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Oxidative stress and chronic inflammation in osteoarthritis

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**Oxidative stress and chronic inflammation in osteoarthritis:
can Nrf2 counteract these partners in crime?**

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3 **Oxidative stress and chronic inflammation in osteoarthritis: can Nrf2 counteract these**
4 **partners in crime?**
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Abstract

Osteoarthritis (OA) is an age-related joint degenerative disease associated with pain, joint deformity and disability. The disease starts with cartilage damage but then progressively involves subchondral bone causing an imbalance between osteoclast-driven bone resorption and osteoblasts-driven remodeling. Herein we summarize the data for the role of oxidative stress and inflammation in OA pathology and discuss how these two processes are integrated during OA progression, as well as, their contribution to abnormalities in cartilage/bone metabolism and integrity. At the cellular level, oxidative stress and inflammation are counteracted by transcription factor nuclear factor-erythroid p45-related factor 2 (Nrf2), and we describe the regulation of Nrf2, highlighting its role in OA pathology. The beneficial effect of some phytonutrients, including the therapeutic potential of Nrf2 activation, in OA is also discussed.

Introduction

Osteoarthritis (OA) is one of the most common degenerative arthropathy in the elderly population. OA is a time- or age-dependent progressive destruction of joints related to articular cartilage damage, synovial lining thickening, subchondral bone alterations, osteophytes formation at the joint margin, and is supported by elevated inflammatory and catabolic responses, which finally result in a loss of joint architecture and deformity.¹⁻⁴ The disease affects mostly individuals over the age of 50 (primary disease), but it can also develop after joint insult and trauma in younger individuals (post-traumatic OA). Several lines of evidences suggest that aging has an impact on the pathogenesis of primary disease. Some OA chondrocytes acquire aging phenotype with increased genomic instability, telomere shortening, epigenetic alterations including decreased genomic global methylation and histone modifications, dysregulated metabolism, mitochondrial dysfunction and altered intercellular networks and cascades.⁵⁻⁷ The disease progresses and the age-dependent alterations in cell signaling and metabolism occur in chondrocytes but also in bone cells (osteoclasts and osteoblasts), causing an imbalance between bone resorption and remodeling and deeper abnormalities in subchondral bone and joint structure. Aging causes OA development, however it is not a sole risk factor. The disease is multi-factorial with both physiological and mechanical processes involved, such as obesity, genetic predisposition, hereditary, metabolic or endocrine alternations, trauma, joint overload and mechanical stress, as well as, long-time exposure to a low-grade chronic inflammation concomitant with a failure in oxidant-antioxidant balance.⁵⁻⁸ Therefore, some aspects of OA pathology are still a subject of an extensive research.^{4,9}

Inflammation and oxidative stress have been increasingly recognized as being closely integrated in OA pathology. Various inflammatory mediators such as cytokines [tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , 8, 6, 15, 17, 18, 21, 33],¹⁰ chemokines, prostaglandins (PGEs)¹¹ and growth factors [transforming growth factor- α (TGF- α)], fibroblast growth factor (FGF) are increased in the joint tissue of OA patients in comparison to healthy individuals.⁹ Local inflammatory response along with aging and/or mechanical load can contribute to increased oxidative stress with accumulation of reactive oxygen species¹² (ROS), superoxide anion, hydrogen peroxide (H₂O₂), nitric oxide (NO) and peroxynitrite and concomitant failure in the expression of antioxidant enzymes and ROS scavenging systems.¹³ At cellular level, oxidative stress causes mitochondrial DNA (mtDNA) and nuclear DNA damage, lipid peroxidation, alterations in cell signaling, transcription and epigenetic changes in gene expression. At the level of the joint as a whole organ, oxidative stress causes

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3 abnormalities in the cartilage and bone metabolism, exacerbating not only degradation but
4 also overall reparative potential of chondrocytes, osteoblasts and their precursors.^{13,14} Thus
5 the regulation of redox homeostasis at cellular level can decrease the severity of OA and limit
6 the disease progression.
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10 The Cap'n'Collar basic leucine zipper transcription factor nuclear factor-erythroid 2
11 p45-related factor 2 (Nrf2, also called Nfe2l2) is the master regulator of the cellular redox
12 homeostasis. Nrf2 regulates the expression of a large network of cytoprotective genes,
13 including enzymes involved in the biosynthesis, utilization and regeneration of reduced
14 glutathione (GSH), thioredoxin and nicotinamide adenine dinucleotide phosphate (NADPH),
15 detoxification enzymes such as glutathione S-transferases (GST), aldo-keto reductases
16 (AKR), and NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), as well
17 as proteins involved in the recognition and clearance of damaged proteins and organelles,
18 such as proteasomal subunits and autophagy-related proteins.¹⁵⁻¹⁶ Collectively, the products of
19 the Nrf2-target genes function to lower the intracellular levels of ROS, reactive nitrogen
20 species (RNS) and electrophiles, thus protecting the cell from the harmful effects these agents.
21 In addition, Nrf2 is involved in the regulation of expression of a number of metabolic genes,
22 most notably those involved in the pentose phosphate pathway, the biosynthesis of purine
23 nucleotides and serine, and enhance fatty acid metabolism and mitochondrial function.¹⁷⁻²¹
24 Overall, the Nrf2 regulatory network provides an interface between redox and intermediary
25 metabolism.
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36 Recently, several studies have revealed the essential role of Nrf2 in preventing the
37 patho-physiological events in joint diseases.²² It has been shown that the appropriate
38 expression and fine balance in Nrf2 activity is absolutely necessary for normal
39 chondrogenesis and proper regulation of cartilage metabolism.^{12,23} There is growing evidence
40 for the involvement of Nrf2 in bone resorption and remodeling indicating a multi-faceted
41 effect in sustaining overall bone integrity. Herein, we attempt to summarize the recent
42 findings for the essential role of Nrf2 in OA. We also discuss the underlying mechanisms of
43 regulation and biochemical crosstalk between Nrf2, ROS signaling and inflammation during
44 OA pathogenesis. The beneficial role of phytonutrients to attenuate cartilage/bone
45 degradation, inflammation and oxidative stress in OA is highlighted.
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55 **Nrf2 function and regulation**

56 Nrf2 is expressed in most cell/tissue types and comprises seven functional domains, arranged
57 as follows from its N- to C- terminus: Neh2, Neh4, Neh5, Neh7, Neh6, Neh1, Neh3 (**Figure**
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3 1). The Neh4 and Neh5 domains form the transactivation domain of the transcription factor
4 that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXR α) represses Nrf2
5 by binding to its Neh7 domain. Neh1 mediates binding to DNA and to the heterodimeric
6 partner of Nrf2, transcription factor of the family of small musculoaponeurotic fibrosarcoma
7 (Maf) proteins. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6).
8 The Neh2 and the Neh6 domains contain specific sequences (degrons) that mediate the
9 degradation of Nrf2.²⁴ Nrf2 is regulated primarily at the level of protein stability. At
10 homeostatic conditions, the levels of Nrf2 are low due to its continuous ubiquitination and
11 proteasomal degradation. In response to stress signals, Nrf2 accumulates, translocates to the
12 nucleus, and as a heterodimer with a small Maf, initiates transcription of its target genes.²⁴

