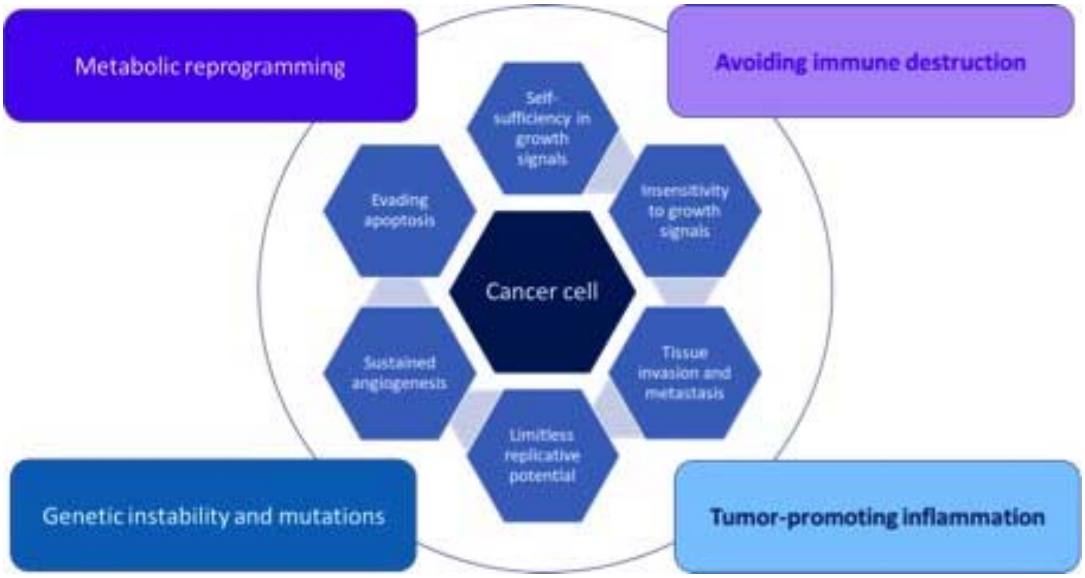

Figure 1
The hallmarks of cancer (Hanahan and Weinberg, 2000; 2011; Hornsveld and Dansen, 2016).

their functional multipotency. Moreover, once differentiated into terminal phenotypes, stem cells will not de-differentiate under physiological conditions (Teng et al., 2011; Teng et al., 2018).

Starting from the survivology theory, in this review we analyse the involvement of the cytoprotective transcription factor NRF2 in tumor biology, with special emphasis on tumor-initiating and radio-resistant cells that we will conventionally term as CSCs. This review argues in favour of classifying NRF2 as an oncogene, considering that it is more or less directly involved in all hallmarks of cancer (Hanahan and Weinberg, 2011), schematically represented in **Figure 1**. The analysis of existing knowledge on NRF2 and CSCs is focused on selective forces driving the survival of tumor cells with particular genotypes and phenotypes, the cytoprotection provided by NRF2 distinctively to normal and tumor cells, the common and distinctive mechanisms accounting for the survival of tumor cells and CSCs in the harsh tumor environment, including those underlying the exquisite radio-resistance of CSCs, also pointing out the involvement of NRF2 in shaping the hallmark features of CSCs.

Physiologic and oncogenic pathways underlying NRF2 activation

The transcription factor NRF2 (nuclear factor erythroid 2-related factor 2), encoded by the NFE2L2 gene, controls the expression of a multitude of cytoprotective molecules involved in oxidative metabolism and drug biotransformation reactions (phase I and II drug-metabolizing enzymes, and phase III drug transporters), as well as in proteostasis, cell proliferation and metabolic reprogramming (Malhotra et al., 2010; Mitsuishi et al., 2012b; Hayes and Dinkova-Kostova, 2014; Pajares et al., 2017). The genes that are under the transcriptional control of NRF2 exhibit in their promoters ARE (Antioxidant Response Elements) that are present in over 250 genes and play a central role in redox homeostasis (Raghunath et al., 2018). The molecular fingerprint of NRF2 is still under development and future studies might unravel new gene targets through which NRF2 is modulating various cellular functions besides redox reactions. Moreover, it has been recently demonstrated using a network medicine approach that NRF2 is a master regulator of multiple cytoprotective responses and a key molecular node within a particular cluster of diseases, representing a promising target for drug development and repurposing (Cuadrado et al., 2018).

Low levels of NRF2 are constitutively expressed in the cytoplasm for sustaining a prompt reaction of cells against stress. The NFE2L2 gene itself contains ARE in its promoter region and therefore NRF2 can control its own basic expression (Kwak et al., 2002). Under non-stressed conditions NRF2 is kept in a transcriptionally inactive form by the interaction of the 29-DLG-31 and 79-ETGE-82 motifs in the Neh2 domain of NRF2 with the Kelch motifs of two molecules of KEAP1 (Kelch-like ECH associated protein 1). Through its bric-a-brac homodimerization (BTB) domain, KEAP1 is an adaptor protein substrate of the CUL3-RBX1 (Cullin 3-Ring Box Protein 1) E3 ubiquitin ligase that promotes ubiquitination of bound NRF2 at several lysine residues in the Neh2 domain, triggering its degradation by the 26S proteasome (McMahon et al., 2003). An alternative mechanism for NRF2 repression under non-stressed conditions is mediated by GSK-3 (glycogen synthase kinase 3) that can directly phosphorylate NRF2 at the level of the 334-DSGIS-338 sequence in the Neh6 domain (Cuadrado, 2015), or indirectly, through Fyn-mediated phosphorylation of NRF2 at Tyr568 (Armagan et al., 2019). A recognition motif for the β-transducin repeat-containing E3 ubiquitin protein ligase (β-TrCP) is thus created, through which NRF2 is presented to CUL1/RBX1, leading to its ubiquitin-dependent proteasomal degradation (Cuadrado, 2015). NRF2 phosphorylation can be also induced by the p38 mitogen activated protein kinase (MAPK) that reinforces NRF2 interaction with KEAP1 (Keum et al., 2006). The third mechanism of NRF2 degradation is mediated by the inositol requiring enzyme (IRE1)/E3 ubiquitin ligase synoviolin (HRD1), whose expression is enhanced by the activation of XBP1–HRD1 from the endoplasmic reticulum (ER) stress pathway (Wu et al., 2014). HRD1 interacts with the Neh4 and Neh5 domains of NRF2 and triggers its degradation independently of KEAP1 (Wu et al., 2014). It is noteworthy that the above-mentioned mechanisms mediate the degradation of NRF2 in specific subcellular compartments (Dodson et al., 2019). Thus, the CUL3-RBX1-KEAP1 complex responds to electrophilic/oxidative stress in the

Table 1

Mechanism	Examples	References
Mutations in NRF2 or KEAP1	These mutations can affect the interaction of NRF2 with its KEAP1 repressor, leading to the activation of the transcriptional program regulated by NRF2.KEAP1 and NFE2L2-mutations, analyzed in 1391 patients with non–small cell lung carcinoma, seem to be predictive markers for chemotherapy resistance.	(Hayes & McMahon, 2009), (Shibata et al., 2008), (Zehir et al., 2017) (Frank et al., 2018)
NRF2 mutations	As shown by the COSMIC Catalogue of Somatic Mutations in Cancer (https://cancer.sanger.ac.uk/cosmic/gene/), 1095 tumor samples out of 19566 exhibited mutations in the Nfe2l2 gene, the most frequent ones being found in penis (11.65%), endometrium (6.08%), esophagus (5.65%), liver (5.29%), upper digestive tract (4.82%) and lung (4.51%). Most of these mutations are missense substitutions (70.23%). Not all these mutations are able to impact the NRF2-KEAP1 interaction and to trigger NRF2 activation. NRF2 gain-of-function mutations in advanced esophageal squamous cancer are associated with tumor recurrence and poor prognosis due to increased proliferation, attachment-independent survival and resistance to chemo- and radiotherapy. In turn, the SNP (-617C › A) in the ARE-like loci of Nfe2l2 abolishes the self-induction of this gene, resulting in increased survival of the sub-group of patients with lung adenocarcinoma exhibiting this SNP.	(Shibata et al., 2011) (Okano et al., 2013)
KEAP1 mutations	By analyzing 40,167 tumor specimens it was found that the prevalence of KEAP1 mutations was 2.7%, with non-small cell lung cancer having the highest levels of KEAP1 mutations (15.8%). Most of the alterations were missense substitutions. The negative prognostic value of KEAP1 mutations in various types of cancer as well as regarding resistance to therapy has been reported.Loss of KEAP1, achieved by a CRISPR–Cas9-based approach, was shown to induce NRF2 hyper-activation and to promote KRAS-driven lung adenocarcinoma in mice.	(Chen et al., 2020) (Konstantinopoulos et al., 2011) (Romero et al., 2017)
Epigenetic mechanisms	Somatic mutations exist in only a small portion of cancer tissues. Epigenetic regulation of KEAP1-NRF2 signaling by DNA methylation, histone modification, and microRNAs seems to be the mechanism underlying the inter-individual variability in the expression of KEAP1 and NRF2 in various types of tumors.	(Guo et al., 2015)
miRNA	A systematic analysis of the NRF2 interactome and regulome revealed that 85 miRNAs are predicted to bind to NRF2 mRNA and to down-regulate its nuclear translation. 63 miRNAs were defined as potential members of negative feedback loops for NRF2 signaling in which an activating transcription factor could increase the level of expression of miRNAs that down-regulate NRF2.miR-507, miR634, miR450a, and miR129-5p, directly targeting NFE2L2, can repress the NRF2-mediated oncogenic pathway in NRF2-stabilized human tumors. A preclinical study evidenced that miR-507 inhibits the growth of A549 tumors in nude mice by targeting NRF2. miR-27a, miR-142-5p, miR-144, and miR-153 were shown to affect in neuronal SH-SY5Y cells the nucleo-cytoplasmic level of NRF2 in a KEAP1-independent manner. Increased miR-93 levels, found in a rat model of estrogen-dependent breast carcinoma, decreased apoptosis, increased colony and mammosphere formation, as well as cell migration and DNA damage in breast epithelial cells.	(Papp et al., 2012) (Yamamoto et al., 2014) (Narasimhan et al., 2012) (Singh et al., 2013)
Methylation	Cytosine methylation in the KEAP1 promoter was registered in lung cancer cells and tissues, but not in their normal counterpart, probably contributing to tumorigenesis.Promoter CpG islands hyper-methylation and aberrant splicing of KEAP1 in prostate DU-145 cells sustained cell growth and enhanced sensitivity to chemotherapeutic drugs as well as to radiation-induced cell death.	(Wang et al., 2008) (Zhang et al., 2010)
Posttransscriptional changes	High levels of the phosphorylated form of NRF2, analyzed in hepatocellular carcinoma specimens, was generally associated with reduced KEAP1 expression and with poor 5-year overall survival of patients.	(Chen et al., 2016a)
Oncogenes and tumor suppressors	Oncogenic alleles of KRAS, c-MYC and BRAF (KRAS G12D, BRAF V619E and c-MYC ERT12) can elevate the levels of NRF2 transcripts, leading to increased antioxidant protection and tumorigenesis.NFE2L2 contains a 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) in its promoter, which is a binding site for the AP-1 transcription factor that integrates multiple oncogenic signals.p21 (Cip1/WAF1), a target of the tumor suppressor p53, can stabilize NRF2 through the interaction of its KRR motif with DLG and ETGE motifs in NRF2, hence competing with KEAP1 for NRF2 binding. In turn, p53 binds to promoter elements activated by NRF2, and acts as a transcriptional repressor of NRF2 target genes. NRF2 can also negatively regulate p53 through its target gene MDM2. Meanwhile, NQO1, which is under NRF2 control, was shown to interact with p53 and to protect it from degradation.	(Rojo de la Vega et al., 2018) (DeNicola et al., 2011) (Tao et al., 2014) (Chen et al., 2009) (Rotblat et al., 2012)

