Keap1/Nrf2 pathway in the frontiers of cancer and non-cancer cell metabolism

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Abstract

Cancer cells adapt their metabolism to their increased needs for energy and substrates for protein, lipid and nucleic acid synthesis. Nuclear erythroid factor 2-like 2 (Nrf2) pathway is usually activated in cancers and has been suggested to promote cancer cell survival mainly by inducing a large battery of cytoprotective genes. This mini review focuses on metabolic pathways, beyond cytoprotection, which can be directly or indirectly regulated by Nrf2 in cancer cells to affect their survival. The pentose phosphate pathway (PPP) is enhanced by Nrf2 in cancers and aids their growth. PPP has also been found to be up-regulated in non-cancer tissues and other pathways, such as *de novo* lipogenesis, have been found to be repressed after activation of the Nrf2 pathway. The importance of these Nrf2-regulated metabolic pathways in cancer compared with non-cancer state remains to be determined. Last but not least, the importance of context about Nrf2 and cancer is highlighted as the Nrf2 pathway may be activated in cancers but its pharmacological activators are useful in chemoprevention.

The Keap1/Nrf2 pathway and its pharmacological manipulation

Nuclear erythroid factor 2-like 2 (Nrf2), a cap n' collar transcription factor with a basic leucine zipper, is a central regulator of the adaptive cytoprotective response against electrophilic or oxidative stressors. Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 in the cytoplasm and facilitates its proteasomal degradation. Keap1 possesses a series of reactive cysteines that can be modified by exposure to oxidants and electrophiles. This modification induces allosteric changes in Keap1 that prevent the marking of Nrf2 for proteasomal degradation. Thus, Nrf2 protein that escapes degradation accumulates and after possible post-translational modifications (e.g., phosphorylation), can enter the nucleus and bind to antioxidant response element (ARE) sequences (5′-TGAG/CNNNGC-3′) that lie on the regulatory regions of its target genes [1]. Consequently, the transcription of a battery of cytoprotective genes is activated such as NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) and *Gst*s. The cytoprotective role of the Keap1/Nrf2 pathway has been the focus of clinical trials employing pharmacological agents that enhance signalling through this pathway: triterpenoids [2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid, methyl ester (CDDO-Me)] in the setting of end-stage diabetic nephropathy[2,3], isothiocyanates (sulforaphane) used in the form of broccoli sprout extracts to detoxify airborne pollutants [4] and dampen autism spectrum symptoms[5] and dimethyl-fumarate (BG-12) to halt the progression of multiple sclerosis [6].

The Keap1/Nrf2 pathway activation status in cancers

The role of the Keap1/Nrf2 cytoprotective pathway in cancer is dependent on context [7]; activation of this pathway may protect and aid an already transformed cancer cell replicate or prevent the transformation of a normal cell to a cancerous one [8]. A recent study on 4742 human cancers revealed that somatic mutations of *Nrf2* or *Keap1* are relatively frequent, highlighting potential important roles of this pathway in cancer [9]. *Keap1* mutations had been described in the past to cause loss of Keap1 function, thus leading to constitutive Nrf2 pathway activation in biliary tract [10] and in non-small cell lung cancer [11]. Interestingly, *Nrf2* mutations occur either in the DLG or in the ETGE motif of the *Nrf2* gene, both leading to constitutive activation of Nrf2. ETGE mutations disrupt the high-affinity binding of Nrf2 to Keap1 whereas mutations in DLG disrupt the low-affinity binding of Nrf2 to Keap1[12]. This enhanced activation of the Nrf2 pathway through mutation promotes the survival of cancer cells by enhancing the expression of antioxidant and xenobiotic detoxification enzymes [11], by mediating cell proliferation [13] and by promoting resistance of cancer cells against drugs due to increased expression of genes that encode efflux pumps [14]. However, Nrf2 has also been shown to be crucial to the prevention of cancer. *Nrf2*-disrupted mice are more sensitive to chemical-induced carcinogenesis

Key words: cancer, Kelch-like ECH-associated protein 1 (Keap1), lipogenesis, metabolism, nuclear erythroid factor 2-like 2 (Nrf2), pentose phosphate pathway.

Abbreviations: Acc1, acetyl-coA carboxylase 1; CDDO-Me, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid, methyl ester; Fasn, fatty acid synthase; G6p, glucose-6-phosphate; G6pd, glucose-6-phosphate dehydrogenase; Idh1, isocitrate dehydrogenase 1; Keap1, Kelch-like ECH-associated protein 1; MCD, methionine–choline deficient; Me1, malic enzyme 1; Nrf2, nuclear erythroid factor 2 like 2; Pgd, phosphogluconate dehydrogenase; Pparγ, peroxisome proliferator activated receptor γ; PPP, pentose phosphate pathway; Srebp-1, sterol regulatory-binding protein 1; Taldo1, transaldolase 1; Tkt, transketolase.