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20 There are three known ubiquitin ligase systems, which have been implicated in the
21 degradation of Nrf2 (**Figure 2**). The major negative regulator of Nrf2 activity is Kelch-like
22 ECH-associated protein 1 (Keap1). Keap1 was identified as a result of studies which followed
23 on from the finding that deletion of the Neh2 domain of Nrf2 (to which Keap1 binds) resulted
24 in its greatly increased activity.²⁵ Subsequent research has elucidated the process by which
25 Keap1 downregulates the Nrf2 activity. Keap1 forms a homodimer via its BTB domain.²⁶⁻²⁸
26 The regions of Keap1 important for interacting with Nrf2 are its Kelch (also known as double
27 glycine repeat, DGR) domains and C-terminal regions, and each Keap1 molecule interacts
28 with one of two proximal portions of Nrf2, the DLG and ETGE motifs (**Figure 1**).²⁹ Both of
29 these motifs lie within the Neh2 domain of Nrf2, separated by an alpha helix, and due to their
30 different electrostatic potentials, the ETGE motif has an approximately 100-fold higher
31 affinity for Keap1 than the DLG motif.³⁰ A “hinge and latch” hypothesis has been proposed
32 whereby the low affinity site (latch) serves to position Nrf2 such that its lysine residues are
33 optimally oriented for ubiquitination, while the high affinity site (hinge) is primarily
34 responsible for keeping Nrf2 bound to Keap1.³¹ Keap1 uses a cyclic mechanism to
35 sequentially bind to the ETGE and the DLG motifs of Nrf2.³² While bound to both of these
36 sites of Keap1, Nrf2 is in a prime position for ubiquitination by Cullin-3 (Cul3)-Rbx1/Roc1
37 ubiquitin ligase (*i.e.*, CRL^{Keap1}), which is recruited to the complex via interactions with the
38 BTB domains of the Keap1 dimer.²⁸ Ubiquitinated Nrf2 is extracted from the Keap1-Cul3 E3
39 complex by the ATP-dependent segregase p97,³³ and degraded by the 26S proteasome - this
40 regulation maintains the half-life of Nrf2 at 10-20 minutes, and ensures low basal Nrf2
41 activity.²⁹

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48 Besides acting as the primary inhibitor of Nrf2 activity, Keap1 serves as the
49 intracellular sensor for electrophiles and oxidants,²⁷ which bring about stabilisation and
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3 activation of Nrf2. This is mediated through specific reactive cysteine residues of Keap1,
4 some of which have lower *pKa* values than most cysteine residues due to their surrounding
5 basic amino acids, meaning they exist as thiolate anions (S⁻, as opposed to SH) making them
6 more reactive with ROS, RNS and electrophiles. Which cysteine residues are modified
7 depends on the type of activator,³⁴ with C151, C273 and C288 being most commonly
8 modified.³⁵⁻³⁷ Small molecule electrophiles and oxidants chemically modify Keap1 cysteine
9 residues, which is hypothesized to alter the structure of the complex, preventing
10 ubiquitination and subsequent degradation of the transcription factor. This results in Keap1
11 becoming saturated with Nrf2 and allowing newly synthesised Nrf2 to accumulate in the
12 cytoplasm, translocate to the nucleus and induce expression of its target genes.³²
13 Alternatively, some toxic metals (As³⁺, Cd²⁺, and Cr⁶⁺) have been found to stabilise Nrf2 by
14 dissociating it from Keap1.³⁸ Other small molecule Nrf2 activators directly disrupt the Keap1
15 : Nrf2 protein : protein interaction.^{39,40}

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25 Upon translocation to the nucleus, Nrf2 forms heterodimers with a small Maf protein,
26 and this dimerization is necessary for efficient DNA binding.⁴¹ Nrf2-Maf heterodimers bind
27 their target gene promoters at antioxidant response elements (ARE), which comprise the core
28 sequence 5'-TGACnnnGC-3', in which the GC dinucleotide is a critical part.⁴² Both of these
29 transcription factors contain a basic leucine zipper (bZIP) motif found within their DNA-
30 binding domains, with the basic region contributing to DNA binding and the leucine zipper
31 facilitating dimerization.³⁸ As mentioned above, the Neh4, Neh5 and Neh3 domains of Nrf2
32 (**Figure 1**) are responsible for mediating transactivation of target genes by recruiting a
33 number of coactivators, whereas the small Maf has no transactivation domain, and does not
34 contribute to this process.⁴³ Coactivators recruited include CREB-binding protein (CBP),
35 coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyl-
36 transferase 1 (PRMT1), which appear to synergise with another recruited coactivator, receptor
37 associated co-activator 3 (RAC3) to facilitate Nrf2-mediated gene expression.⁴⁴ Other studies
38 have unveiled more bZIP binding partners, with which Nrf2 dimerizes, including activating
39 transcription factor 4 (ATF4), Fos and Jun.⁴⁵ While these heterodimers have been shown to
40 bind to the ARE, it has not yet been made clear the extent to which they participate in
41 regulating gene expression. Additionally, Nrf2 transactivation is subjected to negative
42 regulation by retinoic X receptor α (RXR α), which binds Nrf2 and sits at the ARE to prevent
43 binding of Nrf2-Maf heterodimers.⁴⁶ A recent study has shown that Nrf2 binds to MED16,⁴⁷ a
44 tail subunit of Mediator, a highly conserved large conformationally flexible macromolecular
45 complex, which is required for RNA polymerase II-driven transcription of all protein-coding
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3 genes.^{48,49} Pull-down assays have indicated that the Neh4/Neh5 and the Neh1 domains of
4 Nrf2 are involved in this interaction.⁴⁷
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6 In addition to Keap1, Nrf2 is targeted for degradation following glycogen synthase
7 kinase 3 β (GSK3 β)-mediated phosphorylation (**Figure 2**).^{50,51} This kinase phosphorylates
8 serine residues in the Neh6 domain of Nrf2, leading to the recruitment, through DSGIS and
9 DSAPGS motifs (**Figure 1**), of the dimeric β -transducin repeat-containing protein (β -TrCP), a
10 substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (*i.e.*, SCF $^{\beta$ -TrCP}), hence
11 resulting in the degradation of the transcription factor. Activation of certain kinase cascades,
12 including phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase
13 (MAPK) pathways, leads to inhibition of GSK3 β , and certain Nrf2 activators such as the
14 lignan nordihydroguaiaretic acid (NDGA), have been shown to activate Nrf2 via induction of
15 these pathways and GSK3 β inhibition.⁵² A third ubiquitin ligase system, the endoplasmic
16 reticulum (ER)-bound E3 ligase Hrd1 (also known as synoviolin), has been implicated in the
17 degradation of Nrf2 during ER stress (**Figure 2**).⁵³ Acetylation also plays a role in Nrf2
18 regulation – the histone acetyl transferases (HAT) p300 and CBP modify lysine residues of
19 Nrf2 within its Neh1 domain to promote its DNA-binding capacity at certain target genes.⁵⁴
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30 The activity of Nrf2 can be affected by transcriptional and epigenetic mechanisms.
31 Thus, Nrf2 transcription is upregulated by the arylhydrocarbon receptor (AhR) and
32 BRCA1,^{55,56,57} whereas it is downregulated in the livers of peroxisome proliferator-activated
33 receptor α (PPAR α)-null mice following a 24-h period of fasting.⁵⁸ In addition, Nrf2 is
34 upregulated in tumor cells with mutant K-Ras^{G12D} or B-Raf^{V619E}, and it has been proposed that
35 this upregulation is mediated by transcription factors Jun and Myc.⁵⁹ Nrf2 can also activate its
36 own transcription due to the presence of an ARE-like sequence (5'-CTGACTCCGCC-3') in
37 its promoter.⁶⁰ The promoter of the murine *Nrf2* gene contains five CpG sequences, which
38 have been shown to be hypermethylated in a mouse model of prostate cancer, resulting in a
39 transcriptional silencing of Nrf2 and lower target gene expression.⁶¹
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46 Accumulating experimental evidences suggest that micro-RNAs (miRs) also play a
47 role in the regulation of Nrf2. In human MCF-7 breast cancer cells, ectopic expression of
48 miR-28 leads to a decrease in the mRNA and protein levels of Nrf2.⁶² Overexpression of
49 miR-93 has also been shown to reduce the levels of Nrf2 (mRNA and protein) as well as its
50 downstream target gene NQO1 in human MCF-10A and T47D breast cancer cells.⁶³
51 Conversely, the downregulation of miR-93 increased the expression of Nrf2 and its target
52 gene heme oxygenase 1, and had a protective effect in a mouse model of stroke.⁶⁴ In human
53 SH-SY5Y neuroblastoma cells, ectopic expression of miR-27a, miR-142-5p, miR-144 and
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3 miR-153 results in a decrease in the mRNA and protein levels of Nrf2 and the expression of
4 NQO1.⁶⁵ Downregulation of miR-27a increased Nrf2 expression and improved cardiac
5 function in a mouse model of LPS-mediated sepsis.⁶⁶ Interestingly, in homozygous sickle cell
6 disease (HbSS) reticulocytes, increased levels of miR-144 are inversely associated with the
7 levels of Nrf2, and correlate with decreased glutathione regeneration and attenuated
8 antioxidant capacity in HbSS erythrocytes.⁶⁷ A recent study has shown that miR-144 reverses
9 chemoresistance in hepatocellular carcinoma cell lines by targeting the 3'-untranslated region
10 (3'-UTR) of Nrf2 and promoting its mRNA degradation.⁶⁸ By a similar mechanism, miR-153
11 downregulates Nrf2, whereas tanshinone IIA, a diterpene quinone from *Salvia miltiorrhiza*,
12 restores this downregulation, leading to protection of SH-SY5Y cells against 6-
13 hydroxydopamine-induced toxicity.⁶⁹