cytosol, the nuclear or cytosolic GSK-3β-regulated β-TrCP complex responds to metabolic changes, while HRD1 only ubquitylates NRF2 during ER stress.

Under oxidative conditions, critical cysteines of KEAP1 (C151, C273 and C288) get oxidized and inhibit therefore KEAP1-NRF2 interaction by misalignment of lysine residues within the DLG motif of NRF2, leading to NRF2 stabilization. Alternatively, NRF2 stabilization is induced by thiol modifications that produce the dissociation of Cullin3 from KEAP1 (Taguchi et al., 2011). It might be also elicited through non-canonical mechanisms in which various proteins compete with NRF2 or KEAP1, hence disturbing the NRF2-KEAP1 complex. For instance, p21 (Cip1/WAF1) directly interacts with the 29-DLG and 79-ETGE motifs in NRF2 and competes with KEAP1 for NRF2 binding (Chen et al., 2009). Moreover, the autophagy receptor p62, which is encoded by the SQSTM1 target gene of NRF2, competes with NRF2 for binding to KEAP1, hence generating a regulatory loop for controlling the activation of NRF2 in relation with autophagy (Dodson et al., 2015).

While in normal cells NRF2 stabilization and activation is transient in normal cells, in tumor cells it appears to be chronic and relies mostly on disease-specific mechanisms such as somatic mutations in the NFE2L2 gene or in the gene encoding the KEAP1 repressor, complemented by epigenetic changes mediated by microRNAs or aberrant methylation, as well as on oncogene-mediated mechanisms **(Table 1).**

Once stabilized, NRF2 translocates to the nucleus and forms heterodimers with Zip (leucine zipper)-containing small Maf proteins that bind to ARE and initiate a broad transcriptional program of cytoprotective genes (Motohashi et al., 2004). BACH1, which has cytoplasmic localization in resting conditions and nuclear localization under stress, is considered to be the main nuclear repressor of NRF2 transcriptional activity (Dhakshinamoorthy et al., 2005). As ARE overlap with various other enhancers that are recognized by Zip proteins, including AP-1, BACH1, CREB/ATF and Maf homodimers, a strong interference of NRF2 with other signaling pathways has been highlighted in the nucleus (Ma, 2013; Wardyn et al., 2015). For instance, NRF2 and the pro-inflammatory NF-κB transcription factor compete with each other for the transcriptional co-activator CBP (cAMP-responsive element-binding protein (CREB) binding protein) (Ziady et al., 2012). It appears that an intense NF-kB-mediated pro-inflammatory response will restrict the transcriptional activity of NRF2, henceforth promoting an enhanced oxidative stress. In turn, NRF2 suppresses inflammation not only through a redox control, but opposes transcriptional up-regulation of pro-inflammatory cytokine genes (Kobayashi et al., 2016). Moreover, the NFE2L2 gene contains κB sites in its proximal promoter that are targeted by p65-NFkB as a regulatory mechanism aimed at attenuating inflammation (Smale, 2011; Kobayashi et al., 2016).

Intracellular reactive oxygen species levels distinguish between tumor cells and CSCs

Regular tumor cells exhibit increased levels of intracellular ROS

Tumor cells are characterized by higher levels of reactive oxygen species (ROS) than normal cells, deriving from increased ROS production (Manda et al., 2015). The altered mitochondrial metabolism (Weinberg et al., 2010) partly due to oncogenes (i.e. RAS, Bcr–Abl and c-Myc) and loss of tumor suppressors such as p53 (Weinberg et al., 2019), as well as the abnormal activation of various NADPH-oxidases in particular types of CSCs (Diehn et al., 2009) account for the increased production of superoxide anion in tumor cells. Superoxide dismutases (SOD), especially the mitochondrial SOD-2 isoform encoded by a NRF2 target gene, further generate hydrogen peroxide (Szatrowski and Nathan, 1991), a potent second messenger that modulates redox-sensitive oncogenic signalling pathways (Di Marzo et al., 2018).

Apparently, those tumor cells with elevated intracellular ROS levels are selected in the early steps of tumorigenesis if ROS levels fall in a domain that is favouring their survival and proliferation (Panieri and Santoro, 2016). Meanwhile, tumor cells that produce excessive cytotoxic ROS are driven to apoptosis (Simon et al., 2000) or ferroptosis (Chen et al., 2017), and are finally eliminated. ROS can sustain the survival and proliferations of tumor cells through the activity of ERK1/2 (extracellular regulated serine-threonine kinases), one of the members of the MAPK signaling pathway. Meanwhile, ROS-induced cytotoxicity is partly dependent on p38

and JNK (c-Jun NH2-terminal kinase), the two other members of the MAPK family (Son et al., 2011; Son et al., 2013). ERK1/2 can maintain an oncogenic phenotype by stabilizing the proto-oncogenic Myc levels, whereas GSK-3β represses NRF2 and promotes Myc ubiquitination and degradation. Moreover, ERK1/2 triggers the activation of the NF-kB signalling pathway which further provides inflammatory signals to tumor cells, hence enhancing their ability to survive (Braicu et al., 2019).

CSCs exhibit low levels of intracellular ROS

While cancer cells exhibit increased levels of ROS, extensive evidence points out that CSCs are characterized by low intracellular ROS levels. In example, the sub-population of low ROS-producing cells in acute myelogenous leukemia exhibit quiescence and a CSC-specific CD34+/CD38– phenotype that is associated with high levels of BCL-2, an inhibitor of pro-apoptotic proteins promoting cell survival (Lagadinou et al., 2013). Moreover, low ROS-producing cells in head and neck tumors exhibit a CSC-specific phenotype (CD133+, memGrp78+, Glut3+, ALDH+, and increased OCT4 and NANOG expression), that is associated with higher chemo-resistance and tumorigenicity as compared to the high ROS-producing population of regular tumor cells (Chang et al., 2014).

The decreased low levels of ROS in the sub-population of tumorigenic and therapy-resistant cells appear to be attributed to the hyper-activation of the endogenous antioxidant system (Zhou et al., 2014). For instance, it has been shown that the antioxidant GCLM and FOXO1 genes are over-expressed in breast CSCs (Diehn et al., 2009). The GCLM gene is a NRF2 target encoding the regulatory subunit of GCL (glutamate–cysteine ligase) that catalyses the rate-limiting step of glutathione (GSH) biosynthesis, hence increasing the ability of GSH to scavenge ROS. GSH has a broad cytoprotective role, being also involved in nutrient metabolism and regulation of cellular metabolic functions ranging from gene expression, DNA and protein synthesis to signal transduction, cell proliferation and apoptosis (Aquilano et al., 2014). The FOXO gene encodes the forkhead box proteins O (FOXO) family of transcription factors that inhibit the expression of various ROS scavengers (i.e. superoxide dismutase-2 and peroxiredoxins-3,5 in mitochondria, and catalase in peroxisomes), under the negative control of AKT (Klotz et al., 2015). Of note is that the PPARγ (nuclear peroxisome-proliferator activator receptor γ), having a key role in energy and redox homeostasis as well as in inflammation, is at the crossroad of the signaling pathways related to NRF2, FOXO and Wnt/β-catenin, that are involved in various pathologic processes including tumorigenicity and CSCs biology (Polvani et al., 2012).

