Nrf2, the master redox switch: the Achilles’ heel of ovarian cancer?

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Abstract
Ovarian cancer is the most lethal gynecological tumor type in the world due to late stage detection, and resistance to chemotherapy. Therefore, alternative additional therapies are required. The etiology of ovarian cancer remains largely unknown, but risk factors point toward an important role for oxidative stress. Both healthy and tumor cells can cope with oxidative stress by activating the transcription factor Nrf2 (also known as Nfe2l2), the master regulator of antioxidant and cytoprotective genes. Indeed, for most ovarian cancers, aberrant activation of Nrf2 is observed, which is often associated with a copy number loss within the Nrf2-inhibitory complex KEAP1-CUL3-RBX1. A key role for Nrf2 in ovarian carcinogenesis has been validated by siRNA studies. However, to exploit the Nrf2 pathway for therapeutic interventions, potential side-effects should be minimized. In this review, we explore ovarian cancer specific factors with links to aberrant activity of Nrf2, to be exploited in future combination strategies, synergistic with direct Nrf2 inhibitory drugs. Particularly, we propose to stratify patients based on common ovarian cancer mutations (KRAS, BRAF, ERBB2, BRCA1 and its link with estradiol, TP53) for future NRF2 targeting strategies.
1. Oxidative stress

1.1. Introduction
Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and their detoxification by antioxidants. During lifetime, cells are challenged by various levels of oxidative stress, depending on the cell type and cellular location. Two important sources of endogenous ROS production are within the mitochondria and by cells, mainly neutrophils and macrophages, of the immune system. In the mitochondria, during oxidative phosphorylation some electrons will leak out of the electron transport chain and can react with oxygen molecules to generate the superoxide anion (O$_2^-$) (1). O$_2^-$ can further react to hydrogen peroxide (H$_2$O$_2$), and if not neutralized by antioxidants, both these free radicals together can, via the iron-catalyzed Haber-Weiss reaction, be transformed to the most reactive radical among all ROS: the hydroxyl radical (OH$^-$) (2). This implies that in metabolically active cells, such as cancer cells, mitochondria generate a substantial amount of ROS. ROS are not only a harmful side-product of energy production; ROS can also be beneficial, for example as intracellular signaling molecules or in ROS-mediated host defense for the elimination of pathogens (3). Therefore, presence of immune cells at the site of chronic inflammation can, by the presence of chronic oxidative stress, contribute to the pathophysiology of many different diseases (4). In addition to endogenously produced ROS, also several exogenous sources, including UV-light and cigarette smoke, can contribute to the total ROS production within cells. All these sources together will lead to accumulation of ROS which results in oxidative stress. Too much ROS can disturb redox signaling and damage the proteins, lipids and DNA, of which 8-OHdG is an important oxidative DNA modification, within the cell. As such, disturbed redox signaling and ROS-induced damage contributes to aging and is involved in a wide variety of diseases, including Alzheimer’s disease, atherosclerosis and cancer. Reviewed in (5, 6).

As the consequences of long-term oxidative stress can be detrimental, cells have important defense mechanisms against oxidative and xenobiotic stress at their disposal, including the transcription factor Nrf2 (nuclear factor, erythroid 2-like 2; Nfe2l2) (7). Nrf2 is the master regulator of many antioxidant and cytoprotective genes. Under physiological conditions, Nrf2 is present in the cytoplasm where it is bound by Keap1. Keap1 requires the actin cytoskeleton in order to efficiently bind Nrf2 in the cytoplasm (8). Keap1 forms a complex with Cul3 and Rbx1, and this E3 ubiquitin ligase complex is able to bind and
ubiquitinate Nrf2, resulting in Nrf2 being targeted to the proteasomes for degradation. When oxidative stress is present within the cell, the cysteine residues of Keap1 become oxidized, resulting in a conformational change of the Keap1-Nrf2 complex which prevents Keap1 to ubiquitinate Nrf2 (9, 10). At the same time, ROS signaling activates unknown tyrosine kinases that export negative regulators of Nrf2, including Src kinases and Bach1, out of the nucleus (11). As a result, newly formed Nrf2 is then able to translocate to the nucleus and bind, together with small Maf proteins (12), to antioxidant response elements (AREs) resulting in the transcription of its downstream target genes (13). When downstream target genes, like antioxidants, have restored the redox balance, Src kinases will export Nrf2 out of the nucleus again where Nrf2 will be degraded (14) (Fig. 1). Besides the above described Keap1-dependent, redox-sensitive mechanisms of Nrf2 inhibition, also several Keap1-independent mechanisms have been demonstrated. For example, the redox-sensitive transcription factor Bach1 has been shown to compete with Nrf2 for the activation of HMOX1 (a Nrf2 downstream gene) (15, 16), by binding to enhancer ARE sites in the HMOX1 gene. As Bach1 lacks a transcription modulation domain, it will not directly activate/repress the downstream gene, but as its binding prevents binding of Nrf2, Nrf2-induced activation will be prevented by Bach1 binding. However, a similar mechanism could not be clearly defined for other ARE containing Nrf2 downstream genes (15, 16). In contrast to Bach1, a redox-insensitive, Keap1-independent mechanism of Nrf2 inhibition has been discovered (17) and the exact mechanism has recently been unraveled (18). Chowdhry et al. discovered that Nrf2 contains two conserved binding motifs for beta-transducin repeat-containing protein (β-TrCP), which acts as a substrate receptor for the Skp1-Cul1-Rbx1 ubiquitin ligase complex (i.e. SCFβ-TRCP). By GSK3-mediated phosphorylation of one of these binding motifs (DSGIS), binding of β-TrCP to Nrf2 becomes tighter, and as such SCFβ-TRCP-mediated ubiquitination and degradation of Nrf2 increases. These Keap1-independent mechanisms add an extra layer to the transcriptional regulation of Nrf2.

In the following sections we will discuss the role of oxidative stress in carcinogenesis, specifically focusing on ovarian cancer. As Nrf2 is the master regulator in the protection against oxidative stress, modulation of Nrf2 activity can have therapeutic potential. In this review, we will look into the role of aberrant Nrf2 activation during ovarian carcinogenesis, with a special focus on oncogene and tumor suppressor genes herein. The acquired knowledge will be used to strengthen the evidence for an essential role of Nrf2 in ovarian cancer and
to provide therapeutic strategies for the implementation of Nrf2 inhibitors in the treatment of ovarian cancer.

Figure 1. Regulation of Nrf2 by oxidative stress. Nrf2 is the master regulator of many antioxidant and cytoprotective genes. Under normal conditions, Keap1 binds Nrf2 in the cytoplasm. Keap1 mediates ubiquitination of Nrf2, resulting in proteasomal degradation of Nrf2. When oxidative stress is present in the cell, cysteine residues in Keap1 become oxidized, resulting in a conformational change of the Keap1-Nrf2 complex that prevents Keap1 from ubiquitinating Nrf2. As a consequence, newly formed Nrf2 can translocate to the nucleus where it binds together with small Maf proteins to activate transcription from antioxidant response elements (ARE). Downstream Nrf2 target genes are mainly involved in the protection against oxidative stress and xenobiotics.