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as evidenced, for example, by increased benzo(α)pyrene-induced forestomach neoplasia [15], lack of protection against dextran sulfate sodium-induced early phase inflammation in colonic tumorigenesis [16] and increased accumulation of DNA adducts in lung after exposure to diesel exhaust [17]. The cancer chemopreventive actions of triterpenoids, isothiocyanates and other inducers of Nrf2 signalling are often lost in these Nrf2-disrupted mouse models. Conversely, constitutive activation of Nrf2 signalling can be protective as shown in 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis [18]. In many cases, this prevention of tumorigenesis is mediated by inducing antioxidant and cytoprotective genes. But to highlight the importance of context, Nrf2-disruption has been shown to protect against Ras-driven tumorigenesis in the pancreas [19].

Before proceeding with the topic of Nrf2 in cancer cell metabolism, we will summarize first what is known about the role of Nrf2 in the non-cancer state and provide a background on the regulation of cancer cell metabolism.

Summary of the role of Nrf2 signalling in metabolic disease

Most of the effects of Nrf2 described above probably have a common denominator; they are all related to the transcriptional regulation of its cytoprotective and antioxidative gene targets. However, there is increased evidence that Nrf2 can cross-talk with other pathways such as *Ahr* (aryl hydrocarbon receptor) [20], *Notch* [21], peroxisome proliferator-activated receptor γ (*Ppar*γ) [22] and retinoid X receptor α (*Rxr*α) [23]. Interactions with these pathways greatly expand the impact of Nrf2 signalling on cell fate. Other potential Nrf2-dependent pathways have been suggested using screening techniques such as gene expression microarrays [24], ChIP-Seq [25] and proteomic analyses [26]. For example, such approaches have shown that Nrf2 may regulate the expression of lipid and glucose metabolism genes, at least in liver and adipose tissue, where these analyses were mainly performed.

This potential of Nrf2 to play a role in cell metabolism has also become evident from studies in murine models of high-fat diet-induced obesity and methionine–choline-deficient diet (MCD)-induced fatty liver disease. Interestingly, both loss of Nrf2 function models (*Nrf2* knockout mice) and gain of Nrf2 function models (CDDO-Me treated mice or *Keap1* hypomorphic mice, a pharmaco-mimetic model of enhanced Nrf2 pathway activation [27]), have been shown to be at least partially protected from high-fat diet-induced obesity through different potential pathways [28,29]. The repressive effect of Nrf2 on key gluconeogenic genes expression, *Pepck* (phosphoenolpyruvate carboxylase) and *G6Pase* (glucose-6 phosphatase) [29] and on lipogenic gene expression such as fatty acid synthase (*Fasn*) and acetyl-coA carboxylase 1 (*Acc1*) [30] could be one of the mediators of the protective effect of Nrf2 against the metabolic effects of high-fat diet-induced obesity. Moreover, Nrf2-disrupted mice are more susceptible to steatosis and steatohepatitis after MCD diet [31] whereas Keap1 hypomorphic are protected from this liver damage [32]. The emerging role of Nrf2 in metabolism and bioenergetics [33,34] may easily lead to the hypothesis that this function is also important in cancer cells. However, caution should be taken when extrapolating data from non-cancer cells and tissues to the cancer state. Although both normal and cancer cells share the same metabolic pathways, the preference and the utilization rate of metabolites is different; cancer cells usually act in a more cell-autonomous way, being less dependent to endocrine and paracrine signals than normal cells [35]. In the following section, we summarize the special metabolic properties of the cancer cell before considering the roles of the Nrf2 pathway on cancer cell metabolism.