Another rationale for decreased ROS levels in CSCs is the over-expression of particular molecules defined as CSC markers that have the ability to control more or less directly ROS levels. For instance, CD44, a receptor for hyaluronic acid that is considered as marker for several types of CSCs (Yan et al., 2015), limits ROS levels in CSCs by inducing an increased uptake of cystine for GSH biosynthesis through the interaction with the glutamate–cystine antiporter xCT (Ishimoto et al., 2011). Moreover, high CD44 levels can induce p62-mediated NRF2 stabilization in CSCs, resulting in lower ROS levels (Ryoo et al., 2018). This mechanism of NRF2 stabilization proves to have an important contribution to the aggressive phenotype and therapy-resistance of CD44high CSCs. Other CSC markers were also shown to be involved in maintaining low ROS levels in CSCs. Thus, CD13 can protect human liver CSCs against ROS-induced DNA damage probably by excreting ROS, and CD13 inhibition with ubenimex increased ROS levels, abrogated CSCs dormancy and sensitized these cells to anticancer drugs (Haraguchi et al., 2010; Yamashita et al., 2016). Moreover, aldehyde dehydrogenase (ALDH) which is over-expressed in various types of CSCs, metabolizes and detoxifies endogenous and exogenous aldehydes, hence rendering CSCs resistant to anti-cancer therapies due to the decreased ROS levels in ALDHhigh CSCs (Kim et al., 2017).

Other mechanisms might underlie the decreased ROS production in CSCs. It has been shown that breast cancer cells can be reprogrammed to CSC-like cells by epigenetic silencing of the FPB1 gene mediated by the Snail–G9a–Dnmt1 complex (Dong et al., 2013). FPB1 encodes the fructose-1,6-biphosphatase that induces glycolysis and promotes apoptosis resistance in CSCs (Dai et al., 2017). A decreased expression of FPB1 was shown to in-

crease glycolysis and NADPH production by the pentose phosphate pathway, leading to enhanced tumorigenesis accompanied by reduced ROS production due to suppressed activity of the mitochondrial complex I (Dong et al., 2013).

Low ROS levels are critical for CSC biology. For instance, when an experimental increase of ROS was induced in glioma CSCs by the redox modulator cannabidiol, the self-renewal and cell survival ability of CSCs was partly abolished due to decreased ROS-dependent AKT activity, resulting in a significantly increased survival of glioblastoma-bearing mice (Rassool et al., 2007).

Considering that critical levels of ROS are necessary for cell multiplication, the decreased ROS levels in CSCs probably account for their quiescent slow-proliferating state that makes them partly insensitive to anti-proliferating chemo-therapeutics (Gabrielli et al., 2012; Chen et al., 2016b). Low levels of ROS are also critical for maintaining the stemness and the self-renewal ability of CSCs, as these features can be reversed by an experimental increase of ROS production (Shi et al., 2012; Machida, 2018). ROS-mediated differentiation of CSCs can be attributed to: (a) the activation of the p38 MAPK pathway leading to the degradation of the polycomb suppressor protein complex I (BMI) which regulates mitochondrial function and the DNA damage response pathway (Liu et al., 2009), (b) FOXO3 activation which triggers ROS-accumulation as a consequence of transient mitochondrial outer membrane permeabilization (Hagenbuchner and Ausserlechner, 2013). Therefore, a pharmacological increase of ROS levels or the inhibition of critical antioxidant mechanisms might lead in the CSCs population to reduced tumorigenicity and increased sensitivity to anti-cancer therapies. Nevertheless, the functional significance of the ROS status in different types of CSCs, the exact downstream signaling events and the role of ROS in CSC self-renewal is still under investigation. A major drawback resides in the currently available methods for assessing ROS that may produce artifactual results in vitro (Tetz et al., 2013), and the lack of a reliable method to asses ROS in living organisms (Pavelescu, 2015).

NRF2 accompanies tumor cells in their development

Extensive evidence has been brought that persistent activation of the NRF2 transcription factor confers cytoprotection to tumor cells and CSCs, including resistance to anti-cancer therapies, by controlling the transcription of a broad array of antioxidants and other cytoprotective factors. It is possible that the mechanisms underlying NRF2 activation in tumor cells and CSCs are different, considering that tumor cells have an increased oxidative metabolism whereas CSCs are characterized by low ROS levels that would not require, at least theoretically, the activation of endogenous antioxidant mechanisms.

NRF2 activity in tumor cells

The oxidative environment of the tumor, originating not only from tumor cells with higher oxidative metabolism but also from the tumor stroma, including cancer-activated fibroblasts as well as resident and newly recruited immune cells (Weinberg et al., 2019), elicits a persistent activation of the NRF2 system as adaptive mechanism (Kitamura and Motohashi, 2018). The increased ROS levels characterizing regular tumor cells when compared to their normal counterpart, and the oxidative microenvironment to which these cells are constantly exposed, most probably trigger persistent NRF2 activation by oxidizing critical cysteines in the redox sensor KEAP1, hence activating the canonical NRF2 pathway. Additional mechanisms of chronic NRF2 activation are described, that are specifically working in tumor cells and CSCs (Tabel 1), including: (a) somatic gain-of-function mutations in the gene encoding NRF2, and loss-of-function mutations in the gene encoding the KEAP1 repressor; (b) epigenetic regulation of the genes encoding NRF2 or KEAP1; (c) oncogene-mediated NRF2 activation. It has been also shown that loss of PTEN alters the negative regulation of NRF2 mediated by PTEN/GSK-3β-TrCP, accounting for an increased NRF2 signature and tumorigenesis, as observed in 80% of PTEN-negative endometrioid carcinomas that expressed high levels of NRF2 (Rojo et al., 2014).

From the redox metabolism perspective, tumor cells with high NRF2 activity might be selected from normal cells that have been persistently exposed to a chronic oxidative stress that triggers DNA damage with increased frequency of error-prone repair of double-strand breaks

and oncogenic mutations, leading to genomic instability and cell transformation (Rassool et al., 2007). In parallel, these cells develop adaptive antioxidant mechanisms that provide a survival advantage (Menegon et al., 2016). This is the reason why NRF2 activation is essential for inhibiting the early steps of tumorigenesis, but becomes afterward a potent supporter of tumor progression (Milkovic et al., 2017).

A bioinformatic analysis performed on patients-derived gene expression and survival data (665 tumors and 359 normal tissues) from publicly available databases showed that tumors are enriched in genes involved in the response to oxidative stress, and that some of these genes are under the transcriptional control of NRF2 (Rotblat et al., 2013). Thus, G6PD, HMOX1, PRDX4, SRXN1, TXNRD1 were associated with bad prognosis in breast cancer, while GCLC, GSS, NQO1, TXN and TXNRD1 in lung cancer.

There are also human studies showing that not all tumors are characterized by high NRF2 levels / activity as compared to their normal counterpart. Thus, an analysis (Daves et al., 2011) using microarray data from the Oncomine database, revealed that the majority of the investigated tumors exhibited low levels of NRF2 expression. A more comprehensive microarray analysis based on The Cancer Genome Atlas (TCGA) database showed that NRF2 expression was significantly down-regulated in breast, prostate and kidney tumors, excepting colon cancer specimens that exhibited up-regulated NRF2 expression when compared to normal tissue. Down-regulation in the expression of the NRF2 target genes GCLM, GCLC and NQO1 was evidenced in breast, prostate and kidney cancer, respectively. The analysis of survival datasets obtained from the Gene Expression Omnibus (GEO) and TCGA databases evidenced that lower expression of the NFE2L2 gene was associated with poorer outcome in skin cutaneous melanoma and in kidney clear cell carcinoma, while in prostate cancer lower NRF2 expression was associated with recurrence. Discrepancy of results regarding the NRF2 status in tumors requires further systematic investigations and standardized methods for assessing NRF2 levels and its molecular fingerprint in various types of tumors, in correlation with the stage and prognostic of disease. This may bring light if we can classify tumors in NRF2-dependent and -independent, or if NRF2 dynamics during tumor progression is in fact accounting for the observed variability (Dodson et al., 2019).

NRF2 hyper-activation is fine-tuning the distinctive redox metabolism of particular tumor cells that are shifted towards a proliferative phenotype and defective apoptosis. Through its transcriptional program, NRF2 protects tumor cells from oxidative stress and consequent genomic instability, providing a robust shield against ROS-induced mutations and cytotoxicity as well as a survival advantage. Being at the crossroad of various signaling pathways, NRF2 confers to tumor cells broad cytoprotection against the complex web of stressors within tumors, comprising hypoxia (Muz et al., 2015), inflammation (Murata, 2018) and ER stress (Urra et al., 2016), in addition to the aforementioned oxidative stress. Therefore, pharmacologic down-regulation of NRF2 might be a therapeutic option for limiting tumor progression and for increasing sensitivity to therapies based on oxidative stress (radiotherapy, photodynamic therapy), as we will describe later in this review. Meanwhile, pharmacologic activation of NRF2 is seemingly needed as preventive therapy for patients at risk (Krajka-Kuzniak et al., 2017), although it may induce an unwanted redox deregulation if chronic therapy is not highly individualized (Lee et al., 2013).

NRF2 activity in CSCs

CSCs are addicted to NRF2 albeit the fact that they produce only low amounts of ROS. Alternative mechanisms (**Table 1**), other than those related to the oxidation of critical cysteines in the redox-sensitive KEAP1 repressor of NRF2, are most probably accounting for the persistently increased NRF2 activity in CSCs. For instance, it has been shown that the high NRF2 activity in CSC-enriched mammospheres was due to a decreased 26S proteasome activity accompanied by p62/SQSTM1 accumulation, collectively acting for NRF2 activation (Ryoo et al., 2015).

It seems that NRF2 activity reach high levels in CSCs, sustaining not only the resistance to oxidative stress and to anti-cancer therapies, but also aggressive tumorigenicity (Okazaki et al., 2020). In fact, the connection of NRF2 to an increased tumorigenic potential, specific for

CSCs, defines NRF2 addiction in comparison to persistent NRF2 activation that only provides a survival and proliferation advantage to regular cancer cells. Many clinical studies have evidenced strong correlations between NRF2 accumulation and the activation of its transcriptional program in various tumoral tissues, leading to poor clinical outcomes of patients (Kitamura and Motohashi, 2018). Indeed, substantially elevated NRF2 protein levels along with increased expression of some of the antioxidant molecules that are under the transcriptional control of NRF2 (HMOX1, GPX2) were detected in CSCs-enriched MCF7 mammospheres (Ryoo et al., 2015). Moreover, recent proteomic studies performed on highly tumorigenic CSCs and their corresponding non-tumorigenic differentiated cells from human colorectal specimens revealed that the CSC secretome contains many proteins associated with cell survival and protein quality control, including a robust NRF2-specific antioxidant and detoxifying signature (GCLC, GPX2/3 and TXNRD1), as compared to differentiated regular tumor cells (Emmink et al., 2013). The study also highlighted that CSCs are shielded by NRF2 not only against intracellular cues but also against microenvironmental ones.