1.2. The role of Nrf2 in oxidative stress-induced carcinogenesis

Oxidative stress is well recognized for its role during the initiation and progression of cancer (19). The level and duration of the oxidative stress exposure are the main factors that determine the cellular outcome of oxidative stress. On the one hand, a moderate increase in oxidative stress stimulates cell proliferation and may induce a few DNA mutations, while on the other hand, a large increase in oxidative stress results in accumulation of excessive genome-wide DNA damage and this induces either senescence or cell death (20). So, some oxidative stress can be beneficial for cancer cells, but too high levels are cytotoxic. Therefore, a reduction in the level of oxidative stress is essential for the survival of tumor cells. For this purpose, tumor cells can exploit the transcription factor Nrf2 (21, 22). Via continuous activation of Nrf2 and subsequent expression of antioxidant genes, cancer cells aim to maintain a favorable redox balance. This adaptation has indeed been found in a wide variety of cancers, including those of the head and neck (23), lung (24), endometrium (25) and gallbladder (26). Unexpectedly, also constitutive downregulation of Nrf2 has been described to occur in some prostate tumors (27), which in a mouse prostate cancer model (TRAMP mice) could be explained by Nrf2 promoter hypermethylation (28), whereas in human prostate tumors a
similar mechanism causing downregulation of Nrf2 has not been described so far. Downregulation of Nrf2 has also been detected in some tumors of the breast, probably via dysregulated KEAP1- and NRF2-interacting miRNAs (29-31). The constitutive Nrf2 activity generally observed in tumors can partly be explained by (epi)genetic mutations or copy number loss (CNL) in any of the components of the E3 ubiquitin ligase complex (KEAP1-CUL3-RBX1) that degrades Nrf2 or by mutations in NRF2 itself (32-37). Mutations in KEAP1 are spread across the whole gene and only very recently, the functional impact of some of these has been unraveled and clustered in three classes (38): (1) Passenger mutations, which neither affect Nrf2 activity nor Nrf2-Keap1 interactions; (2) Null or near-null mutations, which diminish Nrf2-Keap1 binding and are unable to (or very weakly) repress Nrf2 activity; (3) Hypomorph mutations, which result in either reduced or increased (“superbinders”) Nrf2-Keap1 binding without affecting Nrf2 activity. In contrast to KEAP1, All somatic mutations detected in NRF2 are found in specific “hot-spot” regions: within or near the “ETGE” and “DLG” motifs, which are the Keap1 binding domains of NRF2 (39-41). These mutations highlight the importance of Keap1-dependent Nrf2 regulation by degradation of Nrf2. Most of these mutations do not affect Nrf2 protein levels but protein function, as often these mutated proteins cannot form the Keap1-Nrf2 binding anymore and thereby Nrf2 is not being degraded (38). Also non (epi)genetic mechanisms that modulate Nrf2 activity, such as post-transcriptional modifications of Keap1 or Nrf2 and accumulation of proteins that disturb Nrf2-Keap1 binding, have been discussed before in (42, 43). For example, phosphorylation of Nrf2 (43) or succination of Keap1 are known post-transcriptional modifications that can modulate the activity or Nrf2-Keap1 (44). In a large proportion of the tumors (45), the exact mechanism that causes the observed aberrant activity of Nrf2 remains undiscovered, although for ovarian cancer RBX1 seems to be a key player, as described below.

Ovarian cancer is the most lethal gynecological malignancy in woman worldwide (46). The most common variant (> 90%) is epithelial ovarian cancer (EOC) that is believed to arise from epithelial cells lining the surface of the ovary or the distal fallopian tube (47-49). This variant can be further subdivided into the serous (60-80%), mucinous (3-5%), endometrioid (10-25%) and clear cell type (5-10%) (50, 51). Most tumors, including ovarian tumors, are exposed to high levels of oxidative stress. According to the “incessant menstruation” hypothesis, especially serous, endometrioid and clear cell ovarian cancers are thought to be exposed to large amounts of iron-induced oxidative stress derived from
retrograde menstruation, which is a backward movement of menstrual fluids through the Fallopian tubes to the peritoneal cavity (52, 53). Senthil et al. found increased plasma levels of lipid peroxidation and decreased antioxidant levels in ovarian cancer patients versus healthy controls (54). Furthermore, the amount of 8-OHdG expression in ovarian tissue is positively correlated to malignancy, cancer stage and poor survival (55, 56). Interestingly, oxidative stress levels are also related to chemotherapy resistance, as higher levels of the antioxidant glutathione (GSH) have been found in resistant versus sensitive cells (57, 58). GSH has been described as an important antioxidant that facilitates detoxification and excretion of many chemotherapeutics (59, 60). Despite these high levels of oxidative stress, ovarian cancer cells are obviously able to survive. Also ovarian cancer cells frequently exploit constitutive activation of Nrf2 in order to survive exposure to high ROS levels; nuclear immunostaining for Nrf2 is observed in all types of EOCs (varying between 27-83%), but variation in the amount of constitutive Nrf2 activation is quite large in the studies performed so far (61, 62) (Table 1). The observed constitutive Nrf2 activation was associated with increased expression of several Nrf2 downstream genes (GPX3, SOD2) (62). In one of these publications, they also investigated the frequency of KEAP1/NRF2 mutations in ovarian cancer. They described that 29% of the clear-cell and 8% of the non-clear cell ovarian tumors contain a KEAP1 mutation, and none contain a NRF2 mutation (62) (Table 1). Recently, the low frequency of mutations in either KEAP1 or NRF2 was confirmed for a large set of serous EOC samples of the Cancer Genome Atlas (TCGA) (32) (Table 1). Interestingly, this analysis revealed that the majority of aberrant Nrf2 activity in serous EOCs may be caused by inactivating DNA alterations (inactivating mutations, copy number loss (CNL), hypermethylation) in any of the components of the E3 ubiquitin ligase complex KEAP1-CUL3-RBX1. Indeed, for 90% of these cases the inactivating DNA alteration was associated with increased expression of Nrf2 downstream genes. Most remarkably, heterozygous deletion of RBX1 was the most prominent mechanism that contributed to Nrf2 activation in these serous ovarian cancers (32) (Table 1).

Concluding from this, aberrant activation of Nrf2 is a major event during ovarian carcinogenesis and the majority can be contributed to CNL in the E3 ubiquitin ligase RBX1 (Table 1). As RBX1 is an important E3 ubiquitin ligase in many different complexes, and thereby regulates many important genes besides Nrf2, including NF-κB (63) and hypoxia inducible factor (HIF) (64), it is unlikely that RBX1 will be a tumor specific therapeutic target. Although direct inhibition of Nrf2 seems an option as a therapeutic target, current drugs are not specific for Nrf2
and, in addition, will also affect normal cells. Therefore, in order to minimize the effect of direct Nrf2 inhibition on normal cells, we will explore in this review additional tumor-specific targets that are involved in the aberrant activation of Nrf2. We hypothesize that these tumor-specific targets combined with direct Nrf2 inhibition have the potential to become a new therapeutic strategy to combat ovarian cancer. We will review the evidence that frequently dysregulated oncogenes or tumor suppressor genes (KRAS, BRAF, ERBB2, BRCA1 and TP53) in serous EOC contribute to the observed constitutive activation of Nrf2 and thus provide interesting indirect (tumor-specific) targets. We will focus on serous EOC, as it comprises the largest (sub)type of ovarian cancer and is expected to be exposed to the highest level of oxidative stress (highest 8-OHdG tissue expression (56)).

Table 1. Contribution of gene alterations (epi)genetic mutations, mRNA expression changes) in the negative regulator complex (Keap1-Cul3-Rbx1) of Nrf2 that negatively affect Nrf2 expression or protein function in ovarian (serous) carcinomas in relation to Nrf2 protein expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Martinez et al., 2014 (mutations/CNL/ hypermethylation, N = 316/569/582)</th>
<th>Konstantinopoulos et al., 2011 (N = 30)</th>
<th>Liao et al., 2014 (N = 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene alterations</td>
<td>Mutations, CNL, hypermethylation</td>
<td>Mutations</td>
<td>NA</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>8.5% (only amplifications)</td>
<td>0%</td>
<td>NA</td>
</tr>
<tr>
<td>KEAP1</td>
<td>(0.3% mutations, 32.7% CNL, 0.9% hypermethylation)</td>
<td>8-29% (serous: 9%)</td>
<td>NA</td>
</tr>
<tr>
<td>RBX1</td>
<td>(0% mutations, 81.6% CNL, 7% hypermethylation)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CUL3</td>
<td>(0.3% mutations, 26% CNL, 5.2% hypermethylation)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total contribution</td>
<td>90%</td>
<td>9%</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nrf2 protein</th>
<th>Nrf2 positive</th>
<th>Nrf2 downstream genes</th>
<th>Konstantinopoulos et al., 2011 (N = 30)</th>
<th>Liao et al., 2014 (N = 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2 positive</td>
<td>~81% – increased expression</td>
<td>33-79% (serous: 36%) – nuclear immunostaining</td>
<td>27-83% (serous: 71%) – nuclear immunostaining</td>
<td></td>
</tr>
</tbody>
</table>

CNL, copy number loss; NA, not available.