23 **Pathogenesis of osteoarthritis**

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25 Osteoarthritis is a worldwide problem accounting for 1.1% of global disability-adjusted life
26 years¹⁰ and the fourth leading cause of disability,²² accompanied by pain, and depression, with
27 social impact on the patients' families.^{4,5,9} Currently, available therapies are limited in
28 efficacy because of the complex pathology and attended side effects.⁷⁰ Most therapies only
29 aim to reduce and control OA symptoms and pain, but they fail to provide a cure and thus
30 total joint replacement is often required in the most severe cases of OA.^{22,10,70}

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32 At the early stage of disease the changes in chondrocyte function and survival lead to
33 progressive cartilage disintegration and deterioration. In some patients this process can be
34 accompanied and perpetuated by secondary inflammation and synovitis.⁷¹ Cartilage
35 degeneration accelerates catabolic processes and bone removal by osteoclasts (bone
36 resorption) resulting in bone cysts and sclerotic bone formation. Cartilage and bone loss
37 trigger compensatory repair by osteoblasts with excessive anabolic processes of neocartilage
38 formation at the joint margins and joint edges (osteophytes), periarticular fibrosis and
39 calcified bone formation in advanced disease. The repair at specific site of the joints is a
40 consequence also of generalized alteration in the intrinsic capacity of subchondrial bone to
41 repair the cartilage and in the cross-talk between immune and bone cells.⁸

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43 Despite that OA can be diagnosed quickly by imaging techniques, the disease
44 progresses slowly within years before the first symptoms and a risk score for the individual's
45 susceptibility to OA can be predicted based on analysis of genetics, age and environmental
46 factors in combination with molecular and imaging biomarkers.⁸ Such prediction can be
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3 based on the assumption that the primary disease develops as a *continuum*. Mutations and
4 errors in gene expression of matrix molecules or factors regulating matrix component
5 synthesis can render chondrocytes hypertrophic and dysfunctional and can cause
6 chondrodysplasia at a relatively early age.⁸ Chondrocytes can acquire this altered phenotype
7 progressively especially when they are situated near weight-bearing regions. Over-time (at
8 middle-age; with aging) OA susceptibility can increase because of supplementary
9 mechanical and structural disorders in the joints associated with reduced physical activity
10 and muscle strength, altered bone metabolism, infections, joint injury, inflammation and
11 unhealthy diet.⁸ Finally, age-related changes in growth factor signaling required for
12 morphogenesis and repair contribute to OA onset in the elderly population.⁸ At middle- and
13 late-onset, different cell types can be involved in the pathology – chondrocytes, synovial
14 fibroblasts, bone and immune cells, and all of them may potentially show aberrant gene
15 expression and a failure in oxidant-antioxidant balance. With respect to secondary disease,
16 increased susceptibility to develop OA might be related to altered epigenetic modifications
17 of cartilage after injury. Indeed, more than 1000 differentially methylated regions (DMRs)
18 have been detected in human knee articular cartilage upon damage.⁷² Additional impact in
19 the secondary disease is chronic inflammation often related to dysfunction in immune
20 mechanisms and/or immune cell homeostasis.⁷³

34 **Role of inflammation and oxidative stress in osteoarthritis**

36 **Inflammation and interaction between immune and bone cells in OA**

37 One unresolved link in primary OA is the contribution of inflammation in disease initiation
38 and progression. Several studies have shown a significant association between OA severity
39 and the presence of inflammatory synovitis).^{71,74} Besides OA synovial fibroblasts, OA
40 chondrocytes show altered expression of inflammatory genes, of genes encoding
41 transcription factors or regulatory genes and of genes, associated with the loss of correct cell
42 maturation.^{75,76} Indeed, overexpression and overproduction of pro-inflammatory cytokines
43 have been clearly shown to be part of OA pathology.^{9,10,11} In chondrocytes, cytokines
44 activate PI3K/Akt and NF- κ B signalling pathways, up-regulate matrix metalloproteinases
45 (MMPs)/aggrecanases expression and increase DNA hydroxymethylation via attenuation of
46 ten-eleven translocation 1-3 (TET1-3) enzymes.⁷⁷ OA chondrocytes can also produce
47 chemokines, such as IL-8, that drive neutrophil accumulation.^{78,79}

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3 Indeed, neutrophils can impact seriously OA pathology. It has been found that blood
4 neutrophils from OA patients with active disease respond to in vitro stimulation with elicited
5 pro-inflammatory cytokine production and show elevated expression of bone-damage
6 accelerating receptor activator of nuclear factor- κ B (RANKL).⁸⁰ Mature neutrophils
7 expressing RANKL are found in synovial fluid of mice with OA⁸¹ and disease improvement
8 is associated with inhibition of RANKL expression on neutrophils.⁸² Neutrophils actually
9 endow a destructive phenotype in blood that is further extended and sustained in arthritic
10 synovium.⁸³ In turn, they can drive osteoclast differentiation and maturation from precursor
11 cells directly via RANKL-RANK pathway or they can manipulate chondrocyte and
12 osteoblast functions indirectly via ROS, cytokines and growth factors secretion. Neutrophils
13 can cooperate with NK cells to build the local immune response and can induce general
14 changes in NK homing and activation via secreted chemokines⁷³ that in turn can fail to
15 support normal cartilage and bone repair. It is still unclear if some of the abnormalities in
16 advanced OA are related to age-dependent dysfunction in immune cells and ROS signaling
17 as well as with a shift in the expression of myeloid lineage genes and changes in the
18 composition and quality of the tissue-restricted pools of immune cells.⁸⁴
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31 **Role of oxidative stress in OA pathology**

32 During the past decades, tremendous progress has been made in understanding the role of
33 oxidative stress in health and diseases. ROS including H_2O_2 , $O_2^{\bullet-}$, and $\bullet OH$ are byproducts
34 of normal cellular metabolism. ROS are generated during electron transport chain reactions
35 delivering electrons to molecular oxygen in the mitochondria. The process is restricted by
36 the redox enzyme p66^{Shc}, which is translocated in the mitochondria in response to
37 exogenous signals delivered by growth factor deprivation, oxidative stress or UV
38 irradiation.^{85,86} Generated H_2O_2 can diffuse across the outer mitochondrial membrane to the
39 cytosol where it can modulate the activity of “ROS sensors” such as the thiol functional
40 group of the amino acid cysteine in proteins. Subsequently, the oxidative cysteine
41 modifications can modulate cytosolic protein functions, akin to phosphorylation and can
42 increase mitochondrial permeability and biogenesis.⁸⁶ H_2O_2 also downregulates the activity
43 of the forkhead transcription factor FoxO3, implicated in the expression of mitochondrial
44 antioxidant enzymes, that in turn increase both hydrogen peroxide scavenging and oxidative
45 stress resistance.⁸⁷
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56 **ROS generation in mitochondria can often change during aging resulting in persistent**
57 **endogenous ROS production at small amounts contributing to aging-related mitochondrial**
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dysfunction, swelling and apoptosis together with alterations in chondrocyte senescence and apoptosis. This pathway is considered to play the minor role in the total ROS accumulation but it has the key implication in aging-dependent alterations in OA.^{14,22}