In tumor cells and CSCs, relying for survival on persistent NRF2 activation, it appears that the transient and inducible nature of NRF2 activity for maintaining redox homeostasis (Yamamoto et al., 2018) has been lost, and that cells with persistently increased NRF2 activity have adapted to overcome the associated deleterious effects which are normally driving such cells for elimination (Kucinski et al., 2017). Accordingly, NRF2 addicted tumor cells reach a new level of abnormality. These adaptive mechanisms deserve future in-depth investigations that may highlight additional processes underlying the NRF2 addiction of CSCs, leading to innovative therapeutic approaches for inhibiting the de-differentiation of tumor cells into CSCs under the pressure of the microenvironment.

NRF2 addiction in tumorigenic cells is related to unique metabolic changes induced directly by NRF2 activation that are distinctively displayed in quiescent CSCs and in differentiated proliferative tumor cells. NRF2 mainly contributes to antioxidant and detoxification mechanisms in quiescent slow-growing cells. Meanwhile, it has an important contribution to metabolic reprogramming of proliferating tumor cells by facilitating the pentose phosphate pathway that is branched to aerobic glycolysis and bridges the gap between the glycolytic metabolism and cancer cell proliferation (De Preter et al., 2016; Okazaki et al., 2020). Moreover, enhancement of purine nucleotide synthesis via the pentose phosphate pathway was found to be advantageous for the proliferation and tumorigenic potential of NRF2-addicted cancer cells (Mitsuishi et al., 2012b). New proofs have been recently brought by analysing the NRF2-dependent transcriptome and NRF2 cistrome (genome-wide NRF2 binding sites) in a KEAP1-mutant non-small cell lung cancer cell line (Okazaki et al., 2020). It has been shown that several metabolic genes, such as those involved in the pentose phosphate pathway and NADPH production, as well as some genes involved in GSH biosynthesis are directly under the transcriptional control of NRF2. NRF2 controls the expression the genes encoding the two components of GLC (the catalytic GCLC subunit and the GCLM modifying subunit) that are critically involved in the early steps of GSH biosynthesis. Moreover, NRF2 in conjunction with the transcription factor ATF4 sustain the transcription of the SCL7A11 gene encoding the xCT cystine anti-porter that provides the cellular supply of cystine required for GSH biosynthesis (Mitsuishi et al., 2012b; Ye et al., 2014). In turn, NRF2 activation is sustained by the constitutive activation of the PTEN-PI3K-AKT pathway in various types of cancer cells, inducing nuclear translocation of NRF2 and GSK-3 inactivation, henceforth inhibiting the degradation of NRF2 (Rada et al., 2011; Mitsuishi et al., 2012b). By combining NRF2-dependent transcriptome data with NRF2 antibody ChIP-seq and NRF2-dependent metabolomic data (using 13C-labeled glucose and glutamine) the above mentioned study evidenced that NRF2 activation skewed the metabolite flow in proliferating tumor cells by redirecting glucose and glutamine towards anabolic pathways through a feedforward loop between NRF2 and the PI3K–AKT pathway (Mitsuishi et al., 2012a; Mitsuishi et al., 2012b; Okazaki et al., 2020; Saigusa et al., 2020).

Besides directly activating the transcription of genes encoding glycolytic enzymes, NRF2 activation in CSCs was shown to inhibit the con-

version of pyruvate to acetyl-CoA by directly activating PDK1 (pyruvate dehydrogenase kinase 1), leading to the inhibition of tricarboxylic acid cycle whilst promoting the Warburg effect (Burns and Manda, 2017). This metabolic reprogramming occurred at low ROS levels characteristic for CSCs, through the GRP78/p-PERK/NRF2 signaling pathway involved in maintaining stemness, self-renewal and invasiveness of CSCs (Chang et al., 2018).

Increased GSH and NADPH levels, ensuing from persistent NRF2 activation in addicted cells, and the activation of the NRF2-FoxO3a-Bmi1 axis, shifts the redox balance towards a reductive stress that favours the self-renewal ability of CSCs and their growth in a xenograft mouse model (Kim et al., 2020). The reductive stress may in turn lead to proteotoxicity (Okazaki et al., 2020), but NRF2 is able to regulate the transcription of several genes involved in maintaining proteostasis through autophagy and the unfolded protein response in various pathologic conditions (Pajares et al., 2017). As such, NRF2-addicted cells are endowed with multiple tools for surviving and preserving their phenotype in brutal oxidative or reducing environments, whilst maintaining their addiction to NRF2.

NRF2 is directly involved in maintaining the stemness and self-renewal capacity of CSCs in particular oxidative environments characterized by fine-tuned ROS levels (Kahroba et al., 2019). Thus, the well-known CSC marker CD44 that co-localizes with NRF2 in clinical breast tumor samples proved to increase NRF2 activation through raised levels of p62/SQSTM1 in CD44high breast CSC-like cells, but this effect was not detected in CD44low tumor cells (Ryoo et al., 2018). Moreover, the CSC marker ALDH1A1 (aldehyde dehydrogenase 1 family member A1) triggers increased NRF2 activity through the p62-associated pathway in ALDH1A1high CSC-like ovarian cancer cells, and this contributed decisively to tumor growth and resistance to therapy (Kim et al., 2018a). Final proof regarding the direct involvement of NRF2 in shaping CSCs' features was provided in this study by experiments in which NRF2 was experimentally silenced, leading to the inhibition of CSCs markers expression, colony/sphere formation and tumor growth.

All these observations on CSCs and their tumorigenic potential point towards a possible threshold regarding NRF2 expression/activity and ROS levels, that separates the consequences of NRF2 hyper-activation in quiescent CSCs and differentiated tumor cells, and probably rule the back and forth transition of tumor cells between these states due to their plasticity under the microenvironment influence (Vinogradova et al., 2015). The dynamic levels of NRF2 activity are probably highly dependent on the particular tumor type and the driving forces in the tumor niche. Therefore, standardized investigation protocols and appropriate functional markers have to be defined for improving the prognostic tools related to disease progression and response to therapy.

NRF2 is inter-connected with various CSC-specific signaling pathways. It has been shown that some of the NRF2 target genes are involved in the Wnt and Notch pathways that play a pivotal role in stemness establishment for malignant behaviour of cells, and NRF2 emerged as a central node in cell survival responses (Malhotra et al., 2010; Chorley et al., 2012). It appears that Notch signaling directly activates adaptive NRF2-mediated stress responses through recruitment of the Notch intracellular domain transcriptosome to a conserved Rbpjκ site in the promoter of NRF2 (Wakabayashi et al., 2014). The frequently co-occurring mutations of NFE2L2, KEAP1 and NOTCH1, identified in many types of human cancers, suggest an aberrant NRF2–Notch crosstalk that might specifically enhance tumorigenesis (Lawrence et al., 2014). Moreover, it has been shown that NRF2 takes part in a protein complex with Axin1 that is regulated by the canonical Wnt pathway for redox homeostasis in the liver (Rada et al., 2015). More investigations are needed for establishing the interference between the KEAP1-NRF2 system and signalling pathways specific for CSCs.

NRF2 has also a complex crosstalk with signalling pathways that shape the stress responses of CSCs in their hypoxic and inflammatory niche that maintains stemness, controls the self-renewal of CSCs and their differentiation, as well as the dedifferentiation of non-CSC tumor cells in virtually every step of the tumorigenic cascade (Borovski et al., 2011). Hypoxia is a hallmark of solid tumors that promotes angiogenesis and metastasis, as well as resistance to therapy (Semenza, 2010). CSCs

appear to be the primary regulator of angiogenesis through VEGF, while hypoxia is needed for maintaining the undifferentiated phenotype of CSCs (Heddleston et al., 2010). For surviving in a low-oxygen microenvironment, cancer cells activate the transcriptional program regulated by HIF-1 (hypoxia-inducible factor 1) for increasing angiogenesis and for metabolically reprogramming cancer cells from an oxidative to a glycolytic phenotype (Mole et al., 2009; Toth and Warfel, 2017). Additionally, the PI3K/AKT/mTOR, MAPK (ERK) and NF- B pathways synergistically shape the survival response to hypoxia, addressing cell proliferation, apoptosis, metabolism, migration and inflammation (Muz et al., 2015). The interference of NRF2 with the redox-sensitive HIF-1 have been demonstrated in tumor hypoxia (Toth and Warfel, 2017). Thus, NRF2 knockdown was sufficient to decrease HIF-1α levels (Kim et al., 2011). This can be explained by the fact that NQO1, encoded by a gene that is under the transcriptional control of NRF2, binds to HIF-1α at the level of the oxygen-sensing domain and impedes on is interaction with prolyl hydroxylase domain containing proteins, leading to a decreased proteosomal degradation of HIF-1α (Oh et al., 2016). NRF2 can also induce indirectly enhanced levels of HIF-1α through an increased production of the antioxidant thioredoxin during ROS-producing intermittent hypoxia in A549 adenocarcinoma cells (Hawkins et al., 2016). It seems that HO-1 (hemoxygenase 1), that is regulated by many transcription factors like NRF2, AP-1, HIF-1 and NF-kB (Alam and Cook, 2007), links the protective response developed by cancer cells against hypoxia, oxidative stress and inflammation in the tumor niche (Chau, 2015). It has been also shown that increased levels of HO-1 and carbon monoxide, endogenously generated as a consequence of heme degradation by HO-1, can reinforce specific signaling pathways used by CSCs, such as the Notch pathway (Kim et al., 2018b).