2. The contribution of dysregulated oncogenes and tumor suppressor genes in the continuous activation of Nrf2 in ovarian serous carcinomas

2.1. Pathophysiology of low-grade versus high-grade ovarian serous carcinoma

Serous EOC can be subdivided into low-grade (Type I pathway, 10% of all serous EOC cases) and high-grade (Type II pathway, 90% of all serous EOC cases) tumors
(65, 66). Both types are thought to have a distinct etiology and are characterized by distinct features. Low-grade tumors progress in a slow, step-wise manner and are characterized by mutually exclusive mutations in either KRAS, BRAF or ERBB2 (together covering two-thirds of all low-grade tumors) (67, 68). Only a very small percentage of these tumors harbor a mutation in p53 (<8%) (69, 70). On the other hand, the high-grade tumors are thought to develop very rapidly, and the vast majority of patients diagnosed with these tumors present with late-stage disease. These tumors are characterized by a high level of chromosomal instability and generally harbor a mutation in TP53 (up to 96%) (71). A large proportion of the BRCA1/2 mutant ovarian cancers are of the high-grade serous subtype (72), and these often co-occur with mutations in TP53 (73). Mutations in either KRAS, BRAF or ERBB2 occur very infrequently in this ovarian cancer subtype (65, 67).

In this section, we will elucidate a role for KRAS, BRAF and ERBB2 mutations (low-grade EOC – §2.2) or TP53 and BRCA1 mutations (high-grade EOC – §2.3) in the continuous activation of Nrf2 associated with ovarian serous carcinomas. Based on those insights, we can indicate subpopulations of ovarian cancer patients that might benefit from Nrf2 targeted treatments.

2.2. Relation between aberrant Nrf2 activity and common mutations in low-grade ovarian serous carcinoma

2.2.1. Oncogenic KRAS, BRAF and aberrant Nrf2 activation

The majority of low-grade serous EOCs show mutations in either KRAS or BRAF, of which respectively KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> are the most common variants (68, 74). Both mutations result in the constitutive activation of downstream signaling independently of upstream cues. The major downstream signaling pathway activated by both mutations is the Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) pathway (75). Upon activation of the MAPK/ERK pathway, serial phosphorylation cascades become active and eventually, via phosphorylation, the activity of various downstream proteins, among which transcription factors, can be modulated (76). Constitutive ERK signaling is important during carcinogenesis, as it modulates many different processes, including proliferation, differentiation, survival, migration, angiogenesis and chromatin remodeling (75).

There are indications that oncogenic K-ras or B-raf can constitutively activate Nrf2, although, some seemingly contradicting results have been found for oncogenic RAS proteins; In fibroblasts and keratinocytes, introduction of
overexpressed oncogenic RAS results in increased cellular ROS levels, albeit transient (77-79). However, this is in contrast to the study of DeNicola et al., who found that in a murine model of mutant \( KRAS^{G12D} \) or \( BRAF^{V619E} \) (corresponding to human \( BRAF^{V600E} \)) driven lung and pancreatic cancer, ROS levels were decreased. Further experiments confirmed that this was the direct result of an increase in basal Nrf2 expression up to 60% (80). As shown in this paper, the differential outcome can be explained by the fact that the papers describing increased ROS levels used models in which \( KRAS^{G12D} \) was overexpressed, whereas DeNicola et al. used a model in which \( KRAS^{G12D} \) was expressed at near-physiological levels. DeNicola et al. demonstrated that in both situations total glutathione levels increased, but only near-physiological levels of \( KRAS^{G12D} \) could increase the GSH/GSSG ratio and thereby create a more reduced intracellular environment. These differing results could be explained by the fact that only overexpression of \( KRAS^{G12D} \) stimulated NADPH-oxidase mRNA and activity, and by that ROS production, whereas near-physiological levels of \( KRAS^{G12D} \) did not (81).

As described above, in vivo experiments in mice show that near-physiological levels of oncogenic K-ras and B-raf are able to induce NRF2 expression. Interestingly, also treatment with ARE/Nrf2 inducers in liver cancer cells point toward a role for the MAPK/ERK pathway in the activation of Nrf2 (82, 83). ARE/Nrf2 inducers, such as tert-butylhydroquinone (tBHQ) and sulforaphane, are electrophilic molecules that are able to activate Nrf2 and its downstream genes, often via a mechanism that affects the Keap1-Nrf2 complex formation and thereby Keap1-mediated ubiquitination of Nrf2 (84-87). Each class of ARE/Nrf2 inducers activates a broadly overlapping group of genes, but also shows distinct off-target effects (87). Several mechanisms could explain the role of the MAPK/ERK pathway in the activation of Nrf2. For example, as this pathway can directly modulate downstream proteins via phosphorylation, the direct phosphorylation and thereby stabilization/activation of Nrf2 could be a plausible mechanism. In cell culture experiments (in breast cancer, kidney cells and fibroblasts), upon prevention of Nrf2 phosphorylation via alanine to lysine substitution, only a small reduction in Nrf2 transcriptional activity was seen (88). This indicates that direct phosphorylation of Nrf2 by MAPKs does not play a major role in regulating Nrf2. Therefore, it is more likely that MAPKs indirectly regulate the Nrf2 signaling pathway. Although no specific link with Nrf2 has been described, the MAPK/ERK pathway might for example act via the stimulation of Nrf2 protein synthesis, as this pathway activates several proteins involved in translational assembly, e.g. eIF4E, eIF4B-BPI and eEF2 kinases (89). In MEFs
expressing near-physiological levels of oncogenic K-ras or B-raf, more direct evidence was found for a role of the c-Jun and c-Myc transcription factors downstream of ERK signaling in KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> induced Nrf2 expression. It was shown that c-Jun and c-Myc are directly activated by oncogenic K-ras or B-raf, resulting in elevated c-Jun and c-Myc protein levels, and induction of Nrf2 transcription (80). Immunoprecipitation assays indicate that this is a result of direct binding of both c-Jun and c-Myc to the NRF2 promoter (90). In addition, siRNA knockdown of several downstream MAPK/ERK pathway transcription factors showed that only c-Jun, Fra1 and c-Myc, but not JunD or Elk1 knockdown resulted in decreased expression of Nrf2 in KRAS<sup>G12D</sup> mutant cells, with greatest effects for c-Jun knockdown. Furthermore, near-physiological levels of oncogenic c-Myc have been shown to directly induce Nrf2 expression (80).

Concluding from this, strong evidence points to an important role for oncogenic KRAS or BRAF mutations in permanent activation of Nrf2 expression via the continuous activation of c-Jun and c-Myc transcription factors downstream of ERK signaling (Fig. 2, Table 2).

2.2.2. Overexpressed ERBB2 and aberrant Nrf2 activation
A smaller subset (20-30%) of low-grade serous ovarian cancers shows amplification and/or overexpression of the ERBB2 gene (91-94), which results in increased cell proliferation, altered cell cycle checkpoint and DNA repair mechanisms and is associated with poor prognosis (95-98). Two major downstream pathways of ErbB2 are the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways (99, 100).

As described before for oncogenic K-ras and B-raf, ErbB2 also activates c-Jun and c-Myc via the MAPK pathway, and thereby activate Nrf2 expression (101, 102) (Fig. 2). Moreover, phosphorylation of PI3K via ErbB2 can initiate the PI3K/Akt pathway phosphorylation cascade and this has also been linked to an increased Nrf2 activity. The involvement of the PI3K pathway in Nrf2 activation has mainly been studied with ARE/Nrf2 inducers (103-105).