The significant amounts of ROS are generated via non-mitochondrial pathway, *i.e.* NADPH oxidase (NOX) or dual oxidase (DUOX) assemblies found at the discrete regions at the plasma or endosomal membranes. In phagocytic cells, the active oxidase contains the catalytic subunit (gp91phox), the regulatory subunits (p22phox, p47phox, p40phox, p67phox) and the small signaling G protein kinase. Later it was discovered that non-phagocytic cells from various tissues can express one of the seven homologues of the gp91phox: NOX 1-5 and DUOX 1-2, all containing conserved domain structure of six transmembrane α -helices and an electron transfer center with two haems forming the electron transport channel through the membrane.⁸⁸ The C-terminal domain of the multi-component oxidase binds to the electron-donating pyridine nucleotide NADPH and to the various cofactors such as flavin adenine dinucleotide (FAD) or Ca^{2+} in order to generate superoxide. In a subsequent reaction, two molecules of superoxide can generate H_2O_2 . Similarly to the mitochondrial pathway, H_2O_2 can induce reversible modifications of cysteine containing proteins like tyrosine phosphatases adjacent to receptors for growth factors, cytokines and chemokines or like phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) responsible for the hydrolysis of the bioactive lipid phosphatidylinositol 3,4,5-trisphosphate. In such a way, the NOX enzymes can regulate the downstream signaling for cell activation, differentiation, proliferation and apoptosis in healthy cells. H_2O_2 can diffuse in the cytosol but NOX complexes are present at the lipid rafts⁸⁹ and assemble at focal adhesion regions,⁹⁰ restricting H_2O_2 to specific cellular microdomains and preventing aberrant signaling.

Another system for ROS generation involves the constitutively expressed enzyme xanthine oxidoreductase (XOR). XOR is highly expressed in intestine and liver and also in synovium, lung, kidney and brain. The XOR activity is versatile and tuneable at multiple-levels.⁹¹ At genetic level the expression of the enzyme can be regulated by nutritional factors, oxygen tension, steroid hormones, phorbol esters, regenerative and hyperplastic stimulus, cytokines implicated in inflammation (like $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$)⁹¹. The enzyme can exist in two forms - dehydrogenase and xanthine oxidase (XO) as it undergoes irreversible partial proteolytic cleavage or reversible post-translational modifications via thiol oxidation.^{92,93} During the latter convertible alteration, XOR enzyme is modified from dehydrogenase via intermediate form to reversible XO.⁹¹ The conversion of XOR to XO has been observed in a

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3 variety of hypoxic and ischaemic conditions in the tissues including synovium and has been
4 related with vascular response to oxidative stress and the regulation of pro-inflammatory and
5 pro-thrombotic activities of endothelial cells.⁹¹ The mechanisms include the metabolic
6 alterations and the activation of transcriptional factors, including hypoxia-inducible factor-1
7 (HIF-1) and protein kinases.⁹⁴ XOR can catalyze the oxidation of hypoxanthine to xanthine,
8 can act as a NADH oxidase (the XO form) to catalyze the subsequent oxidation of purines to
9 urate and can catalyze the reduction of nitrates and nitrites, being the rate-limiting enzyme in
10 purine catabolism as well as being the constant source of ROS, NO and peroxynitrite.^{92,93,95,96}
11 XOR level in synovial fluid is elevated in OA patients but in a lesser extent in comparison to
12 that in patients with rheumatoid arthritis,⁹⁷ and its amount in synovium correlates with the
13 severity of joint injury⁹⁷⁻⁹⁹ and inflammasome activation driving OA progression.¹⁰⁰ More
14 than 50% of synovial XOR exists in oxidase form and is expressed in synovial tissue upon
15 injury.^{97,101} Increased XO activity is associated also with altered adhesion and accumulation
16 of leukocytes in microvasculature and elevated ROS and cytokine production by infiltrating
17 and locally activated monocytes and neutrophils.⁹¹

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19 Several scavenging systems are available in the cells to detoxify ROS: the enzymes
20 superoxide dismutases (SOD), catalase, glutathione peroxidase and glutathione reductase.¹⁰²
21 SOD1 is a dimeric cytosolic enzyme that binds copper and zinc (Cu/ZN-SOD), whereas
22 SOD2 is a mitochondrial homo-tetramer that binds one manganese ion per subunit (Mn-
23 SOD). Both enzymes convert superoxide to H₂O₂ and diatomic oxygen. Catalase is a
24 tetrameric protein that converts H₂O₂ to H₂O and gaseous O₂. In the cytosol glutathione
25 peroxidase/glutathione reductase system maintains the reducing environment in the cells.
26 When ROS production escapes the antioxidant systems and mechanisms, the cells are
27 exposed to oxidative stress and become sensitive to the activation of apoptotic pathways.
28 ROS-mediated damage by oxidative stress can be often reversed by repair, replacement,
29 degradation or sequestration of the damaged macromolecules, but in some cases the stress
30 can be sustained driving mitochondrial and cell death or mutagenesis.^{85,86} The biological
31 effects of ROS in all cell types are due to hyperperoxidation, protein carbonylation, direct
32 DNA damage, telomere shortening, epigenetic alterations of gene expression and failure in
33 DNA repair, changes in receptor and metabolic pathways and autophagy. However, the
34 sources of ROS may differ in various cell types and may be dependent on cell type
35 functional and metabolic state.

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37 Abnormal ROS signaling has been described in OA synovial tissue, fibroblasts
38 chondrocytes, osteoblasts and osteoclasts, concomitant with spatiotemporal progression of
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3 injury from the articular surface to the subchondral bone. ROS generation can be influenced
4 by underlying chronic inflammation involving immune cells or by generalized alterations in
5 cell-driven repair affecting nuclear reprogramming (**Figure 3**). Indeed low level of ROS is
6 important for normal osteoclastogenesis and osteoblastogenesis to sustain bone integrity,
7 whereas high level of ROS may contribute to mitochondrial dysfunction and alterations in
8 signaling pathways and gene expression that in turn cause chondrocyte apoptosis and
9 senescence, subsequent cartilage degradation, and changes in subchondral bone and
10 processes of bone resorption and remodeling.

17 ***ROS signaling and nuclear programming***

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19 Recently several studies have drawn a clear link between ROS signaling and nuclear cell
20 programming. At physiological levels, ROS can alter nuclear reprogramming of somatic
21 cells to pluripotency, restricting cellular and genomic damage during self-renewal or
22 preventing inappropriate differentiation of pluripotent cells.^{103,104} The mechanism can
23 involve the inhibition of Nox family members, which increases genomic stability and/or the
24 activation of innate immunity as a positive feedback cycle of ROS generation.¹⁰⁵ During
25 early reprogramming, antioxidant enzymes are upregulated, Nrf2 expression gradually
26 increased in coordination with changes in mitochondrial biogenesis and bioenergetic
27 functions.¹⁰⁵ Supplementary mechanism to affect pluripotency is the regulation of H₂O₂
28 production from superoxide and consequent cysteine oxidation of transcriptional factors or
29 epigenetic modifiers by H₂O₂.¹⁰⁶ Such findings encourage the researchers to use inducible
30 pluripotent stem cells (or adult stem cells) for the treatment of degenerative diseases like
31 OA, where an alteration of the cartilage and bone tissue reparative capacity has been
32 described. Experiments with undifferentiated human mesenchymal stem cells (hMSCs) have
33 shown higher levels of glycolytic enzymes, increased rate of glycolysis for energy supply
34 and of ROS signaling.¹⁰⁷ During the differentiation of hMSCs to osteoblast cells however,
35 the ability to produce ROS is lost because of increased ROS scavenging and upregulation of
36 antioxidant enzymes, and of transition from glycolysis to oxidative phosphorylation.¹⁰⁸ The
37 underlying mechanism involves initial increase of Nrf2, NF- κ B and AP-1 activity, and
38 induction of hypoxia-inducible factor expression (HIF- α).¹⁰⁸