NRF2 confers radio-resistance to tumor cells

The role of NRF2 in shielding CSCs against oxidative stress is best highlighted when these cells face the attack of radiotherapy. Generally, the main issues regarding therapy efficacy in tumors are related to the heterogeneity and plasticity of tumor and stromal cells in the tumor niche that drives distinctive therapy outcomes. In response to the therapeutic challenge, the development of powerful adaptive responses by particular tumor clones, including CSCs, may result in a more resistant, aggressive, and invasive phenotype that accounts for disease recurrence and metastasis (Kreso et al., 2013).

NRF2 and photon radiotherapy

Radiotherapy (RT), more specifically photon radiotherapy (Ph-RT) with X and γ rays, remains the gold standard for oncologic treatments due to its exquisite ability to induce irreversible oxidative DNA damages and consequent death of tumor cells. The therapeutic potential of Ph-RT is highly dependent on the radiation quality, its dose and dose rate. Ph-RT is mainly acting through the generation of a deleterious ROS burst produced by water radiolysis and consequent disruption of mitochondrial functions (Pilie et al., 2019) that inflicts in time extensive oxidative alterations of proteins, lipids and nucleic acids, irreversibly alters redox signaling and drives cell death mainly through mitotic catastrophe (Ryter et al., 2007). This is an ideal scenario that is working well in non-CSC tumor cells exhibiting elevated ROS levels, hence being able to overpass more easily the ROS threshold separating cell survival from cell death (Day and Suzuki, 2006). The concomitantly elevated NRF2 activity of tumor cells provides a survival advantage in regular conditions, but is generally insufficient for protecting regular tumor cells against the deleterious oxidative stress inflicted by Ph-RT. Meanwhile, the therapeutic efficacy of Ph-RT is compromised in the case of CSCs that have low ROS levels and are highly shielded against oxidative damages by increased NRF2 levels and activity (Ding et al., 2015). Additionally, CSCs exhibit altered DNA damage responses and repair pathways that are mainly mediated by the two serine threonine protein kinases ataxia–telangiectasia mutated (ATM) and Rad3-Related (ATR), as well as by their two downstream checkpoint kinases Chk1 and Chk2 (Bao et al., 2006; Carruthers et al., 2015). Therefore, CSCs are endowed with an exquisite resistance to exogenously induced oxidative DNA damages that can decisively lead to the failure of Ph-RT (Schulz et al., 2019). The crosstalk of ATM/ATR and NRF2 connects antioxidant and DNA damage responses. NRF2 silencing in ovarian cancer cells

was able to induce a deficient DNA damage response leading to enhanced cytotoxicity (Khalil and Deeni, 2015). Meanwhile, ATM plays a critical role in increasing ROS levels in stem cells via modulation of the AMPK-mTOR pathway or via increased NADPH production (Ito et al., 2004; Cosentino et al., 2011), but no such evidence exists in the case of CSCs.

NRF2 seems to be a promising marker reflecting the radio-resistance of tumors, as higher NRF2 activity in human tumors is associated with lower responses to Ph-RT. For instance, it was shown that the mutational status of KEAP1/NFE2L2 can predict the risk of local tumor recurrence after Ph-RT in patients with non–small lung cancer and could be non-invasively identified in circulating tumor DNA (Jeong et al., 2017). The study also documented a critical role of TP53 and KEAP1 mutations during post-irradiation oncogenesis of laryngeal squamous cell carcinoma, as deletion of either gene was able to increase the self-renewal of originating CSCs.

In head and neck squamous carcinoma cells an elevated activity of NRF2 and increased expression of several of its target genes (HMOX1, NQO1 and GST) was evidenced in the tumors from 48 Ph-RT-tolerant patients, as compared to 69 Ph-RT-sensitive tumors (Wang et al., 2017). In turn, a recent data mining study (Zimta et al., 2019), that was performed on lung adenocarcinoma patients receiving Ph-RT, highlighted using clinical and RNA-sequencing data that recurrent tumors did not show significant expression changes of the Nfe2l2 gene and for most of the 117 investigated NRF2 target genes, excepting the gene encoding methylenetetrahydrofolate dehydrogenase. Moreover, some NRF2 gene targets were even found down-regulated, such as ADH1A, ADH1B, ALDH3A1, ALDH3A2, ALDH6A1, GPX2, ADH1C, AKR1C3 and NQO1. Under-expression of several genes encoding aldehyde dehydrogenase (ALDH3A1, ALDH3A2, ALDH6A1), a marker extensively used to identify and isolate various types CSCs, including those related to lung adenocarcinoma (Sullivan et al., 2010; Ma and Allan, 2011), indicates that recurrent tumors developing after Ph-RT might not exhibit an enrichment in CSCs in more advanced stages of development. We may not rule out that in early stages of tumor development, CSCs might have had an important role in tumor initiation, but CSCs get differentiated into non-CSC tumor cells in later stages and contribute to tumor expansion (Eramo et al., 2008; Huang et al., 2015).

Taking into account that tumors can potentially arise from a single CSC, a failure of radiation treatment might be attributed to the incomplete eradication of the small CSC population that endows tumors with an intrinsic radio-resistance. The correlation between clonogenicity in vitro and stemness in vivo highlights the importance of intrinsic radio-sensitivity of CSCs for radio-curability of tumors with different histologies (Krause et al., 2017). Moreover, RT can induce a state of acquired radio-resistance due to a strong selection pressure that favor the survival of radio-resistant cells with advantageous mutations (Bertrand et al., 2014). Additionally, following RT, tumors become enriched in CSCs through the reprogramming of differentiated polyploid tumor cells that survive to the oxidative challenge elicited by Ph-RT, and probably get shielded against oxidative stress by increased NRF2 activity. For instance, non-CSC cells isolated from human breast tumors were shown to acquire following experimental Ph-RT stem-like properties (re-expression of the specific transcription factors Oct4, Sox-2, Nanog and Klf4) and to exhibit increased tumorigenicity (Lagadec et al., 2012; Martins-Neves et al., 2018). Moreover, it was found that sub-toxic ex vivo radiation exposure of differentiated glioblastoma cells isolated from patient resections can increase tumorigenicity through re-acquisition of stemness markers and stem-associated properties of glioblastoma cells, in part via a survivin-dependent pathway (Dahan et al., 2014). Apparently, the transcription factor Sox2 is central in tumor cell plasticity by regulating de-differentiation and acquisition of CSCs properties through the transcription of a distinct gene set in differentiated tumor cells and CSCs (Berezovsky et al., 2014). Its reprogramming action seems to be reinforced by the hyper-activation of c-Met signaling (Li et al., 2011), as demonstrated in glioblastoma cells.

Of utmost practical importance seems to be the dynamic gene and protein expression upon irradiation, indicative for future tumor radio-resistance (Kurth et al., 2015). The antioxidant shielding can be reinforced by Ph-RT itself in therapy-surviving tumor cells. Thus, it was

shown that radio-resistance of CD24−/low breast cancer cells derived partly from over-expression of genes involved in GSH biosynthesis (FOXO1, GSS, GCLM, Gpx1) and in other antioxidant and repair mechanisms (Prdx3, Prdx4, MT3, Apoe, Nme5, Bnip3). Induction of antioxidant and DNA repair mechanisms impaired apoptosis and hypoxia-triggered responses within the tumor CSC niche (Krause et al., 2017), hence endowing tumor cells with multiple tools for escaping to a second exposure to Ph-RT. This redox re-setting of irradiated cells towards stronger cytoprotective responses was shown to rely, at least partly, on the up-regulation of antioxidant genes targeted by NRF2 as well as on miRNAs, and NRF2 silencing clearly restrained the ability of tumor cells to limit the oxidative stress generated directly or indirectly by irradiation (Marampon et al., 2019). Additionally, it was demonstrated that single doses and daily dose fractions of ionizing radiation (2-8 Gy) can induce in a dose-dependent manner and with a 5 days-delay the transcription of NRF2 target genes (HMOX1) in breast tumor cell lines (McDonald et al., 2010). The study also showed that sub-lethal whole-body irradiation in the context of NRF2 deletion increased radio-sensitivity. The reported delayed activation of NRF2 might be in fact the consequence of a late Ph-RT-driven activation of endogenous ROS producers, such as NADPH-oxidases (Manda et al., 2019), and not the direct effect of the initial ROS burst triggered by radiation through water radiolysis. It has been previously shown that radiation induces MnSOD and thioredoxin expression in the intestinal mucosa within 6 hrs after abdominal exposure, this response vanishes by four days, and a second wave of induced antioxidant genes, including GSH peroxidases and metallothioneins, is emerging later (Haton et al., 2007).

Results point towards a distinctive cell- and dose-dependent dynamics of the NRF2-related mechanisms through which tumor cells counteract the deleterious oxidative changes inflicted by Ph-RT, hence limiting therapy efficacy. Besides targeting tumor cells, therapeutic destabilization of cancer-associated fibroblasts in the tumor niche through Ph-RT-induced growth arrest and cell senescence can greatly contribute to tumor regression, but an inflammatory phenotype may be induced in particular conditions, supporting further tumor progression and metastasis (Wang et al., 2019).

The increase of radiation dose for destroying radio-resistant cells is apparently not an option. Besides the need to protect the surrounding normal tissue, life-threatening systemic effects, such as myelosuppression (Wang et al., 2006) and cardiotoxicity (Ping et al., 2020), may be induced even by radiation beams that are well focused spatially on the tumor.