Interestingly, one study in urothelial carcinoma showed that there is a link between ErbB2 activity and activation of Nrf2 via the PI3K/Akt pathway; chemical inhibition of either ErbB2 or Akt resulted in decreased Nrf2 activation (106). Moreover, Chowdhry et al. observed in A549 lung cancer cells with KEAP1 loss-of-function mediated activation of Nrf2, a markedly reduced expression of Nrf2 and its downstream genes upon inhibition of PI3K or Akt (18). The activity of PI3K is negatively regulated by the phosphatase PTEN, and as such, inhibition of PTEN
activity should counteract the PI3K-mediated activation of Nrf2. Indeed, several studies have shown this is the case (107, 108). For example, Pitha-Rowe et al showed that treatment with synthetic triterpenoids could activate Nrf2 signaling via inhibition of PTEN activity, which stimulated Akt phosphorylation (107). Several mechanisms of action have been proposed to explain the link between PI3K activity and Nrf2 activation. The first explanation is that activated Akt1 stimulates the stability of Nrf2 via phosphorylation (104). However, unlike for the MAPKs, no consensus phosphorylation sequences have been found for Akt in either Nrf2 or Keap1. This makes indirect regulation of Nrf2 protein stability by activated Akt more likely. For example, activated Akt is able to inactivate GSK3β by phosphorylation (109). GSK3β is responsible for the activation and nuclear translocation of the Src-A subfamily kinases Src, Fyn, Yes and Fgr, which in their turn are important for nuclear export of Nrf2 (14, 110). So by inactivation of GSK3β, Akt can stimulate nuclear accumulation of Nrf2, thereby preventing the cytoplasmic degradation of Nrf2 by Keap1. In addition, inactivation of GSK3 can
prevent phosphorylation of the DSGIS motif of Nrf2, thereby preventing the binding of β-TrCP to Nrf2 and β-TrCP-mediated degradation of Nrf2 (18, 108). Alternatively, Kang et al. suggest that also a different mechanism can be involved in PI3K stimulated Nrf2 activity (111). Previously, they observed activation of the PI3K pathway upon treating cells with the ARE/Nrf2 inducer tBHQ (103). The subsequent activation of Nrf2 appeared to be the result of increased nuclear actin levels. Normally, Keap1 binds the actin cytoskeleton to efficiently sequester Nrf2 in the cytoplasm (8). Upon tBHQ treatment, activation of the PI3K pathway resulted in depolymerization of actin microfilaments, after which Nrf2 formed a complex with the depolymerized actin which translocated into the nucleus (111). Taken together, ERBB2 overexpression results in the activation of at least two major downstream signaling pathways that can activate Nrf2: the MAPK and the PI3K/Akt pathway (Fig. 3, Table 2). The first seems to regulate Nrf2 activity on the transcriptional level, whereas the second seems to act on the protein level. As most of these data are collected in other cancer and normal cell types, further studies need to be performed showing that this link between ErbB2 and persistent Nrf2 activity also holds true in ovarian cancer. Furthermore, future research should determine the relative importance of the MAPK and PI3K/Akt pathway on the continuous activation of Nrf2 in ERBB2 mutant cells.

2.3. Relation between aberrant Nrf2 activity and common mutations in high-grade ovarian serous carcinoma

2.3.1. BRCA1 mutations and aberrant Nrf2 activation
Mutations in BRCA1, a ubiquitously expressed DNA repair enzyme, are almost exclusively found in high- and not low-grade ovarian serous carcinomas. Up to about 50% of the high-grade ovarian serous carcinomas contain a BRCA1 (epi)genetic mutation. Of all mutations in this subtype of ovarian cancer, approximately 10% can be attributed to germline BRCA1 mutations, about 6% to sporadic BRCA1 mutations, and 13-31% to hypermethylation of the BRCA1 promoter (112-114). Remarkably, germline mutations in BRCA1, predispose only to breast and ovarian tumors, and not others (115). This seems to imply that hormones are important players during BRCA1 mutant tumorigenesis. In this section, we will discuss the effects the female sex hormone estradiol and its receptor (ER, estrogen receptor) have on Nrf2 activity, and how these effects might play a role in BRCA1 mutant carcinogenesis.
Chapter 5

Figure 3. Overexpressed ErbB2 can aberrantly activate Nrf2 via the PI3K/Akt pathway (and the MAPK/ERK signaling pathway, as shown in Fig. 2). Overexpressed ErbB2 preferentially forms heterodimers with its family members ErbB1/3/4. Upon ligand binding, the ErbB2 heterodimer becomes active and can activate PI3K. PI3K in its turn, converts PIP2 to PIP3, which then activates Akt. Akt is able to modulate the nuclear accumulation of Nrf2 by three different mechanisms: (1) via inhibition of GSK3β, thereby preventing the nuclear accumulation of Src-subfamily kinases and as such prevent their binding to and their nuclear export of Nrf2; (2) via inhibition of GSK3, thereby inhibiting phosphorylation of the DSGIS motif of Nrf2 and as such prevents binding of β-TrCP and β-TrCP-mediated degradation of Nrf2; (3) via depolymerization of actin, which results in the formation and nuclear translocation of Nrf2-actin complexes.
Table 2. Mechanisms by which common oncogene and tumor suppressor gene mutations in ovarian serous carcinomas can constitutively activate Nrf2.

<table>
<thead>
<tr>
<th>Oncogene/tumor suppressor gene (epi)genetic mutation</th>
<th>% of total subpopulation (low-/high-grade)</th>
<th>(Putative) mechanism of constitutive Nrf2 activation</th>
<th>Level of regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low-grade ovarian serous carcinoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS and BRAF: Gain-of-function</td>
<td>Both ~30%</td>
<td>c-Myc and c-Jun transcription factors</td>
<td>Transcription</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Direct phosphorylation by ERK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased Nrf2 protein synthesis (via activation of elf4E, elf4B-BPI and eEF2 kinases)</td>
<td>Translation</td>
</tr>
<tr>
<td>ERBB2: Amplification, overexpression</td>
<td>20-30%</td>
<td>c-Myc and c-Jun transcription factors</td>
<td>Transcription</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Direct phosphorylation by Akt</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decrease nuclear export (via Src-A subfamily kinases)</td>
<td>Protein-protein interactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Nrf2 degradation via β-TrCP mediated ubiquitination</td>
<td>Protein-protein interactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased nuclear import (via complex formation Nrf2 and depolymerized actin)</td>
<td>Protein-protein interactions</td>
</tr>
<tr>
<td><strong>High-grade ovarian serous carcinoma</strong></td>
<td>26-47%</td>
<td>Estrogen-induced ROS production in combination with</td>
<td>Post-translational modification (Keap1)</td>
</tr>
<tr>
<td>BRCA1: Loss-of-function</td>
<td></td>
<td>Absence of ER-induced repression of Nrf2 and its downstream genes</td>
<td>Transcription and protein-protein interactions</td>
</tr>
<tr>
<td>TP53: Loss-of-function, 80% total TP53 mutations</td>
<td>&gt;80%</td>
<td>No evidence found</td>
<td>N/A</td>
</tr>
<tr>
<td>Oncomorphic transformation, 20% total TP53 mutations</td>
<td></td>
<td>c-Myc transcription factor</td>
<td>Transcription</td>
</tr>
</tbody>
</table>

β-TrCP, beta-transducin repeat-containing protein; ER, Estrogen receptor; ROS, Reactive oxygen species.
As there is a close regulation between BRCA1 and Nrf2, BRCA1 mutations are expected to result in changes in Nrf2 activity. BRCA1 has been shown to directly bind Nrf2, thereby interfering with Keap1 binding, and enhancing stability and activation of Nrf2 (116). As a consequence, loss-of-function mutations in BRCA1 are expected to result in decreased Nrf2 activity and thus, increased sensitivity towards ROS-induced damage. Gorrini et al. could confirm this in BRCA1-null premalignant mammary epithelial cells. These cells accumulated high levels of ROS and exhibited enhanced sensitivity toward oxidative stress, which resulted in the accumulation of DNA damage and senescence (116). This could be overcome by constitutive activation of Nrf2 via the use of KEAP1 shRNA. This shows that Nrf2 activation is essential for the survival of BRCA1 mutant cells during (prolonged) exposure to oxidative stress.