54 ***ROS signaling and OA synovial fibroblasts***

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56 Increased expression and protein content of the pro-oxidant enzymes Nox2 and XO have
57 been shown in synovial tissue of patients with knee OA.¹⁰⁹ The level of the enzymes
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3 correlates with local expression of prolydase, an imidodipeptidase with a role in the
4 recycling of collagen.¹¹⁰ Prolidase is regulated at the post-transcriptional level by integrin-
5 dependent signaling delivered from stimulatory (*i.e.*, thrombin) or inhibitory (*i.e.*, echistatin)
6 β_1 -integrin ligands or by nitric oxide donors.¹¹⁰ Indeed in serum of patients with knee OA,
7 increased oxidant (measured by ROS, total peroxide and lipid hydroperoxide levels) and
8 decreased antioxidant state (measured by antioxidant capacity, thiol level and catalase
9 enzyme activity) are related with elevated serum prolydase activity¹¹¹ and elevated
10 inflammasome activity in synovium, indicating a link between ROS signaling, inflammation
11 and cartilage collagen metabolism.¹⁰⁹

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OA synovial fibroblasts show higher production of chemokines, IL-1 β , TNF- α and
increased expression of thrombin.^{112,113} Thrombin contributes to fibrin deposition,
angiogenesis, proliferation and proinflammatory processes in OA synovium involving the
intensive cleavage of osteopontin¹¹⁴ and the activation of HO-1.¹¹³ The latter enzyme is a
stress-inducible rate-limiting enzyme in heme degradation responsible for cytoprotection
against oxidative injury and is upregulated via the activation of PKC δ , c-Src, and Nrf2.¹¹³
Thrombin secreted by OA fibroblasts can also alter the metabolism of cartilage
chondrocytes via HO-1. The mechanism involves an increased production of insulin-like
growth factor-1, a reduction of insulin-like growth factor binding protein-3, a decreased
activation of ERK 1/2, an elevated activity of MMPs.¹¹⁵ Alterations in OA synovial
fibroblasts have been also related to their increased sensitivity to local NO donors which
directly contribute to elevated ROS production and oxidative stress.¹¹⁶ Oxidative stress in
OA fibroblasts trigger MAPKs phosphorylation, TAK1-mediated NF- κ B activation and
Cox-2/PGE2 expression, both reversible by antioxidants such as N-acetylcysteine and
hyaluronic acid.¹¹⁶ The production of local inflammatory mediators and ROS in OA
synovium sustains the proliferative ability of OA fibroblasts and alters the regulatory
mechanisms of MMPs, cytokine and growth factors expression. For example IL-1 β as well
as ROS can induce the expression of particular miRNAs, which control NF- κ B activation,
translocation and acetylation. On the other hand, miRNA expression can be controlled by
histone deacetylases (HDACs), enzymes sensitive to ROS signaling. Recent studies have
shown that HDAC inhibitors can directly suppress gene expression of iNOS and MMPs in
OA fibroblasts. Indirect effects of their administration include the alteration of NF- κ B
activity and translocation, changes in phosphorylation of p38 and ERK, and control of miR-
146a expression.^{117,118}

ROS signaling and OA chondrocytes

Articular human normal cartilage is composed of a hydrated extensive extracellular matrix (ECM) in which a small number of cells (2-3% of the total tissue volume) named chondrocytes are embedded. These cells are the only cell type in the normal mature articular cartilage, which are metabolically very active and normally do not divide after adolescence. They maintain homeostasis and balance of matrix synthesis and degradation, as well as repair damaged cartilage tissue in OA.^{10,119} Cartilage catabolism comprises secretion of proteases, suppression of matrix synthesis and inhibition of chondrocyte proliferation, while the anabolism is associated with secretion of antagonistic cytokines, synthesis of protease inhibitors, production of extracellular matrix and cell replication. Early changes in the articular surface include fragmentation, fibrillation and gradual thinning of cartilage and loss of viscosity of the synovial fluid with alteration in the biochemical composition.^{10,119}

Articular chondrocytes from OA patients are deteriorated and have irregularly orientated mitochondria. Indeed chondrocytes near the fibrillated regions show swollen mitochondria with disoriented cristae and nearby glycogen vacuoles, indicative of altered cell metabolism.¹²⁰ In culture, OA chondrocytes show increased mitochondrial mass [as a result of increased citrate synthase (CS) activity] but reduced mitochondrial respiratory activity and changes in mitochondrial membrane potential in comparison to normal chondrocytes.^{14,121} The collapse of the mitochondrial membrane potential results in mitochondrial swelling, disruption of outer mitochondrial membrane and release of pro-apoptotic factors, such as cytochrome c, apoptosis-inducing factor and pro-caspases.¹⁴ A proteomic study has demonstrated that enzymes involved in glycolysis significantly decrease in OA chondrocytes¹¹⁹ along with a deficiency in mitochondrial SOD, catalase and glutathione scavenging systems; overproduction of ROS, NO, detrimental peroxynitrite and reduction of the ATP pool.¹¹⁹ Several genomic, lipidomic¹²² and high-resolution mass spectrometry analysis¹²³ have identified enrichment in pathways related to lipid (eicosanoids) metabolism and oxidative stress in OA chondrocytes. It is assumed that excessive cartilage load can trigger abnormalities in ROS release, mitochondrial functions and energy/metabolic state in some predisposed individuals and in some areas of cartilage. In those individuals altered ROS signaling can be sustained by aging, itself, resulting in the senescence secretory phenotype of chondrocytes. This phenotype includes the upregulation of the expression of matrix metalloproteinases (MMPs) and aggrecanases, cytokines and growth factors and the integration with the PI3K-Akt, p38, ERK and NF-kB signaling linking cell receptor with metabolic pathways.¹²⁴ Consequently, more chondrocytes at the

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3 superficial zone acquire alterations in total DNA methylation profile,¹²⁵ in histone
4 acetylation of anabolic genes (like SOX9 gene) and in particular miRNA leading to a failure
5 in regulation of transcription and stability of antioxidant, inflammatory and metabolic
6 genes.¹²⁶ The persistent ROS generation in combination with altered ROS scavenging in
7 cartilage cause a progressive mitochondrial damage in most of the chondrocytes with
8 changes in oxidative phosphorylation, redox signaling, miRNAs-regulated mitochondrial
9 biogenesis¹²⁷ and expression of mitochondrial ROS antagonist chaperones.¹¹⁹ As a result,
10 chondrocyte apoptosis at superficial cartilage areas increases, and chondrocytes at deeper
11 zone become hypertrophic disrupting normal endochondral ossification and matrix repair.
12 Hypertrophic chondrocytes usually have metabolic and proliferation defects that can be
13 overcome by the antioxidant treatment with N-acetylcysteine¹²⁸ or the administration of 17 β -
14 estradiol.¹²⁹ They also show alteration in HO-1. The enzyme is responsible for the
15 degradation of heme to carbon monoxide (CO), free iron and biliverdin-IX. In mammals,
16 biliverdin-IX is further converted to bilirubin-IX, and endogenous radical scavenger with
17 recognized anti-inflammatory properties. On the other hand free iron is rapidly sequestered
18 into the iron-storage protein ferritin, leading to additional antioxidant and antiapoptotic
19 effects. CO has also several biological functions, with antiapoptotic and anti-inflammatory
20 properties.¹¹³

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22 Recently it has been shown that the accumulation of ROS in chondrocytes can lead to
23 mitochondrial DNA (mtDNA) mutations. Researchers have even identified the specific
24 mitochondrial DNA haplogroups J and T that have been associated with variations in the
25 prevalence and progression of cartilage loss in some European populations.¹³⁰⁻¹³³ In
26 particular, J haplotype has been linked to lower serum levels of markers of collagen type-II
27 degradation and of matrix metalloproteinases and higher ATP levels in the Spanish
28 population, while T haplotype correlates with lower disease risk in a small UK cohort.¹³¹⁻¹³³