Dose fractionation, even ultra-hypo-fractionation, is a convenient alternative in terms of failure-free survival and limited side-effects (Widmark et al., 2019). Nevertheless, we should be aware that radio-resistance of tumor cells exposed to low-dose irradiation may occur, and is accounts for increased recurrence and treatment failure in many patients (Tang and Loke, 2015). For instance, ROS elevation elicited by 5 cGy alpha particles beam promoted autophagy and an increase in NRF2/HO-1, inducing radio-resistance in the human adenocarcinoma alveolar basal epithelial cells A549 when cells were subsequently exposed to a much higher therapeutic radiation dose (Chen et al., 2015). Therefore, a main issue to be tackled when using fractionated Ph-RT is the therapy scheme in terms of total dose, fractionated dose, total time and interval between fractions (Hall Eric J, 2006; Cummings et al., 2007). In turn, an initial exposure of normal cells to low-dose radiation might increase their antioxidant shielding against subsequent therapeutic radiation hits, hence being less sensitive to the damaging effects of therapy. For instance, an early ERK1/2-dependent activation of NRF2 was evidenced within the first 24 hrs post-irradiation in mouse RAW 264.7 macrophages exposed to Ph-RT at doses as low as 0.1 Gy (Tsukimoto et al., 2010). Radio-adaptation of normal cells in response to low-dose radiation might rely on the ATM/ERK/NF-κB pathway (Ahmed and Li, 2008) as well as on PPARγ activation (Zhang et al., 2008), both of these protective signalling pathways being intimately connected to the KEAP1/NRF2 axis (Polvani et al., 2012; Wardyn et al., 2015).

NRF2 and hadron therapy

For many tumors, current Ph-RT approaches using photons yields insufficient benefits for local tumor control and patient survival, probably because tumor cells are radio-resistant

against low linear energy transfer radiation due to intrinsic tumor characteristics impinged by hypoxia and ROS levels (Thariat et al., 2019). Radiotherapy using particles (hadron therapy – HT), such as neutrons and protons, is a new option to overcome the DNA repair ability of CSCs. While neutron HT is not therapeutically convenient due to severe side-effects, proton HT proved to be superior to conventional Ph-RT, being used now worldwide for the treatment of many types of tumors. Protons have a specific Bragg peak related to dose deposition that is characterized by radiation dose fall-off at the end of the range as well as a sharp lateral dose fall-off, with maximum energy deposition in the target region. Therefore, proton therapy is able to better spare normal tissues surrounding the tumor target, hence allowing the safe delivery of higher radiation doses that have an enhanced cytotoxic action on tumor cells, including CSCs (Zhang et al., 2013; Hu et al., 2018). For instance, the reduction of the irradiated normal brain volume enables a drastic reduction of the deleterious effects in the early cognitive outcome at one year after proton HT in pediatric patients, as compared to Ph-RT (Pulsifer et al., 2015). Moreover, the physical characteristics of proton beam therapy are suited for repeated irradiation, hence providing higher chance for long-term disease control in head and neck cancer (Phan et al., 2016).

Radiotherapy is dependent on oxygen availability and its efficacy is drastically hampered in hypoxic areas (Thomlinson, 1971; Bennett et al., 2018). Particle beams with high linear energy transfer, such as carbon ion beams, have a decreased oxygen enhancement ratio that is highly needed for successful therapeutic action in hypoxic radio-resistant tumors, especially in the CSCs niches (Gatenby et al., 1988; Nakano et al., 2006). A possible explanation of this biological effect is that ROS localised in the particle track might be insufficient to stabilise HIF-1α (Wozny et al., 2019) and to activate STAT3, MAPK and AKT/mTOR signaling pathways (Ferrandon et al., 2013), important repair mechanisms being thus less active (Wozny et al., 2017). In aqueous samples, sparse hydroxyl radical levels decreased with increasing linear energy transfer, and significantly higher levels of hydrogen peroxide were generated by >100 keV/µm carbon-ion beams as compared to 20 keV/µm carbon-ion beam and X rays (Matsumoto et al., 2019). In tissues, the differences between Ph-RT and carbon HT are apparently deriving from ROS distribution (Wozny et al., 2019). Thus, X-rays produce ionizations and ROS uniformly inside cells, while carbon ions generate a very high ionization and ROS density along individual tracks, and are not able to activate HIF1α. Therefore, complex and unrepairable DNA damage is produced, leading to the death of tumor cells exposed to carbon-ion beams. Moreover, carbon ions activate the upstream signaling pathways accounting for the decrease in MMP-2 expression, hence inhibiting invasion and migration of tumor cells. Due to the advantage brought by carbon HT in protecting normal tissues, research on NRF2 was for the moment focused more on its involvement in the response of normal cells to radiation. Thus, in vivo exposure of mice hippocampus to carbon-ion beams at relatively high doses (4 Gy) resulted in impaired cognitive performance, neurodegeneration and neuronal cell death, as well as in disrupted activities of tricarboxylic acid cycle flux and electron transport chain, reduced mitochondrial integrity, and decreased antioxidant activity. As a consequence, a decline of ATP production and persistent oxidative damage in the hippocampus occurred, most probably related to altered or insufficient activity of NRF2/PINK1 (PTEN-induced kinase 1) (Liu et al., 2018). Although this experimental setting reproduced an extreme irradiation condition, it emphasized that co-therapies that up-regulate NRF2 activity are necessary for better protecting normal tissues against carbon HT.

Chemoradiotherapy

Besides radio-resistance, NRF2 can induce chemo-resistance despite the fact that this type of therapy is less dependent on ROS and their death-inducing action, as compared to RT (Yang et al., 2018). Apparently, elevated cellular ROS production during chemotherapy derives from ROS generation in mitochondria and inhibition of the cellular antioxidant system, mainly related to GSH and superoxide dismutases (Marullo et al., 2013). In turn, prolonged exposure to the chemotherapy-elicited ROS burst induces drug resistance through various mechanisms, hence compromising the therapeutic benefit of repeated treatment session (Maiti, 2010). For instance, it was demonstrated that NRF2 hyper-

activation is associated with chemo-resistance to anti-cancer drugs, mediated by detoxification of drug-induced ROS and electrophiles, decrease of drug accumulation in cells via up-regulation of ABC (ATP-binding cassette) transporters, and inhibition of apoptotic responses (Choi and Kwak, 2016). Moreover, NRF2 can be upregulated by multiple anti-cancer drugs (Tonelli et al., 2018), suggesting an important role of NRF2 in inducing an immediate and reversible drug tolerant state as well as the in the acquisition of permanent resistance.

The disappointing rates of survival and local control associated with single-modality therapy (RT or chemotherapy) led to the development of chemoradiotherapy (Neuner et al., 2009) that could reinforce cytotoxicity against therapy-resistant cells through additive or synergistic effects, as well as by increasing the radio-sensitivity of tumor cells. Chemoradiotherapy has demonstrated superior outcomes in patients with particular types of radio-resistant tumors (i.e. esophageal cancer and non- small-cell lung carcinoma) when compared to RT alone (Neuner et al., 2009; Gray et al., 2020).

FLASH radiotherapy

FLASH radiotherapy (FLASH-RT) lately emerged as a revolutionizing type of RT that can markedly increase the differential effect of Ph-RT and proton HT in tumors and normal tissues, whilst preserving the ability of these therapies to destroy tumor cells (Bourhis et al., 2019a). FLASH-RT is based on an ultra-fast delivery of photons, electrons or protons in the tumor, at dose rates several orders of magnitude higher than those currently used (≥100 Gy/s versus 0.01 Gy/s). As such, FLASH-RT can generate an overwhelming amount of ionizations at the level of tumor cells in an extremely short time (less than tenth of seconds as compared to minutes-long sessions that are repeated over weeks), this accounting for its therapeutic efficacy. The molecular effects of radiation pulses delivered to normal and tumor cells on these timescales are anticipated to be different from those produced by conventional Ph-RT in terms of oxygen availability and ROS generation (Raschke et al., 2016). A decrease in the radio-sensitivity of normal cells is expected to be obtained due to transient oxygen depletion following a short pulse irradiation (Wilson et al., 2012), that cannot be compensated by diffusion and re-oxygenation (Vozenin et al., 2019b), and therefore damaging oxidative effects in normal tissues are significantly limited. For the moment, FLASH-RT demonstrations were made mostly at pre-clinical level (Vozenin et al., 2019a; Diffenderfer et al., 2020). The first patient with T-cell cutaneous lymphoma disseminated throughout the whole skin surface has been successfully treated (Bourhis et al., 2019b), and several clinical trials using FLASH-RT are ongoing (Zilli et al., 2018; Chan et al., 2019). Preliminary results indicate that this innovative radiotherapeutic approach holds great promises for increasing the safety of Ph-RT and HT, whilst providing important therapeutic benefits. Being in its infancy, there are no available data for the moment on the NRF2-mediated effect of FLASH-RT on CSCs.

Photodynamic therapy

Another type of anti-cancer therapy that is mainly based on oxidative stress is photodynamic therapy (PDT). PDT (Agostinis et al., 2011) consists in the administration of an inactive and minimally toxic photosensitizer (PS), followed by its activation through precise tumor illumination with harmless visible light of well-defined wavelength, and generation of a localized oxidative burst that inflicts damages to tumors. PDT relies on the cytotoxic effects of a peculiar type of ROS, singlet oxygen, that is generated only rarely and in very small amounts in mammalian cells, and therefore these cells are less prepared to detoxify it. PDT is often more effective in inducing tumor cell death than RT, considering that Ph-RT is acting mainly via radiation-induced hydrogen peroxide against which cells are endowed with a powerful weapons comprising catalase, peroxyredoxins and GSH peroxidases. Moreover, PDT has the advantage of a safer toxicological profile in comparison with chemotherapy and RT, and can be repeatedly applied in case of tumor recurrence even to immunosuppressed patients. Whilst not being able to detoxify singlet oxygen, mammalian cells can counteract the oxidative damages inflicted by PDT through the endogenous antioxidant system controlled by NRF2 (Manda et al., 2018). Therefore, CSCs endowed with high NRF2 activity might be highly efficacious in protecting themselves against PDT. Additionally, NRF2 controls the expression of the human ABC transporter ABCG2 gene

whose product is critically involved in extruding porphyrinic PS, hence greatly limiting PDT efficacy in tumor cells with high NRF2 activity, including CSCs (Ishikawa et al., 2013).