BRCA1-induced Nrf2 activation is thus abolished in BRCA1 mutant cells. Therefore, in order to survive prolonged exposure to oxidative stress, other factors should take over this function of BRCA1. Recent studies indicate that estradiol can activate Nrf2 in a wide range of cell types, including the prostate cancer cell line PC3, the breast cancer cell line MCF7, and wild-type mouse primary B cells (116, 117). Estradiol treatment increased only Nrf2 protein and not mRNA levels, suggesting that estradiol stabilizes the Nrf2 protein. Based on current literature, the most likely mechanism in which estradiol (or its metabolites) can stabilize the Nrf2 protein, is via the generation of oxidative stress. Two studies in ovarian cancer have indeed shown the link between estradiol treatment and increased ROS (61, 118). Both studies examined the effect of estradiol after a 24h treatment either on ROS production in the estrogen receptor negative (ER –) ovarian cancer cell line Hey or on oxidative DNA damage (8-OHdG) in mouse ovarian surface epithelial cells. Estradiol treatment indeed increased ROS production, oxidative DNA damage and Nrf2 protein levels. However, in several studies using breast cancer cell lines, this relation could not be reproduced (116, 119). In these studies, ROS production was either determined earlier after the addition of estradiol (4h), or they used cellular protein lysates treated with estradiol instead of intact cells. This apparent discrepancy might be explained by the fact that the breast cancer studies looked at the direct effect of estradiol itself, while the ovarian cancer studies likely observed an indirect effect of estradiol’s metabolites; indeed, in contrast to estradiol itself, these metabolites were shown to generate ROS production in breast cancer cells (119). Estrogen is known to be metabolized to catechols, and upon further oxidation, to DNA-reactive quinones and semiquiones, which have
been shown to be highly mutagenic (120). The catechol metabolites, but not their parent estrogens (estrone, estradiol and estriol), were shown to be highly redox active and to generate ROS. Strikingly, this ROS-production was independent of ERα expression and was both seen in normal and tumorigenic breast cell lines (119). Very recently, by using a PI3K inhibitor, it was shown for mouse mammary epithelial cells that the estradiol-induced oxidative stress and Nrf2 accumulation were dependent on activation of the PI3K/Akt pathway (117). Interestingly, further experiments elucidated that mammalian target of rapamycin (mTOR), one of the downstream targets of the PI3K/Akt pathway, turned out to be involved in the estradiol-induced Nrf2 accumulation (117). Further studies should determine whether this finding can be translated to ovarian cells.

Interestingly, estradiol seems to play a dual role in modulating Nrf2 activity. On the one hand its metabolites activate Nrf2 via the generation of ROS (independent of ER), but on the other hand estradiol itself can inhibit the Nrf2 downstream genes via binding to ERα (dependent of ER). Ansell et al. found that ERα, but not ERβ can undergo an estradiol-dependent interaction with Nrf2, and thereby represses Nrf2-mediated transcription (121). Others revealed by chromatin immunoprecipitation in MCF-7 breast cancer cells that, in the presence of estradiol, both ERα and the NAD+ dependent deacetylase Sirt1, directly bind to the ARE within the promoter region of NQO1 (downstream gene of Nrf2). As a result, Nrf2 binding to the ARE of NQO1 was reduced and this resulted in reduced expression of NQO1 (122). So for estradiol to result in activation of Nrf2, either the repression of Nrf2 activity by ERα needs to be minimized or ERα should be absent (ERα−). In breast tumors, the last option seems to occur most often, as breast tumors with a BRCA1 mutation are so-called triple negative (ER−/PR−/HER2−) (negative for estrogen receptor progesterone receptor /human epidermal growth factor receptor 2) in 80-90% of the cases (123-125). The minority of BRCA1 mutant breast tumors that are ERα+ have a clear distinct etiology (126). In contrast to breast cancer, in a small ovarian cancer patient study (N = 22), only 23% of the BRCA1 mutant tumors was ER− and this frequency was similar in the non-BRCA1 mutant tumors. Interestingly, in BRCA1 mutant ovarian tumors ERα expression was inversely correlated with ERβ expression compared to a positive correlation in non-BRCA1 mutant tumors (127). This study indicates that, in contrast to most breast tumors, absence of ERα does not occur in most ovarian tumors, and therefore other unknown mechanisms might be responsible for minimizing ERα-mediated repression of Nrf2 activity (128, 129). In addition to ERα-mediated Nrf2 repression, a recent study by Singh et al. discovered that in
primary breast epithelial cells and in an estrogen-induced breast carcinogenesis rat model, E2 can activate miR-93, resulting in hypermethylation of Nrf2 (130). By treating these rats with resveratrol, E2-mediated methylation changes of Nrf2 could be reversed. This resulted in decreased tumor incidence and proliferation, and increased latency of E2-induced breast tumors. So, in contrast to previous studies (116, 117), this model describes a negative effect of E2 on Nrf2 expression. However, even if hypermethylation of Nrf2 would be induced by E2, it is unlikely that BRCA1 mutant cells would survive, as they already possess higher levels of ROS and would go into senescence or apoptosis before E2-induced carcinogenesis would occur (116). Furthermore, these interesting results could not be confirmed with data of the TCGA, as Nrf2 hypermethylation does virtually not occur in either breast or ovarian cancer (http://cancergenome.nih.gov/). To determine whether during BRCA1 mutant ovarian compared to breast carcinogenesis similar mechanisms regulate Nrf2 activity, more research is required.

In conclusion, recent studies suggest that estradiol and/or its metabolites, via the generation of oxidative stress and the activation of the PI3K/Akt pathway, stimulate the accumulation of Nrf2. This might be contributing to the tissue-specificity of BRCA1 mutant tumors. However, to fully activate Nrf2, the ERα-mediated repression of Nrf2 activity should be minimized in these cells as well. When both requirements are fulfilled, the increased Nrf2 activity can alleviate the higher levels of oxidative stress in BRCA1 mutant cells just enough to keep the cells below the threshold for inducing cell death while still accumulating DNA damage over time, as their ability to repair damaged DNA is partly affected. In this manner, the BRCA1 mutant cells can acquire additional mutations, which eventually could result in the full transformation into a cancer cell (Fig. 4, Table 2).