29 30 31 ***ROS signaling and OA osteoclasts and osteoblasts***

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33 The osteoclasts drive the processes of bone resorption and bone matrix loss. The osteoclasts
34 are differentiated from bone marrow monocyte precursors. The process is dependent on NF-
35 kB signaling driven by RANKL-RANK or is regulated by IL-17, GM-CSF and/or IFN- γ .
36 Osteoblasts, stem cells, stromal cells, neutrophils, NK cells and even migrated in the bone
37 marrow CD4⁺ T cells can drive osteoclastogenesis via direct contact. It has been shown that
38 RANKL-RANK system stimulates ROS generation in osteoclasts.¹³⁴ The administration of
39 the antioxidants N-acetylcysteine and glutathione attenuates RANKL-mediated Akt and
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3 ERK activation and NF- κ B signaling (via inhibition of IKK activity and I κ Ba
4 phosphorylation) as well as it reduces bone resorbing activity and regulates osteoclasts
5 apoptosis.¹³⁵ RANKL-mediated ROS production and osteoclast differentiation is abrogated
6 and blocked when bone marrow precursors are depleted of Nox1 by siRNA or by expression
7 of a dominant-negative mutant of Rac1.¹³⁴ The same group has shown that the role of ROS
8 signaling for osteoclast differentiation is dependent on activation of JNK. The osteoblasts
9 drive the opposite process of bone resorption, bone remodeling and repair. In OA the
10 balance between activation of osteoclasts and osteoblasts leads to abnormal remodeling and
11 formation of osteophytes. Similarly to osteoclasts and p38 and transcription factor NF-
12 ATc1.¹³⁴ RANKL can attenuate Nox2 mRNA expression but it can upregulate Nox1 and
13 Nox3 transcription during the differentiation of macrophage lineage to osteoclasts.¹³⁶ Indeed
14 siRNA targeting of p67(phox) or p22(phox) in macrophage lineage downregulates ROS
15 generation, suppresses RANKL-induced osteoclastogenesis, inhibits the expression of
16 osteoclast marker genes (TRAP, cathepsin K, Atp6i, CIC-7, and NF-ATc1).¹³⁶

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Osteoblasts drive the opposite process of bone resorption, bone remodeling and repair. In OA the balance between activation of osteoclasts and osteoblast leads to abnormal remodeling and formation of osteophytes. Similarly, to osteoclasts, osteoblasts are sensitive to ROS signaling during their differentiation from mesenchymal cells. Osteoblastogenesis involves Runx2-dependent and -independent mechanisms of gene expression and is dependent on growth factor signaling pathways triggered by bone morphogenic proteins (BMPs), transforming factor (TGF)- β and Wnt cascade. Recently it has been shown that BMP-2 induces a rapid generation of ROS and activation of NADPH oxidase and that Nox4 activation is a key factor for osteoblast differentiation in response to BMP-2.¹³⁷ In differentiated OA osteoblasts however oxidative stress can have several impacts on their functions. The administration of the antioxidant N-acetylcysteine elevates osteocalcin and type I collagen synthesis and decreases alkaline phosphatase activity in OA osteoblasts. The mechanisms involve activation of p38 and JNK1/2, but not ERK1/2 kinases, induction of ATF-2/CREB activation, inhibition of NF- κ B, and activation of COX-2 promoter activity via CRE site. Consequently, the antioxidant increases COX-2 and PGE₂ levels but decreases TNF- α induced IL-6 production.¹³⁸

Nrf2 and OA pathogenesis

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3 As a master regulator of the cellular redox homeostasis, Nrf2 induction may play a key role in
4 OA pathophysiology. Nrf2 is responsible for the activation of glutathione-S-transferase,
5 NQO1,¹² and glutathione peroxidase activities¹³⁹ thus attenuating early joint pain.¹⁴⁰ Nrf2 is a
6 promoter of HO-1 expression,^{102,141} prevents 4-hydroxynonenal (HNE)-induced mitochondrial
7 damage,³ and its deficiency in mice leads to more severe OA development.¹⁴¹ HO-1 and γ -
8 glutamylcysteine ligase (γ -GCLC) act synergistically as phase 2 antioxidants and were found
9 in higher levels due to Nrf2 activation.¹⁴² In addition, Nrf2 is involved in the regulation of
10 ROS production by mitochondria and NADPH oxidase.¹⁴³

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16 Studies in Nrf2-deficient mice have shown that they are highly susceptible to
17 chemical-induced toxicity and oxidative burden, as a result of dysregulation/loss of
18 expression of detoxification, xenobiotic metabolism and redox genes.^{144,145} However other
19 genes including those involved in protein transport, ubiquitination, phosphorylation, cell
20 cycle, growth and apoptosis have been identified to be also Nrf2-dependant.¹⁴⁶ By using
21 ChIP-Seq and microarray analyses, 645 basal and 654 inducible direct targets of Nrf2 were
22 identified and confirmed to be related with stress response and cell proliferation in mouse
23 embryonic fibroblasts.¹⁴⁷ In humans, polymorphisms in the ARE sequences, to which Nrf2
24 binds, have been reported.¹⁴⁸ Concerning inflammatory responses, a recent review discussed
25 the role of Nrf2 in the regulation of the expression of HO-1 during hematopoiesis,
26 development and activation of lymphoid cells and macrophages as well the interaction
27 between Nrf2 and NF- κ B and the mechanism of Nrf2-regulated NLP3 inflammasomal
28 activity.¹⁴⁹

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38 Abnormal ROS signaling in OA synovial fibroblasts by cytokines, thrombin or stress
39 is associated with increased Nrf2 activity. Oxidative stress in cartilage may trigger activation
40 of Nox4 and mitochondrial dysfunction. However, both factors may be counteracted by
41 increased Nrf2 activation in fibroblasts.¹⁵⁰ Nrf2 regulates thrombin-induced production of
42 HO-1 in synovial fibroblasts and consequently, inhibition of the Nrf2-dependent signaling
43 pathway by Nrf2 siRNA, diminishes HO-1 expression. Thrombin increases the Nrf2
44 phosphorylation and binding to the ARE element of HO-1 gene promoter. Nrf2 activity is
45 attenuated by inhibitors of PAR1/PAR3, PKC δ , and c-Src pathways indicating that all of
46 them are involved in thrombin-induced Nrf2 activation in human synovial fibroblasts.¹¹³
47 Nrf2 activation antagonizes cytokine-induced NF- κ B signaling by limiting I κ B α
48 degradation, suggesting that a failure in this reciprocal interaction may also occur in OA
49 synovial fibroblasts as they over-produce inflammatory cytokines.¹⁴⁹ However, the specific
50 environment in OA cartilage may contribute to a different role of Nrf2 in OA fibroblasts in
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3 comparison to that in fibroblasts from other tissues. For example skin fibroblasts from
4 patients with systemic sclerosis show decreased Nrf2 activity along with elevated
5 inflammatory gene expression and fibrosis. In skin fibroblasts, the Nrf2 silencing
6 constitutively elevates collagen synthesis, spontaneous myofibroblast differentiation, and
7 enhances TGF- β responses¹⁵¹ showing that Nrf2 may be a key cell-intrinsic anti-fibrotic
8 factor sustaining extracellular matrix homeostasis in systemic sclerosis.¹⁵¹ In another study
9 using human periodontal ligament fibroblasts, it has been demonstrated that H₂O₂-mediated
10 oxidative stress decreases the osteogenic differentiation of fibroblasts via interference with
11 Wnt/ β -catenin signaling and Nrf2 expression at protein and mRNA levels.¹⁵²
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18 Nrf2 plays a major chondroprotective role in the progression of OA and suppresses IL-
19 1 β -induced MMP-1, MMP-3 and MMP-13 expression at both mRNA and protein levels, and
20 induces the expression of phase 2 antioxidant enzymes, such as NQO1 and HO-1,^{9,11,141} as
21 well as suppresses the PGE2 and NO production and inhibition of the phosphorylation of NF-
22 κ B p65 and I κ B α in human OA.¹¹ It has been demonstrated that histone deacetylase inhibitors
23 (HDACi),¹⁴¹ physical activity,¹⁴² phytochemicals, such as protandim, 6-gingerol,³ ascorbic
24 acid,¹⁵³ sulforaphane,¹⁵⁴ curcumin,¹⁵⁵ carotenoids,¹⁵⁶ as well as broccoli¹⁵⁷ and sesame oil are
25 protective in models of OA by activation of Nrf2.¹³⁹ Downregulation of Nrf2 could result in
26 inhibition of chondrogenesis through apoptotic cell death, but on the other hand stable
27 overexpression of Nrf2 caused inhibition of chondral differentiation by reduction of the
28 characteristic markers, such as type II collagen (Coll II), type X collagen (Coll X) and
29 osteopontin. This means that appropriate expression and fine balance in Nrf2 activity is
30 absolutely necessary for normal chondrogenesis and proper regulation of OA.^{12,23} Nrf2 can
31 also regulate indirectly chondrocyte apoptosis and senescence via the expression of
32 glyoxalase I (an enzyme that detoxifies methylglyoxal).¹⁰ This enzyme regulates the levels of
33 the advanced glycation products (AGEs).¹⁰ The levels of AGE such as urinary pentosidine
34 correlate with the degree of cartilage damage and chondrocyte loss.^{158,159} Thus, the
35 overexpression of the receptors of AGEs in OA might suppress Nrf2 and glyoxalase I,
36 respectively.¹⁰
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50 Nrf2 has been reported to play important roles in the regulation of inflammatory
51 processes. The induced expression of IL-1 β and TNF- α results in higher expression of NO,
52 which inhibits chondrocytes cytoskeletal actin polymerization and beta-1 integrin-dependent
53 signaling, decreased expression of IL-1 β receptor antagonist and TGF- β , suppression of
54 chondrocyte collagen and proteoglycan synthesis, activation of MMPs and suppression of
55 proliferation, as well as, promotion of apoptosis. NO suppresses energy metabolism and
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3 directly induces calcification of articular cartilage. During apoptosis mitochondria sequester
4 Ca^{2+} in providing energy to the cells. The excessive Ca^{2+} concentrations lead to the opening of
5 the mitochondrial permeability, which depolarizes the mitochondria and leads to
6 mitochondrial swelling and apoptosis.¹⁴ Nrf2 inhibits NF- κ B activation and the production of
7 inflammatory mediators, such as IL-1 β and PGE2, as well as, MMP-1, MMP-3 and MMP-13,
8 which are important risk factors that have the ability to inhibit type II collagen synthesis in
9 ECM.^{11,154} The mechanisms of this inhibition are just beginning to be understood, and include
10 repression of transcription of pro-inflammatory genes.¹⁶⁰