Pharmacologic NRF2 inhibitors for decreasing radio-resistance

Considering the extensive evidence that CSCs are addicted to NRF2 that sustains tumor progression, recurrence and resistance to oxidative stress-based therapies, there is a strong rationale behind the intensive research for developing small molecules for targeted NRF2 inhibition.

Despite huge progress in deciphering the mechanisms involved in NRF2 stabilization and activation of its transcriptional activity (Robledinos-Anton et al., 2019), for the moment there are no clinically approved NRF2 inhibitors.

Promising results were obtained at preclinical level with various compounds and natural products that proved, mostly at preclinical level, the ability to inhibit the protective activity of NRF2 in various types of tumor cells (**Table 2**). Nevertheless, their clinical translation is limited by important side-effects mainly related to "off target" actions on cysteine residues. Therefore, derivatives are under construction for improving efficacy while limiting side-effects. A high-throughput screening performed on 400 000 small molecules (Molecular Libraries Small Molecule Repository Library, MLSMR) was used for identifying new NRF2 inhibitors, but it did not provide compounds with significantly improved selectivity (Robledinos-Anton et al., 2019). It should be also taken in consideration that long-term systemic NRF2 inhibition should be avoided, considering the cytoprotective role of NRF2 which is critical for maintaining the homeostasis of normal tissues and for providing protection against the attack of ROS and xenobiotics (Yamamoto et al., 2018).

Knowing the mechanisms of NRF2 activation, the logical way to reach inhibition appears to target its interaction with co-factors that sustain the transcriptional activity of NRF2. Thus, a

Table 2

NRF2 inhibitor	Origin and action	References
Brusatol	Plant-derived natural quassinoid that exhibits broad cytotoxicity in cancer cells by inhibiting *de novo* synthesis of cellular proteins, including NRF2. Due to its toxicity, brusatol derivatives were designed, but are under investigation for anti-inflammatory action.	(Olayanju et al., 2015) (Sun et al., 2016) (Tang et al., 2014)
Halofuginone	The toxic febrifugine derivative (alkaloid) rapidly triggers at low doses a cellular aminoacid starvation response that represses global protein synthesis, including NRF2. It inhibits radiation-induced epithelial-to-mesenchymal transition (EMT), possibly through TGF-β1 signaling, and suppresses cellular repair mechanisms.	(Tsuchida et al., 2017) (Follo et al., 2019) (Cook et al., 2010)
Trigonelline	This plant alkaloid (also extracted from coffee) decreases NRF2 activity in tumor cell lines by inhibiting its nuclear accumulation. Concurrently, it blocks the NRF2-dependent expression of proteasomal genes.	(Arlt et al., 2013) (Boettler et al., 2011)
Luteolin	The dietary flavone acts as an anti-cancer agent against lung, breast, glioblastoma, prostate, colon, and pancreatic cancers. It inhibits critical events associated with tumor progression, including cell invasion, metastasis, transformation and angiogenesis, by inhibiting transcription factors and kinase-induced modifications. It inhibits cell cycle arrest and induces apoptosis. Luteolin increases ROS by inhibiting NRF2 activity as well as by activating a lethal endoplasmic reticulum stress response and mitochondrial dysfunction.	(Chian et al., 2014) (Lin et al., 2008) (Imran et al., 2019) (Tang et al., 2011)
Wogonin	This natural flavonoid is able to reverse multidrug resistance by inhibiting NRF2 (in cisplatin-resistant head and neck cancer cells), resulting in increased ROS accumulation and activation of selective cell death pathways (PUMA and PARP).	(Kim et al., 2016) (Zhong et al., 2013)
Malabaricone-A	The plant derived diarylnonanoid hash pro-oxidant and anti-cancer activity in leukemic cell lines, and inhibits NRF2 transcriptional activity.	(Manna et al., 2015)

promising therapeutic approach for decreasing NRF2 activity in tumor cells is, at least theoretically, the pharmacologic stabilization of BACH1, the nuclear repressor of NRF2. Through the C-terminal bZip domain, BACH1 heterodimerizes with small Maf proteins and binds to Maf recognition elements in the promoters of targeted genes, thus competing with NRF2 for the binding to oxidative-stress-responsive genes (Zhang et al., 2018). As a result, the transcription of many antioxidant genes is inhibited, and this may sensitize tumor cells to therapies based on oxidative stress. Nevertheless, we should be aware that BACH1 is a pleiotropic basic leucine zipper transcription factor that has a broad transcriptional and functional impact. For instance, by regulating the expression of several functional metastasis genes, including MMP1, CXCR4 and HMGA1, BACH1 seems to increase the risk of bone metastasis in breast cancer, in addition to HIF1 and Smad4 (Liang et al., 2012). Moreover, it has been shown that BACH1 stabilization triggers the transcription of hexokinase 2 and glyceraldehyde 3-phosphate dehydrogenase, leading to an enhancement of glucose uptake, glycolysis rates and lactate secretion, thereby stimulating glycolysis-dependent metastasis of mouse and human lung cancer cells (Wiel et al., 2019). Moreover, it has been shown that NRF2 activation triggers, as regulatory mechanism, BACH1 stabilization in lung adenocarcinoma cells by inducing the expression of the HMOX1 gene encoding HO-1. Therefore, NRF2 limits the heme-mediated interaction of BACH1 with the degrading ubiquitin ligase Fbxo22 (Lignitto et al., 2019), and specific HO-1 inhibitors hold promise for restraining BACH1 activity aiming to decrease the metastatic potential of various types of tumor cells (Pittala et al., 2013).

Resulting from the high throughput screening on 400 000 small molecules (Molecular Libraries Small Molecule Repository Library, MLSMR) mentioned above, ML385 emerged as a potent and reasonably specific NRF2 inhibitor in tumor cells with KEAP1 mutation. MLR385 exhibits significant anti-tumor activity in combination with chemotherapeutics (Singh et al., 2016). It binds to the Neh1 domain of NRF2 and interferes with the binding of the MafG-NRF2 complex to regulatory DNA binding sequences (Singh et al., 2016). Further studies should be performed for clearly establishing if ML385 is indeed selective for NRF2 or if it also inhibits other bZip transcription factors involved in chemoresistance. The AEM1 compound was also identified as NRF2 inhibitor that sensitizes human adenocarcinoma A549 cells to various chemotherapeutic agents, and can inhibit their growth both in vitro and in vivo (Bollong et al., 2015). Additionally, AEM1 was shown to inhibit Sirtuin 2-mediated p53 deacetylation, and to sensitise p53-proficient tumor cells (Hoffmann et al., 2014).

Interestingly, several therapeutic approaches for decreasing NRF2 activity in tumor cells by modulating down-stream effectors are underdevelopment. Agonists of several nuclear receptors, such as the glucocorticoid receptors and retinoic acid receptors, were shown to bind to NRF2 and to inhibit the transcriptional activity of NRF2 by hindering its interaction with ARE (Namani et al., 2014). In the search for specific NRF2 inhibitors using a drug-repurposing approach, around 4000 clinical compounds were analysed in vitro on A549 tumor cells with high NRF2 activity due to KEAP1 mutations (Choi et al., 2017). The study indicated clobetasol propionate (CP) as the most potent NRF2 inhibitor that prevented nuclear accumulation of NRF2 by promoting its β-TrCP-mediated degradation, dependant on glucocorticoid receptors and GSK-3. Consequently, CP triggered oxidative stress and strongly suppressed the anchorage-independent growth of KEAP1-mutated tumors. In the same context of therapeutic targeting of nuclear receptors, retinoid X receptor alpha (RXRα) was shown to inhibit the NRF2 signaling pathway through a direct interaction with the Neh7 domain of NRF2 (Wang et al., 2013). Consequently, the all-trans retinoic acid (ATRA), an agonist of retinoic acid receptors, was able to decrease the therapy resistance of ovarian and lung CSCs. ATRA induces CSC differentiation and stemness inhibition, partly due to the decrease of NRF2-mediated transcription as a downstream event of ALDH1A1 suppression occurring selectively in ALDH1high CSCs (Moreb et al., 2017; Kim et al., 2018a). Unexpected results were obtained by using agonists of PPARγ in particular tumors addicted to the IGF (i growth factor) axis or occurring in hyper-insulinemic patients (Vella et al., 2017). PPARγ may act directly or through upstream pathways to activate

NRF2, and NRF2 can induce the expression of PPARγ through a positive feedback loop (Polvani et al., 2012). However, PPARγ activation by anti-diabetic thiazolidinediones was able to inhibit tumor cell growth (Vella et al., 2017) through other mechanisms then those related to NRF2, such as by lowering the levels of circulating insulin and key pathways of the insulin/IGF axis (i.e. PI3K/mTOR, MAPK and GSK-3β/Wnt/β-catenin pathways) that regulate cancer cell survival, reprogramming and differentiation. In general, therapeutic targeting of nuclear receptors for inhibiting tumor progression raises clinical issues, considering the complex network of signaling pathways associated with nuclear receptors and the multitude of consequences at functional level.

It has been shown that NRF2 can negatively regulate the signaling molecule stimulator of interferon genes (STING), and this greatly impedes on the anti-tumor immune response modulated by cytokines and interferons (Gunderstofte et al., 2019; Su et al., 2019). In turn, type I interferons that are produced upon STING activation abrogate NRF2 function and could have beneficial effects by increasing the sensitivity to therapy of NRF2-addicted tumor cells (Olagnier et al., 2018). It has been shown that STING agonists, such as the newly developed dimeric aminobenzimidazole, can elicit a strong anti-tumor activity, resulting in complete and lasting regression of mouse syngeneic colon tumors (Ramanjulu et al., 2018). Moreover, STINF agonists sensitize melanoma cells to BRAF inhibitors by facilitating anti-tumor adaptive T cell responses (Chipurupalli et al., 2020). Several STING activators are now in clinical trials (Berger et al., 2019).