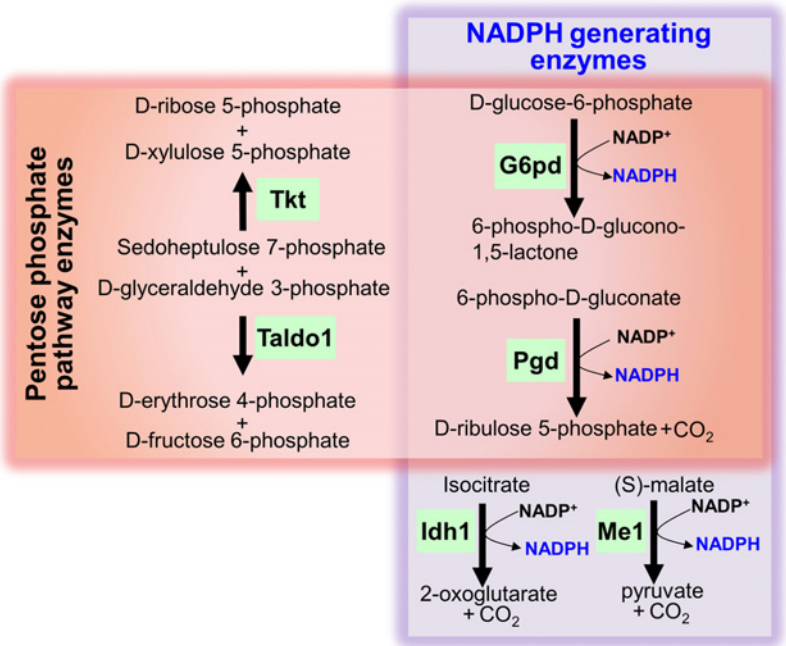
Overview of cancer cell metabolic regulation

Cancer cells can be described as 'hungry' for energy as they strive to meet the increased demands of their rapid growth and replication. Cancer cells alter their metabolism to accommodate these special metabolic needs. The most prominent change in cancer cell metabolism is the increased uptake of glucose and utilization of aerobic glycolysis instead of the more efficient process, as far as ATP production is concerned, of oxidative phosphorylation, the so called Warburg effect [36]. Of course, cancer cells also show increased use of glutamine as a key source for nitrogen [37] and have increased DNA and lipid synthesis [38]. These metabolic properties of the cancer cells, which are usually common among various types of cancer, have come under the spotlight as potential drug targets with the prospect of reducing the energy and nutrients cancer cells need to thrive. Pathways that have been targeted at least experimentally are the glutamine pathway (glutaminase), the pentose phosphate pathway (PPP) (phosphoglycerate mutase), lipid biosynthesis (monoglyceride lipase) and glycolysis [glucose transporter 1 (Glut1) glucose transporter and monocarboxylate transporter]. All these are anabolic pathways that provide cancer cells the necessary energy and substrates to synthesize lipids, proteins and nucleic acids. Given that all these metabolic pathways also function in normal cells, it is always challenging to find agents with adequate therapeutic indices so as to prevent toxicity to normal tissues.

Elucidation of the differential regulation of enzymes in these pathways in the cancer cell is also under investigation. The transcriptional, translational or post-translational regulation of metabolic enzymes can be pharmaceutically targeted affecting more than one metabolic pathway at the same time. For example, myelocytomatosis oncogene (*Myc*), a well described oncogenic transcription factor that controls cell proliferation, has been shown to regulate *Ldha* (lactate dehydrogenase A) [39], which converts pyruvate to lactate. *Myc* also increases the expression of a *Glut1* and hexokinase 2 which converts glucose to glucose-6-phosphate (*G6p*) and then *G6p* can enter glycolysis

Figure 1 | **PPP and NADPH generating enzymes**

The enzymes that are highlighted with green colour have been described to be regulated by Nrf2. Information about the depicted reactions was collected from UniProt.



to produce ATP or the PPP that leads to NADPH and ribose production [40]. *Hif-1* (hypoxia-inducible factor-1) can also transactivate these genes under hypoxic conditions [41]. Another well characterized tumour suppressor gene, *p53*, has also been implicated in the regulation of cancer cell metabolism [42] by activating hexokinase 2. Octamer-binding protein 1 (*Oct-1*), another transcription factor that is up-regulated in cancers, can co-operate with *p53* in the regulation of glycolytic enzymes [43].

Apart from these well-described oncogenes, other transcription factors such as sterol regulatory-binding protein 1 (Srebp-1) and Pparγ that are well described to regulate lipogenesis and adipogenesis respectively in non-cancer cells, have been found to be overexpressed in some cancers. For example, Srebp-1 is overexpressed in prostate cancer [44] and breast cancer [45] promoting their growth whereas Pparγ is highly expressed in colon adenocarcinomas [46] and gastric cancers [47], inhibiting their growth. From these examples it is apparent that transcription factors can regulate the expression of genes encoding metabolic enzymes in cancer cells. Based on this concept, the role of Nrf2, as a transcription factor, in the regulation of the cancer cell metabolism also needs to be considered.