2.3.2. TP53 mutations and aberrant Nrf2 activation

The vast majority of high-grade serous ovarian tumors contain a mutation in TP53 (96%) (71, 131), but it is very uncommon in low-grade serous ovarian tumors (<8%) (69). Mutations in TP53 result either in a complete or partial loss of wt function (occurring in 80% of all TP53 mutated ovarian tumors) or a gain-of-function/oncomorphic transformation of p53 (132). Oncomorphic mutations require the loss of the second WT allele and result in the production of p53 proteins with prolonged half-life and oncogenic functions, without remaining wt p53 function. What are the indications that alterations in the function of p53 also
Figure 4. BRCA1 mutant cells are more sensitive toward (estradiol-induced) oxidative stress. BRCA1 is a dsDNA repair enzyme. BRCA1 binds Nrf2, thereby preventing Keap1 binding and degradation of Nrf2. Both functions of BRCA1 are important for protection against oxidative stress. When cells are exposed to estradiol, its metabolites can cause DNA damage either directly or indirectly via ROS. Upon exposure to ROS, Nrf2 translocates to the nucleus and can bind AREs to activate antioxidants and detoxifying enzymes like NQO1, which converts DNA-reactive quinones back to catechols. However, estradiol-bound ER\(\alpha\) can inhibit transcription of Nrf2 downstream genes either via direct inhibition of Nrf2 activity or via attraction of the histone deacetylase SIRT1 to the promoter region of NQO1. Therefore, in BRCA1 mutant, ER\(\alpha^+\) cells, a high, continuous exposure to estradiol results in lethal amounts of ROS and DNA damage. To prevent this lethal damage, BRCA1 mutant cells should overcome or minimize the ER\(\alpha\)-mediated repression of Nrf2 activity, resulting in sufficient activation of Nrf2 to overcome lethal DNA damage. Due to impaired dsDNA repair, some DNA mutations will accumulate in these BRCA1 mutant cells and this could result in carcinogenesis.
modulate Nrf2 activity? As each oncomorphic p53 mutation can have other transcriptional targets and can undergo interactions with different proteins (132), it is difficult to predict their exact role in the modulation of Nrf2 activity. Nevertheless, it is known that several of these p53 oncomorphs can activate c-myc (132), which has been shown to directly bind to the NRF2 promoter to activate transcription (90). In the next paragraph, we will shortly discuss the normal function of p53 in relation to oxidative stress and then, we will focus on the effects of loss-of-function TP53 mutations on Nrf2 activity, as this type of mutations are more frequent compared to the oncomorphic p53 mutations.

As a transcription factor, p53 plays a role in many different processes, from apoptosis and autophagy to cell cycle arrest and senescence (133). Under unstressed/low stress conditions, p53 protein levels are kept low, as p53 is continuously being degraded via mdm2-mediated proteasomal degradation. On the other hand, when cells are under severe stress, including oxidative stress (induced damage), phosphorylation of both mdm2 and p53 prevents their interaction, and thus prevents the degradation of p53 (134).

There is a close interaction between the redox-active transcription factors p53 and Nrf2 in the regulation of oxidative stress and the cellular response toward it (Fig. 5). In a low oxidative stress environment, p53 activates several target genes involved in the protection against ROS that partly overlap with downstream genes of Nrf2, including SESN1, SESN2, HMOX1 and GPX1 (135-137). This maintains the reduced environment, in which Nrf2 remains inactive. In contrast, during oxidative stress, Nrf2 will be activated and induce an antioxidant response. When ROS-induced DNA damage accumulates within the cell, p53 becomes active and either induces cell cycle arrest which provides extra time to repair the damaged DNA, or induces apoptosis. In the case cell cycle arrest is induced by p53, p53 activates its downstream target p21, a cell cycle inhibitor. At the same time, p21 also inhibits apoptosis (138). In order to prevent apoptosis, p21 has to prevent an apoptosis-inducing pro-oxidative environment. Toward this end, p21 binds Nrf2 and thereby prevents Keap1 binding, Keap1-mediated ubiquitination and thus proteasomal degradation of Nrf2 (139). So by activating Nrf2, p21 can indirectly prevent a pro-oxidative environment, and as such inhibits apoptosis. In the case where p53 induces apoptosis, the cell requires a pro-oxidative environment. In this case, p21 will not become active as now the Nrf2-induced anti-oxidative response needs to be suppressed and oxidative stress needs to be generated (140). Under these circumstances, the Nrf2-induced anti-oxidative response can be suppressed via direct binding of p53 to AREs in several of Nrf2
downstream genes, including x-CT, MnSOD, NQO1 and GST-α1, thereby preventing their activation (141). Polyack et al. discovered by using serial analysis of gene expression (SAGE), a method that measures absolute levels of RNA abundance, that only 0.19% (14 out of 7,202 identified transcripts) of all p53 induced transcripts are more than 10 fold increased versus control cells before apoptosis commences (142). Remarkably, most of these genes were predicted to play a role in the generation of, or the response to, oxidative stress. These studies suggest that p53 induces apoptosis via the transcriptional induction of pro-oxidative genes and the prevention of activation of anti-oxidative genes, resulting in accumulation of ROS which damage the mitochondria. Leakage of calcium from these damaged mitochondria results in activation of caspases, and thereby the induction of apoptosis.

In conclusion, in a physiological situation, p53 plays a dual role in the regulation of oxidative stress and the Nrf2 response: at low stress levels, p53 seems to have anti-oxidative properties, while at high stress levels, p53 seems to have pro-oxidative properties (Fig. 5). Whether it is one or the other response is probably dependent on several factors, including qualitative and/or quantitative changes to p53, and the co-expression of other genes (133, 143). Intriguingly, the other way around, Nrf2 also seems to play a dual role in the regulation of p53. Several downstream genes of Nrf2 can modulate p53 activity, including but not limited to NQO1 and MDM2. NQO1 has been shown to activate p53, as its interaction with p53 can prevent the mdm2-mediated p53 degradation (144). Mdm2 on the other hand, inhibits p53 activity directly as it ubiquitinates p53 and thereby targets p53 to the proteasomes for degradation (145). Whether Nrf2 inhibits or activates p53 activity is probably cell- and context-dependent. Several consequences of p53 loss-of-function/null mutations on Nrf2 activity can be deduced from the literature as describe above. To start with, the tightly controlled regulation between both p53 and Nrf2 is lost without functional p53. This will affect the normal cytoprotective responses toward oxidative stress. In the case of ovarian cancer, for example increased metabolic activity or estradiol metabolites could result in accumulation of ROS, after which Nrf2 becomes active. However, without functional p53, neither cell cycle inhibition via induction of p21, nor suppression of the anti-oxidative response via p53 binding toward AREs can take place anymore. This might result in the survival of cells that under normal physiological conditions would go into senescence or apoptosis. So, in contrast to certain p53 oncomorphs which can directly activate Nrf2 via active c-myc, loss-of-function p53 mutations do not directly activate Nrf2, but rather contribute to the
survival of cells that are exposed to prolonged oxidative stress (Table 2). This continuous exposure to oxidative stress could then permanently activate Nrf2.

Figure 5. Crosstalk between p53 and Nrf2 during (oxidative stress-induced) DNA damage accumulation. Under conditions with low DNA damage, p53 levels are kept low by mdm2-mediated ubiquitination and degradation of p53. At these low levels, p53 can activate several antioxidants (partly overlapping with downstream genes of Nrf2). When cells are exposed to increasing levels of DNA damage, p53 levels increase, as the phosphorylation of both mdm2 and p53 prevents their interaction. Depending on cell type and biological context, p53 can either decide to (1) induce cell cycle arrest and thereby increase the time necessary to repair damaged DNA or to (2) induce apoptosis. In order to induce apoptosis, a pro-oxidative environment is necessary. As such, p53 blocks the Nrf2-mediated antioxidant response by direct binding to antioxidant response elements (AREs) of downstream Nrf2 genes. The other way around, when cell cycle arrest is induced via p21, an apoptosis response should be prevented. P21 can do so by direct binding to Nrf2, thereby preventing keap1-mediated degradation of Nrf2.

2.4. **Potential translation of cell line and animal studies into patients**
As described in section 2.2., several potential mechanisms link KRAS, BRAF and ERBB2 mutations to persistent Nrf2 activity in low-grade ovarian serous
carcinomas. However, as mentioned in section 2.3., in high-grade ovarian serous carcinomas no direct involvement of *BRCA1* and *TP53* (loss-of-function) mutations by themselves was found that could explain aberrant activation of Nrf2 (summarized in Table 2). Interestingly, *BRCA1* mutations by themselves can even reduce Nrf2 activity, because, as a consequence of a loss-of-function mutation, *BRCA1* cannot stabilize Nrf2 anymore (116, 146). As discussed in section 2.3.1., there are indications, although not completely proven yet, that in the context of *BRCA1* mutant tumors, a combination of high estradiol levels and minimal ERα-mediated repression of Nrf2 activity are important factors that result in continuous Nrf2 activity in *BRCA1* mutant tumors.