Knowing the complex array of interaction between NRF2 and other signaling pathways (Cuadrado et al., 2018), drug repurposing is a promising strategy to speed-up the discovery of NRF2 inhibitors and to translate them in the clinical practice for increasing the radio-sensitivity of tumor cells. Several currently used drugs are under study for their ability to inhibit NRF2 activation and/or NRF2 expression (Panieri and Saso, 2019): ATRA and RARα agonists, used for neuroblastoma and acute promyelocytic leukemia treatment, the topoisomerase inhibitor Camptothecin, used for chemotherapy in lung, breast, colon, ovarian and brain cancer, the multi-tyrosine kinase inhibitor Sorafenib, used as anti-angiogenic agent. NRF2-inhibiting activity was evidenced also for several drugs used for the treatment of other diseases than cancer, such as the antidiabetic drug Metformin, the anti-rheumatic drug Auranofin, and the histone deacetylase inhibitor Varfarin, used in epilepsy and seizure disorders. Although, radio-sensitization is needed for a limited time, shortly before and/or after RT, the use of these approved drugs raises issues related to their pleiotropic action on various cellular targets.

Surprisingly, molecules known as NRF2 activators, like the synthetic triterpenoid bardoxolone methyl (CDDO-Me), were shown to exert anti-cancer activity in tumor cell lines and tumor-bearing mice (Wang et al., 2014). It is presumed that low concentrations of CDDO-Me are preferentially interacting with KEAP1 to release NRF2 and activate the phase 2 cytoprotective pathway. Meanwhile, CDDO-Me at higher concentrations might interact with lower binding affinities with various other target proteins (i.e. tubulin, IκB kinase), hence inhibiting proliferation and inducing apoptosis (Liby et al., 2007). It may also occur that CDDO-Me loses its ability to activate NRF2 in cells with high levels of NRF2 activity (Mitsuishi et al., 2012a). Three clinical trials on CDDO-Me in cancer have been conducted in the last years according to clinicaltrials.gov, but no data have been communicated yet. From the same perspective, it has been shown that ascorbic acid, a well-known antioxidant, was found to sensitize leukemia KCL22 cells that are resistant to imatinib, a BCR/ABL tyrosine kinase inhibitor, by lowering NRF2 levels in the nucleus (Tarumoto et al., 2004). This was due to the decrease of GSH levels consequent to the decline of NRF2-mediated expression of GCLC. Additionally, it has been shown that ascorbate, at concentrations achieved only by intravenous (iv) administration, may be a prodrug for hydrogen peroxide formation (Chen et al., 2005). The ascorbate-induced increase of ROS levels seems to be a promising strategy for radio-sensitization of CSCs across multiple tumor types, and iv-administered ascorbate proved to be safe in most patients, with virtually no toxicity compared to most currently available chemotherapeutic agents. The occurrence of one predicted complication, oxalate kidney stones, is still controver-

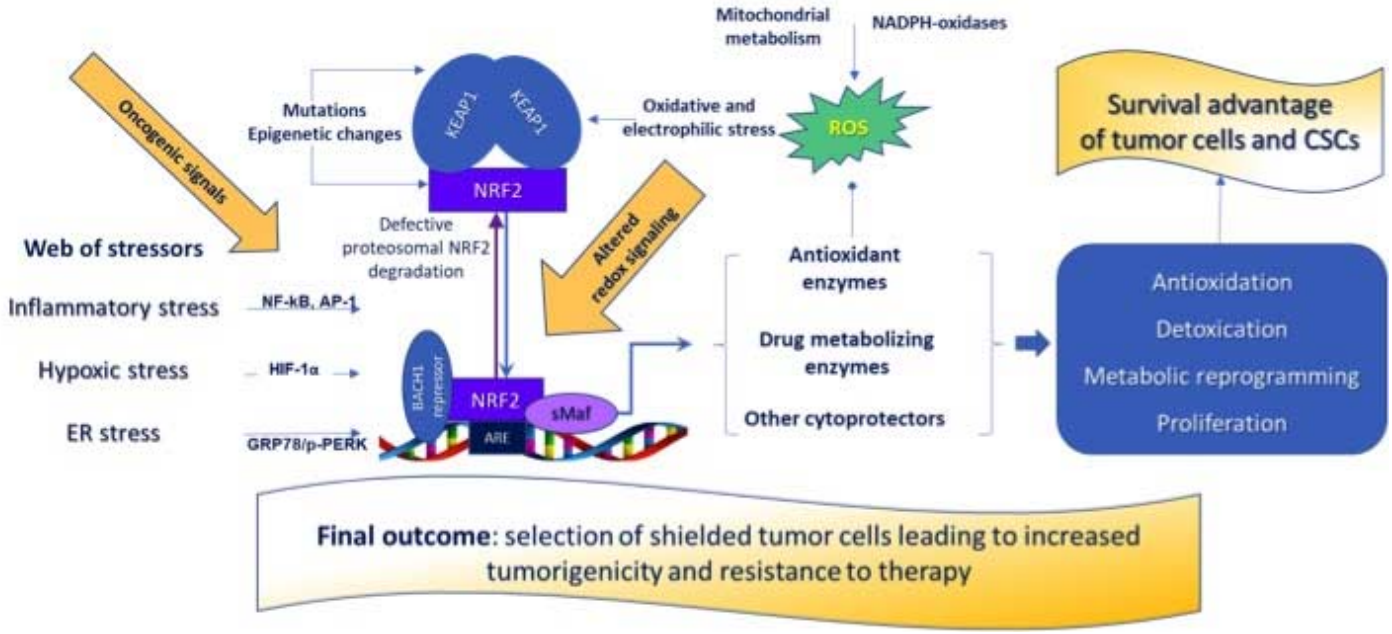

Figure 2
The network of NRF2-related processes sustaining tumorigenicity and therapy resistance in tumor cells.

sial (Levine et al., 1999). Clinical evidence is that at a minimum 1 g/kg ascorbate should be administered intravenously twice weekly, and the absolute minimum treatment duration is two months for reaching radio-sensitization (Shenoy et al., 2018). Concluding, the pro-oxidative action of some of the NRF2 activators might be dose-dependent, requiring therefore highly personalized dose regimens for driving the redox status in each patient towards the desired profile that sustains a therapeutic benefit. Moreover, NRF2 activators might be therapeutically required sometime after combined therapy with RT and NRF2 inhibitors for restoring redox homeostasis in normal tissues (Cuadrado et al., 2019), albeit there are concerns that an increase in NRF2 performed at an improper time point after RT might shield tumor cells against the long-term effects of RT.

NRF2 regulates the redox status and signalling with close and distant consequences, complemented by the perspective provided by the newly-generated NRF2 interactome evidencing a multitude of NRF2-centered interaction networks (Cuadrado et al., 2018). For instance, it has been recently shown in 721 gliomas from The Cancer Genome Atlas database that NRF2 propels tumorigenesis by inducing the expression of the transcriptional co-activator TAZ from the Hippo signaling pathway that promotes tumor growth (Escoll et al., 2020). This complex network of interactions between NRF2 and other signalling pathways is probably the main drawback of NRF2-targeted therapeutics from the point of view of predictable drug action. Nonetheless, radio-sensitization of CSCs using NRF2 inhibitors is a short-term adjuvant therapy that will only transiently impair NRF2 activity in tumor and normal cells, with acceptable and reversible side-effects regarding the alteration of redox homeostasis in normal cells. Accordingly, for the moment the main focus in drug discovery or repurposing is to identify targeted NRF2 inhibitors with as less as possible of off-target effects, able to bring NRF2 activity to well-defined levels appropriate for radio-sensitization.

Conclusion and future perspectives

As exemplified in this review, constitutive activation of the NRF2 pathway has a critical role in sustaining tumor progression and recurrence, and accounts for resistance to therapy, especially to therapies primarily based on oxidative stress such as radiotherapy and photodynamic therapy. This appears to be a potent adaptation mechanism through which tumor cells adapt for surviving under the attack of insidious cues that drive oncogenesis and act in the turbulent tumor niche, in close crosstalk with other signaling pathways that protect tumors cells against a web of stressors. Stress shielding promotes the selection of particular tumor cells that are kept in a quiescent state until driving forces make them to proliferate, invade the organ and migrate to distant sites, resulting in tumor recurrence, metastasis and resistance to therapy (**Figure 2**).

From an academic point of view, in-depth investigation of the inter-related mechanisms underlying tumor progression using advanced concepts and tools of network medicine and systems biology is highly important for better defining the players in cancer. Without intending to over-simplify, from the clinical point of view it is most important to investigate tumor specimens from a more general perspective related to tumor progression, recurrence potential and individualized therapeutic responses. A promising approach is to correlate the currently available tumor markers with markers reflecting NRF2 activity in various types of tumors. Coordinated clinical research on new cohorts selected by well-defined criteria is needed for systematic investigations of these aspects in well-characterized tumors from patients whose evolution is monitored in time. Moreover, the methodological approach for assessing NRF2 levels and the transcriptional activity of NRF2 in tumor specimens has to be refined for future clinical translation. Such a focused large-scale study will most probably provide reliable evidence if NRF2 can be considered an oncogenic marker for improved disease prognosis and response to therapy. It is expected to obtain a set of genes under the transcriptional control of NRF2, that correlate well with disease stage, or the survival prognostic, or the response to therapy in particular types of tumors. Additional knowledge or a new perspective on NRF2 biology in cancer can identify new molecular targets or mechanisms for transiently decreasing NRF2 activity in tumors, hence fostering drug development or repurposing for radio-sensitization of tumor cells, with limited off-target effects. ✔

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Competing interests/conflict of interests

No competing interests to declare.

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