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16 Several lines of evidences indicate that Nrf2 is an important factor for osteoclast and
17 osteoblast differentiation as well as to sustain normal skeletal and bone metabolism. Nrf2-
18 deficient mice have lower bone mass and bone strength.¹⁶¹ The same authors described that
19 the deletion of Nrf2 inhibits the load-driven gene expression of antioxidant enzymes and
20 Wnt5a in cultured primary osteoblasts¹⁶¹ indicating that Nrf2 regulates the overall bone
21 homeostasis. Nrf2 is a key factor in the maintenance of bone microarchitecture as observed
22 in an animal model of postmenopausal osteoporosis.¹⁶² Nrf2 deficiency promotes the
23 RANKL-induced activation of p38, c-Jun, ERK, induces c-Fos and NF- κ B activation and
24 osteoclastogenesis, likely due to dysfunction in the production of antioxidant enzymes and
25 glutathione.¹⁶³ The overexpression of Nrf2 enhances the expression of anti-oxidant enzymes,
26 inhibits osteoclast differentiation and attenuates lipopolysaccharide-mediated RANKL-
27 dependent cranial bone destruction *in vivo*.¹⁶⁴ The same authors showed that during
28 osteoclastogenesis, the precursor cells lose Nrf2, fail to translocate Nrf2 in the nucleus
29 because of Keap1 upregulation, and downregulate cytoprotective enzymes (HO-1, γ -
30 glutamylcysteine synthetase, and glucose-6-phosphate dehydrogenase), elevating ROS
31 production.¹⁶⁴ Consistent with this report is a study demonstrating increased oxidative stress
32 in bone marrow-derived cells from ovariectomized Nrf2-deficient mice and a higher
33 responsiveness of bone marrow-derived cells to osteoclastogenic stimuli *in vitro*.¹⁶²
34 Knockout mice of BTB and CNC homology 1 (Bach1)—the competitor of Nrf2 in
35 transcriptional regulation attenuate RANKL-mediated osteoclastogenesis.¹⁶⁵ The mechanism
36 involves RANKL-dependent nuclear import of Bach1 and Nrf2 nuclear export, which
37 attenuates Nrf2-mediated antioxidant enzymes and ROS signaling in osteoclasts. Moreover,
38 the induction of Bach1 nuclear export inhibited bone destruction *in vivo*.¹⁶⁵ Nrf2 knockdown
39 or deletion increases osteoclastic differentiation from bone marrow-derived macrophages via
40 upregulation of transcription factor NFATc1.¹⁶⁶ Interestingly, in this study Nrf2 inhibits
41 osteoblast differentiation and mineralization via suppression of Runx2, osteocalcin, and
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3 osterix.¹⁶⁶ The number of osteoblasts in bone is increased in Nrf2-knockout mice in
4 comparison to wild-type mice, indicating that both osteoclastogenesis and
5 osteoblastogenesis are dependent of Nrf2 or at least require its presence.¹⁶⁶ When Nrf2-
6 deficient mice are irradiated, the bone loss and RANKL expression markedly increase, but
7 the mineralization of calvarial osteoblasts decreases, suggesting that loss of Nrf2 can
8 activate ROS signaling, altering the sensitivity to radiation-induced bone damage.
9 Importantly, all of these effects are restored by the antioxidant N-acetylcysteine.¹⁶⁷ The role
10 of Nrf2 in osteoblast-driven bone formation has been demonstrated in a model of fracture
11 healing. The underlying mechanism is related to elevated expression of vascular endothelial
12 growth factor (VEGF) and diminished production of osteocalcin in Nrf2-deficient mice.¹⁶⁸
13 Together, these studies have shown that Nrf2 plays a key role in regulation of the balance
14 between osteoclast-driven bone resorption and osteoblast-driven remodeling (**Figure 4**).
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23 However, in Nrf2-deficient mice, altered bone metabolism has not been studied with
24 respect to a dysfunctional immunity or/and cytokine production imposing the need for
25 further investigations. It has not been yet considered whether Nrf2 has a critical role in
26 maintaining hematopoietic stem cell function, including effects on cell quiescence and self-
27 renewal potential. Nrf2 favors the commitment of both myeloid and lymphoid lineages
28 suggesting that Nrf2 imposes a general regulatory role in bone marrow niches.¹⁶⁹ Thus, Nrf2
29 may regulate osteoclastogenesis or osteoblastogenesis by stabilization of bone marrow niches
30 with progenitors. It has been shown that Nrf2-deficient mice have more severe cartilage
31 damage in two models of OA, destabilization of the medial meniscus (DMM) and
32 monosodium iodoacetate (MIA) models.¹⁴¹ The chondroprotective role of Nrf2 in these
33 models was mediated by the use of HDAC inhibitors inhibiting OA-associated proteins
34 MMP1, 3, 13 and pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. Because inhibition
35 of HDAC fails to improve the disease in Nrf2-deficient mice,¹⁴¹ this supports the notion for
36 a more general cell dysfunction that is likely mediated by epigenetic mechanisms.
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48 **The manipulation of stem cells via Nrf2**

49 The manipulation of stem cells (from different origin; stem cells or adipose-driven) is a new
50 strategy exploiting the key role of Nrf2 in cell differentiation, senescence and apoptosis. It
51 has been shown that the generation of osteoblast cells from stem cells can be achieved by
52 silencing of Nrf2 and autophagy. *In vivo*, bone formation is enhanced by injection of Nrf2
53 knockdown stem cells.¹⁷⁰ Moreover, therapy with adipose-driven stem cells can be improved
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3 by promoting endothelial progenitor cell migration and angiogenesis under hypoxia via
4 TLR4-mediated NF-kB signaling and antioxidant-driven Nrf2 activation.¹⁷¹
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8 **Anti-inflammatory drugs in OA therapy**