The potential mechanisms by which common oncogene and tumor suppressor gene mutations in ovarian serous carcinomas can constitutively activate Nrf2 are mainly based on studies using cancer cell lines. To our knowledge, so far, no studies have been performed in ovarian cancer patient-derived material to determine whether mutations in any of these genes are linked to Nrf2 expression. Nevertheless, in various other tumors the mutational status of *KRAS*, *BRAF* and *ERBB2* has been indirectly related to Nrf2 expression (via *KEAP1* mutations/activity). Gallbladder tumors (n=13) showed *KEAP1* alterations exclusively co-occurring with p53 overexpression and not with *KRAS* mutations (34). In another study, it was found that in lung tumors (n=76) *KEAP1* mutations do not co-occur with other (common) mutations such as *EGFR*, *KRAS*, *ERBB2* and *NRF2*. These studies give us an indication that either *KEAP1* mutated tumors give rise to a completely different subset of cancers, or that both *KEAP1* mutations and *KRAS*, *BRAF* or *ERBB2* mutations have in common that Nrf2 becomes aberrantly active, and therefore, there will not be a positive selection for additional mutations that result in aberrant activity of Nrf2. Either way, both explanations do not exclude a role in vivo for *KRAS*, *BRAF* or *ERBB2* mutations in the continuous activation of Nrf2. In addition, no relationship was found between expression of cytoplasmic Keap1 and the *KRAS* mutation status (147). This is in line, with the literature described in section 2.2.1., stating that *KRAS* mutations can induce *NRF2* on the transcriptional level rather than the protein level/via modulation of Nrf2 stability regulated by Keap1. As the link between estradiol, the PI3K/Akt pathway and Nrf2 activity has been discovered only very recently (117), no patient studies have been performed yet to confirm this. Future research should confirm that this link also exists in patients. In addition, no patient studies have been done to confirm the mechanisms, found in cell and animal studies, linking *KRAS*, *BRAF* and *ERBB2* mutations to constitutive Nrf2 activation. However, these mechanisms
have been studied in detail in both cell culture and animal models, and therefore they are likely to occur as well in ovarian cancer patients. These studies provide promising indications to pursue further studies in ovarian cancer patients that investigate Nrf2 inhibition as potential target in ovarian cancer treatment.

3. **Nrf2 inhibition as promising target in ovarian cancer treatment**

Our poor understanding of the underlying biology, together with the late stage detection and the commonly acquired chemotherapy resistance, make ovarian cancer a difficult disease to treat. Currently, chemotherapeutics and surgery are still the most common first-line treatment option for ovarian cancer, but especially in advanced stage patients, these treatments are often inadequate (148). Therefore, there is a pressing need for better therapies against ovarian cancer. Currently, the development of new anti-cancer drugs has put a major focus on neutralizing the direct effects of mutations in oncogenes or tumor suppressor genes, for example by modulating the activity of the oncogene or tumor suppressor gene directly or by modulating downstream pathways (149). Instead of focusing on new drugs that modulate activity of oncogenes or tumor suppressor genes, we propose that targeting the underlying stress adaptations can be a potent direction in the development of new anti-cancer drugs. As discussed in section 1.2., one of these underlying stress adaptations ovarian cancer cells can acquire is the protection against high levels of ROS via constitutive activation of Nrf2. Therefore, as Nrf2 determines whether ovarian cancer cells will survive or not, this might be the ignored Achilles’ heel of ovarian cancer. An important advantage of Nrf2 inhibition, is the expected therapeutic window. Both normal and cancer cells require Nrf2 activity to maintain redox balance. However, as baseline ROS levels are much higher in cancer cells, they require higher Nrf2 activity and are more dependent on this activity for their survival. As such, Nrf2 inhibition is expected to increase ROS levels both in cancer and normal cells, but because baseline ROS levels in cancer cells are much higher, the ROS levels will only in cancer cells increase beyond a threshold that triggers cell death. This might create a therapeutic window in which cancer cells will die while normal cells stay largely unaffected. Despite this, normal tissues that are commonly exposed to oxidative stress or xenobiotics, such as the liver and the kidney, require a relatively high Nrf2 activity in order to protect them from a wide variety of toxic insults (150). In addition, Nrf2 is also important in regulating processes that are independent of its cytoprotective functions; Nrf2 can stimulate angiogenesis (151), limit inflammation responses (152, 153) and modulate
metabolism (e.g. inhibition lipid synthesis, support β-oxidation of fatty-acids, stimulate carbohydrate metabolism and NADPH generation) (154, 155). Furthermore, Nrf2 is important in hematopoietic stem cells for balancing between quiescence and proliferation, self-renewal and differentiation, and homing and retention of cells in the bone marrow niche (156). Therefore, any toxicity in normal tissues against inhibition of Nrf2 is expected to arise first in tissues requiring high Nrf2 activity. By combining direct Nrf2 inhibitors with drugs that inhibit the tumor-specific cause of aberrant Nrf2 activation, dosage of each drug can be reduced without compromising treatment efficacy. Furthermore, the potential toxicity in normal cells is reduced, because normal cells are less dependent on both pathways for their survival. Taken all this together, inhibition of Nrf2 seems an interesting therapeutic target that has the potential to bring us one step closer toward making ovarian cancer a curable disease.

3.1. Effects of Nrf2 inhibition in anti-cancer treatment
So far, the potential of (specific) Nrf2 inhibition in anti-cancer treatment has been explored using siRNAs/shRNAs targeting Nrf2 or by overexpressing KEAP1. Knockdown of Nrf2 decreased cell proliferation in several cancer cell lines, including those of the lung(157), pancreas(158) and cervix(159). In addition, Nrf2 inhibition resulted in reduced anchorage-independent growth in several lung cancer cell lines (160). Translated to the in vivo situation, intratumoral injection of Nrf2 siRNA/shRNA has shown potency in reducing tumor growth in mouse xenograft models (159, 160) and reduced tumor angiogenesis in the chicken embryo chorioallantoic membrane (161). Interestingly, in combination with other anti-cancer treatments, NRF2 knockdown turns out to be an even more effective anti-cancer treatment: resistance to a wide range of chemotherapeutic drugs, including doxorubicin, etoposide, gemcitabine, 5-FU, bleomycin and cisplatin, can be reduced by inhibition of Nrf2 activity (157, 158, 160, 162, 163). Recently, it was discovered that ROS production was increased in 5-FU resistant colon cancer cells, and this resulted in increased TET1 expression, associated with hypomethylation of NRF2. When these cells were transfected with NRF2 or HO1 shRNA and subcutaneously implanted in the back of nude mice, tumor volume, size and weight were significantly reduced compared to control shRNA transfected cells (164). Furthermore, in pancreatic cancer cell lines, sensitivity to gamma-radiation was further increased upon inhibition of Nrf2 (158). In summary, these studies clearly show the potential of Nrf2 as therapeutic target, as its inhibition results in decreased tumor growth and enhanced sensitivity to many commonly used
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chemotherapeutics, including cisplatin which is often used in the treatment of EOCs, and gamma-radiation. However, as the level of Nrf2 expression is negatively correlated with chemotherapy-induced myelosuppression, it is expected that inhibition of Nrf2 further exarcabates this serious side-effect (165). This severe limitation again underlines the need for a combination strategy in which ovarian cancer specific factors with links to aberrant Nrf2 activity are combined with direct Nrf2 inhibitory drugs.

3.2. Therapeutic options to inhibit Nrf2 activity in patients

To achieve inhibition of Nrf2 as promising therapeutic option in ovarian cancer treatment, selective Nrf2 inhibitors are required. Currently only two (non-specific) Nrf2 inhibitors have been described: brusatol from Brucea javanica and procyanidins from Cinnamomi Cortex extract (166, 167). Both natural compounds have been shown in cancer cells to inhibit Nrf2 activity and increase sensitivity to commonly used anti-cancer drugs, such as cisplatin and doxorubicin. However, both substances have broader (unknown) activities (168), brusatol for example can also inhibit DNA, RNA and protein synthesis (169) and activates NF-κB (170).