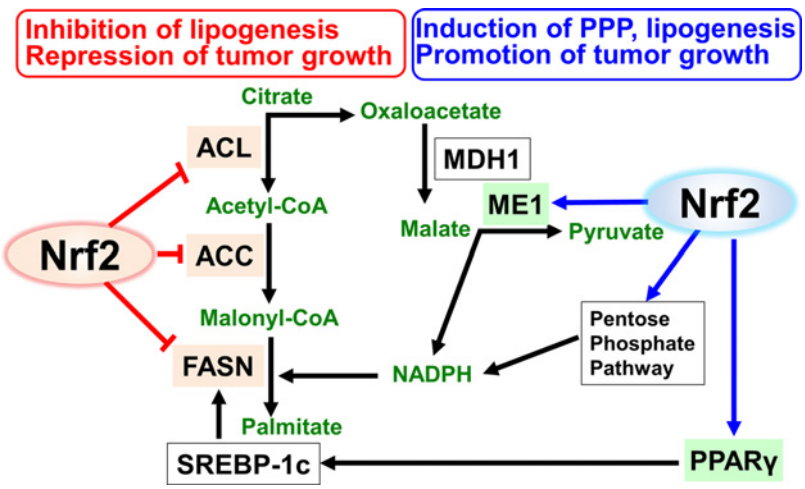
The transcriptional regulation of cancer cell metabolism by the Keap1/Nrf2 pathway

Some well-described oncogenes or tumour suppressor genes can affect the metabolic status of the cancer cells not only by regulating their cell cycle but also by directly or indirectly affecting the expression of metabolic enzymes. In this context, as the Keap1/Nrf2 pathway is usually found to be activated in cancers and appears to affect metabolic responses in normal tissues, the next logical step was to investigate the potential role of Nrf2 in the regulation of the metabolism of cancer cells. The only study to date that directly investigated this role of Nrf2 in cancer employed *in vitro* models (human lung carcinoma cell lines H2126, LK2, EBC1 and mainly focused on A549 cells that carry a somatic mutation in the Keap1 gene and in which the Keap1 promoter is hypermethylated leading to constitutive Nrf2 activation) and *in vivo* models (mice with xenotransplantation of A549 lung cancer cells) [48]. Of course, results from other studies have been supportive of the overall conclusions and are also discussed below. These models are also of a direct clinical relevance as *Keap1* mutations were among the most frequent ones in the lung cancers [9].

Silencing Nrf2 in A549 cells led to the down-regulation of a series of genes involved in the PPP and include glucose-6-phosphate dehydrogenase (*G6pd*), phosphogluconate dehydrogenase (*Pgd*), transketolase (*Tkt*) and transaldolase 1 (*Taldo1*). Moreover, genes involved in NADPH synthesis also showed reduced expression after Nrf2 knockdown: malic enzyme 1 (*Me1*) and isocitrate dehydrogenase 1 (*Idh1*). The reactions catalysed by these enzymes are summarized in Figure 1. Other anabolic genes were also down-regulated: phosphoribosyl pyrophosphate amidotransferase (*Ppat*) and methylenetetrahydrofolate dehydrogenase 2

Figure 2 | Nrf2-regulated metabolic pathways and potential effects on tumour growth

Nrf2 induces genes that encode enzymes of the PPP and other NADPH generating enzymes (Me1). Pparγ induction by Nrf2 can potentially lead indirectly to elevated Srebp-1c expression and increased lipogenesis. On the other hand, activation of Nrf2 pathway leads to reduced expression of the lipogenic enzymes Acl, Acc and Fasn. The molecular mechanisms of this repressed expression are not fully known yet. Abbreviations: ACL, ATP citrate lyase; MDH1, malate dehydrogenase 1. ➔ Denotes induction; ┤ Denotes repression.



(*Mthfd2*) [48]. ChiP-Seq analysis confirmed these results. The importance of these results became evident by performing xenotransplantation of A549 cells in nude mice. Mice developed tumours whose growth and end-point mass were significantly lower if Nrf2 had been silenced in the A549 cells before transplantation. This retardation of tumour growth was replicated by silencing both *G6pd* and *Tkt* (known Nrf2 target genes). Thus, the Nrf2-regulated expression of these PPP genes is important for tumour growth. Interestingly, *G6pd* has also been confirmed as an Nrf2-regulated gene in studies using another cancer cell line, Hepa 1c1c7 cells (a mouse hepatoma cell line) [25] and non-cancerous cells such as mouse embryonic fibroblasts [49] and also in mouse liver [50] and small intestine [51]. Besides *G6pd*, other NADPH generating enzymes have been shown to be regulated by Nrf2 in non-cancer states as well: *Me1* in small intestine [51] and in liver of mice fed a standard [50] or a high-fat diet [52], *Idh1* in mouse liver [50] and mouse embryonic fibroblasts [49], *Pgd* and *Taldo1* in mouse liver [50].