In our lab, we are exploiting the artificial transcription factor (ATF)-technology in order to realize the “druggable genome” concept by specifically modulating the expression of any given gene (171). In order to permanently repress Nrf2 expression, we propose a different therapeutic intervention, called “Epigenetic Editing” (172). “Epigenetic Editing” is the removal or addition of epigenetic marks in a gene-specific manner. Epigenetic marks are heritable, yet reversible modifications of the DNA or histones that affect gene expression without changing the DNA-sequence (173). We and others used engineered epigenetic editors (EEEs), consisting of engineered DNA binding domains fused to the catalytic domain of epigenetic enzymes, to silence (174-178) or activate (179-181) (depending on the epigenetic enzyme) any gene of interest (182). In the case of serous EOCs, permanent silencing of NRF2 is an interesting therapeutic option. Opposed to currently known (non-specific) Nrf2 inhibitors and siRNA, tumor-targeted delivery (183-185) of EEEs is expected to give less off-target effects in the cancer cell and repeated treatments will be additive to result in permanent changes in the epigenetic regulation of Nrf2, and thereby silence its expression. By treating the cancer cells with an EEE that inhibits Nrf2, not only the cancer cells that are directly hit by the treatment are affected, but also their daughter cells. Therefore, EEEs that inhibit Nrf2 provide a revolutionizing, new therapeutic approach that has great potential in the treatment of serous EOCs.
3.3. Possible opportunities for the implementation of Nrf2 inhibitors in the treatment of ovarian serous carcinoma

As discussed in this review, mutations in *BRAF*, *KRAS* and *ERBB2*, and high levels of estradiol in combination with mutations in *BRCA1*, are directly linked to the aberrant activation of Nrf2 in serous EOCs. Therefore, patients harboring any of these mutations might specifically benefit from a strategy that combines the inhibition of Nrf2 by itself with inhibiting the tumor-specific cause of the aberrant Nrf2 activity in these patients.

For example, in low-grade serous EOCs harboring a mutation in *BRAF* or *KRAS*, we could think of combining a MEK inhibitor, a downstream kinase in the MAPK/ERK pathway, with a Nrf2 inhibitors. Low-grade serous EOCs that have a mutation in *ERBB2* can use both the PI3K/Akt and MAPK/ERK pathway to aberrantly active Nrf2. In these cancers, the relative importance of both pathways in the aberrant activation of Nrf2 should be determined in order to design a rational therapeutic strategy containing inhibitors of the PI3K/Akt or MAPK/ERK pathway alone or together in combination with Nrf2 inhibitors.

In high-grade serous EOCs harboring a *BRCA1* mutation a completely different drug combination might be required. In this group of cancers, not the *BRCA1* mutation by itself, but the combination of a *BRCA1* mutation, high estradiol levels and minimal ERα-mediated Nrf2 repression are important in the aberrant activation of Nrf2. Therefore, a new therapeutic strategy should take into account that Nrf2 activity is regulated at many different levels, and that changing one level of regulation might affect the others. As the estradiol-induced Nrf2 accumulation is dependent on PI3K/Akt signaling, a straight-forward approach would be to combine Nrf2 and PI3K inhibitors. Another option in *BRCA1* mutant EOCs would be to increase the repressive capacity of ERα on Nrf2 activity. By combining drugs that increase ERα expression and inhibit Nrf2 expression at the same time, double effectiveness is expected. As mentioned in section 3.2., the ATF-technology could be very suitable for specifically upregulating ERα and downregulating Nrf2 expression in BRCA1 mutant ER- ovarian cancer cells. A completely different approach is to hit the BRCA1 mutant cells at their weakest spot by combining Nrf2 inhibitors with drugs that increase sensitivity for ROS-induced DNA damage specifically in the tumor. As an example, we could think of combining poly ADP ribose polymerase (PARP) and Nrf2 inhibitors in *BRCA1* mutant ovarian cancers (186). In response to single strand DNA (ssDNA) breaks, PARP enzymes are essential in base (BER) and nucleotide excision repair (NER). Upon PARP inhibition, ssDNA breaks accumulate and, if left unrepaired until DNA
is replicated, the replication itself can convert these ssDNA into double strand DNA (dsDNA) breaks (187). In order to repair these dsDNA breaks, a functional homologous recombination (HR) pathway, of which BRCA1 is an essential component, is required. Upon PARP inhibition, healthy cells can exploit HR as a back-up DNA repair systems, but in contrast to healthy cells, BRCA1 mutant cells do not have functional HR and therefore become extra sensitive to DNA damage (188, 189). Interestingly, ROS mainly generate ssDNA breaks that are repaired by BER and NER (190). Therefore, inhibition of Nrf2, resulting in the accumulation of ROS, has the potential to synergize with PARP inhibitors. As a result, this combination is able to specifically kill the cancer cell and act synergistically, which enables the use of a lower dosage and thereby reduce potential side-effects, while remaining similar efficacy as monotherapy. In ER+ BRCA1 mutant cells, it is unknown whether constitutive Nrf2 activation is present and if so, which mechanisms are involved. Therefore, more research is required to determine whether there is a potential for Nrf2 inhibitors as well in this small subcategory of BRCA1 mutant ovarian cancers.

As described above, many different therapeutic strategies can be thought of that combine the inhibition of Nrf2 by itself with inhibiting the tumor-specific cause of the aberrant Nrf2 activity. Also, in cancers which harbor mutations that are not linked to the aberrant activation of Nrf2, such as the TP53 mutant serous EOCs, Nrf2 inhibition can be effective as well. For example, in ovarian cancer, TP53 mutations are often linked to platinum-based chemotherapy resistance (191, 192). In this context, inhibition of Nrf2 might sensitize platinum-resistant ovarian cancer cells, as Nrf2 inhibition is expected to both increase the amount of DNA damage and create a more pro-oxidative environment, important for the induction of apoptosis (193).

4. Concluding remarks
Cancer cells are exposed to high levels of ROS and therefore protection against oxidative stress is essential for their survival. Constitutive activation of the transcription factor Nrf2 is an important mechanism for many different tumors, among which ovarian tumors, to adapt to this pro-oxidative environment. Furthermore, besides regulating the oxidative stress levels, Nrf2 can regulate many more essential processes within the tumor, including DNA repair (194, 195), anabolism (196), angiogenesis (197), and acquiring chemoresistance (62, 163, 198). In this review, we have discussed the role of dysregulated tumor suppressor genes (BRCA1, TP53), oncogenes (KRAS, BRAF, ERBB2) and estrogen levels as
underlying causes of the continuous activation of Nrf2 in serous EOC. Continuous Nrf2 activity is important during carcinogenesis of many serous EOCs. It turns out that in serous EOC, dysregulation of estrogen levels and the oncogenes KRAS, BRAF and ERBB2 can contribute to the continuous activation of Nrf2, but no evidence for a direct link between BRCA1 and TP53 mutations by themselves could be found. Therefore, inhibition of Nrf2 can be a potent therapeutic option in ovarian cancer treatment, but so far, only a few (non-specific) Nrf2 inhibitors, brusatol and procyanidins from Cinnamomi Cortex extract, have been described (166, 167). We propose that “Epigenetic Editing” (engineered DNA binding domains fused to the catalytic domain of epigenetic enzymes) (172) could be interesting as a therapeutic modality in order to gain specific and permanent Nrf2 inhibition. Obviously, to move toward implementation of Nrf2 inhibition in current ovarian cancer treatment, more insights into the beneficial effects of Nrf2 inhibition in combination with other therapies are required. With the acquired knowledge, we are one step further toward a fully patient-tailored combination therapy including Nrf2 inhibition, which has the potential to become a successful therapeutic option for ovarian cancer patients, both in first-line treatment and after development of resistance. This would help us to make big steps forwards in ovarian cancer treatment, thereby improving survival rates tremendously and making ovarian cancer a more curable disease.

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