From these lines of evidence, Nrf2 seems to be important for the proliferation of cancer cells by up-regulating the expression of a series of enzymes involved in the PPP and the production of NADPH. However, this role of Nrf2 seems to be at least partially present in non-cancer tissues (liver, small intestine) where NADPH can also be used as a scavenger for oxidative stress and for anabolic pathways such as fatty acid synthesis. In this case, at the cellular level, this can have protective effects. However, if Nrf2 is significantly overexpressed, as happens in the *Keap1* knockout mice, this can also have adverse effects for the organism. Indeed, the *Keap1* knockout mice die approximately 20 days after birth as they cannot get adequately fed due to the development of hyperkeratosis in the oesophagus and forestomach [53]. This phenomenon can be attributed to hyperproliferation and hyperkeratinization of epithelial cells as it was shown by increased bromodeoxyuridine (Brdu) incorporation in mice with very low Keap1 expression [48] (one allele *Keap1* knockout and the other allele hypomorphic; $Keap1^{flox/-}$) [27].

Conclusions and perspective

The role of the Keap1/Nrf2 pathway as an orchestrator of cytoprotective genes expression is well described. Most of the studies on the role of Nrf2 in a variety of settings (drug toxicity, cancer, obesity) were performed based on the hypothesis that activation of Nrf2 pathway can reduce levels of reactive oxygen species and other toxic species (e.g., lipid peroxides) that have deleterious effects on the cells and where their production often increases in disease settings. Indeed, most of these hypotheses were proved to be correct as Nrf2 appeared to have a protective effect. However, in the case of cancer, already transformed malignant cells may benefit from these cytoprotective effects and enhance their survival.

Beyond those notions that are widely held, Nrf2 can exert other functions as well by controlling the expression of genes that encode other transcription factors or other enzymes that possess a variety of properties. Such genes are the ones that can affect metabolism. The importance of anabolic metabolic pathways (e.g., PPP, lipid and nucleic acid synthesis) for the growth of cancer cells is well established. From this point of view, herein Nrf2 was described as a transcription factor that enhances the PPP and NADPH generation and in this way it helps cancer cells proliferate.

However, the involvement of Nrf2 in metabolic regulation in the non-cancer state is also emerging beyond the PPP and NADPH generation. A metabolic pathway that can also be potentially important in cancer is *de novo* lipogenesis. As already mentioned, activation of the Nrf2 pathway leads to a repressed lipogenic programme in the liver of mice fed high-fat [30] or standard diet [24]. Specifically, Nrf2 has been found to have a repressive effect on ATP citrate lyase [26] that catalyses the conversion of citrate to acetyl-coA, on *Acc1* that converts acetyl-coA to malonyl-coA [30] and on *Fasn* that catalyses the formation of palmitic acid from malonyl-CoA [24,30] (Figure 2). This reduced lipogenesis in the liver after activation of the Nrf2 pathway leading to lower hepatic triglycerides, is beneficial in the context of fatty liver disease. This effect could also be inhibitory to cancer growth as cancer cells require increased lipogenesis so as to meet their augmented demands [54]. In Figure 2, we summarize what is already known about the role of Nrf2 in the lipogenic pathway and propose a potential role of this pathway in cancers. Similarly, in the same figure we have included the Pparγ pathway that is known to promote adipogenesis and lipogenesis [55] and we speculate on its potential relevance in cancers.

To better assess these Nrf2-regulated pathways in cancers, further studies are warranted starting from the cancers that have been shown to have an activated Nrf2 pathway. Analysing biopsies of these cancers for the expression of genes that are potential Nrf2 targets in non-cancer metabolism, such as the ones that regulate *de novo* lipogenesis, should make it possible to identify correlations or at least associations of the activated Nrf2 pathway and overexpression of these genes. The next step would be to confirm these observations using, for example, mouse models in which modification of these pathways can be performed genetically.

In conclusion, Nrf2 appears to act as a hormetic factor as both its extremely low or its extremely high levels can have negative effects [7]. Thus, in the case of cancer, Nrf2 pathway activation can be beneficial for cancer prevention but absence of Nrf2 can increase the susceptibility of cells to carcinogenic agents whereas increased Nrf2 signalling may aid cancer cell survival and proliferation.

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