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10 Currently, there is no enough satisfactory drug for effective OA treatment and hence OA
11 cannot be cured totally. Moreover, the existing treatment strategies aim only to control the
12 symptom and reduce the pain.²² Although there are promising candidates they are limited in
13 efficacy and associated with certain toxicity.⁷⁰ The current management of OA includes: first,
14 behavioral interventions (patient education, exercise and change in life style); second, simple
15 analgesic, such as acetaminophen (paracetamol); third, application of nonsteroidal anti-
16 inflammatory drugs (e.g., COX-2 inhibitors); fourth, intraarticular injections of
17 corticosteroids, hyaluronic acid and glucosamine; and fifth, total joint replacement in the most
18 severe cases of OA.^{22,70} Bisphosphonates might have symptomatic and structural benefits for
19 patients with OA. Pamidronate disodium was able to prevent completely OA pathology in
20 rabbits through osteoprotegerin receptor activator of NF-kB ligand. Aldendronate reduced
21 osteophyte formation and articular cartilage degeneration.⁹ HDACi have emerged as potential
22 therapeutic strategy of OA treatment. Its effect is linked with the acetylation of Nrf2, which
23 enhances its functions in OA.¹⁴¹
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35 **Effect of plant derived or synthetic antioxidants on Nrf2 expression in** 36 **osteoarthritis**

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38 Plants-derived antioxidants have a great potential to modulate Nrf2 expression. For example,
39 curcumin has well proven anti-oxidant activity and can protect human chondrocytes from
40 apoptosis and dysfunction by inhibiting AP-1/NF-kB signalling and consequent production of
41 IL-1 β and activation of MMP-3.¹⁷² The inhibitory effect on NF-kB signaling by curcumin
42 may be, at least in part, due to the activation of Nrf2. Curcumin stimulates HO-1 gene activity
43 by promoting inactivation of the Nrf2–Keap1 complex, leading to increased Nrf2 binding to
44 the resident HO-1 AREs.¹⁷³ Calycosin, a compound derived from the Chinese medicinal herb
45 *Radix Astragali*, has similar effect as it activates HO-1 and Nrf2 in synovial fibroblasts from
46 arthritic patients, and inhibits pro-inflammatory factors.¹⁷⁴ The widely applied antioxidant
47 ascorbic acid (AA) was able to reduce apoptosis and loss of viability, and markedly decreased
48 H₂O₂-mediated senescence of chondrocytes. AA stimulated the expression of collagens and
49 proteoglycans, as well as inhibited chondrocyte differentiation under oxidative stress through
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3 decreasing the activity of Nrf2, NF-kB, AP1 and MMP-3.¹⁵³ Sesame oil treatment for seven
4 days significantly decreased OA-associated joint pain, by decreasing IL-6, increasing CS
5 activity and myosin heavy chain Ila mRNA expression. Sesame oil decreased muscular lipid
6 peroxidation, activated Nrf2, inhibited ROS and increased glutathione production and
7 glutathione peroxidase activity.¹³⁹ Protandim[®] (plant-derived science-based formulation) and
8 6-gingerol preserved chondrocytes viability and mitochondrial metabolism through Nrf2
9 upregulation and subsequent reduction of IL-1 β and MMP-13.³ Wogonin, a naturally
10 occurring flavonoid, revealed potent anti-inflammatory and chondroprotective effects through
11 the activation of ROS/ERK/Nrf2 signaling pathways in human osteoarthritis chondrocytes.
12 This molecule completely suppressed the expression and production of inflammatory
13 mediators (IL-6, COX-2, PGE2, iNOS and NO), as well as, matrix degrading proteases
14 including MMP-13, MMP-3, MMP-9.¹⁷⁵

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16 It is noteworthy that the dose responses of exposures to some of the phytochemicals
17 discussed above are often non-linear J-shaped or inverted U-shaped.^{176,177} Dose responses of
18 this type are known as hormetic dose responses, and are characterized by a low-dose
19 stimulation and a high-dose inhibition.¹⁷⁸ The initial event is mild stress, which subsequently
20 engages cellular stress response mechanisms, including those regulated by Nrf2. The
21 transcriptional upregulation of Nrf2-dependent as well as other inducible cytoprotective
22 proteins (also known as vitagenes) allows adaptation and survival, and enhanced resistance to
23 subsequent challenges, such as those caused by oxidative stress and chronic inflammation.¹⁷⁹

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25 In addition, natural and synthetic compounds can modulate NLRP3 inflammasome
26 activation via Nrf2. For example treatment of mice with lupus nephritis with
27 epigallocatechin-3-gallate (EGCG) or with citral (3,7-dimethyl-2,6-octadienal; a major
28 active compound in a Chinese herbal medicine *Litsea cubeba*) attenuates NLRP3
29 inflammasome activation via Nrf2 signaling.^{180,181} By a similar mechanism, mangiferin (2-
30 C- β -d-gluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone) protects mice with sepsis-induced
31 acute kidney-injury,¹⁸² biochanin inhibits acute liver injury,¹⁸³ and antroquinonol (active
32 compound from *Antrodia camphorata*) mitigates IgA nephropathy in mice.¹⁸⁴

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34 Several studies have pointed to the cytoprotective potential of Nrf2 against
35 glucocorticoid-induced apoptosis of osteoblastic cells. Dexamethasone-induced ROS
36 production is suppressed by Nrf2 activation and antioxidant enzymes expression by indole-
37 3-carbinol¹⁸⁵ and by sulforaphane (a naturally occurring isothiocyanate)¹⁸⁶ and icaraside
38 II.¹⁸⁷ Notably, sulforaphane, one of the most potent naturally occurring Nrf2 activators
39 known to date, has been shown to suppress pro-inflammatory responses and protect cultured
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3 human chondrocytes against various pro-inflammatory and pro-apoptotic stimuli, including
4 shear stress.^{188,189} When included in the diet, sulforaphane (3 μ M/day) decreased the arthritis
5 score in a mouse model of osteoarthritis.¹⁵⁴ The cytotoxic effect of methylglyoxal on
6 osteoblastic cells was reversed by glabridin by Nrf2 activation, increases in HO-1 and
7 glyoxalase I, and normalization of mitochondrial function.¹⁹⁰ Icariside II, synthetic SC79
8 and OSU53 can also prevent dexamethasone-induced apoptosis of osteoblasts via Akt
9 activation downstream of Nrf2.^{187,191,192} All of these compounds may have potential to limit
10 osteoblast cell death, which could lead to osteoporosis or osteonecrosis.
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20 **Conclusions and future directions**

21 ROS signaling may play a dual role in OA. At low physiological levels, ROS can be
22 important for cartilage and bone integrity by sustaining nuclear programming during
23 chondrogenesis, osteoclasts and osteoblasts differentiation probably via Nrf2-sequestration
24 mechanisms. Aging, mechanical load and inflammation can increase ROS production and can
25 trigger oxidative stress with mitochondrial dysfunction, disturbances in cell signaling and
26 alterations in epigenetic control of gene expression. Consequently, high level of ROS
27 signaling and altered Nrf2 activity can increase chondrocyte apoptosis with cartilage
28 degradation, induce chondrocyte hypertrophy and dysfunction in subchondral bone.
29 Decreased Nrf2 activity may be a result of a failure in its homeostatic post-translational
30 regulation and/or altered epigenetic and transcriptional regulatory mechanisms. Experimental
31 evidence suggests that the role of Nrf2 in bone integrity is complex. Low Nrf2 activity may
32 sustain bone resorption favoring osteoclastogenesis at early stages of OA, whereas high Nrf2
33 activity may enhance osteoblastogenesis in advanced disease stage, inducing extensive bone
34 remodeling. Therapeutic approaches to increase Nrf2 activity by pharmacological agents may
35 counteract oxidative stress and inflammation in OA thus effectively limiting cartilage
36 degradation and bone resorption, whereas overall restoration of homeostatic Nrf2 activity may
37 be required to normalize imbalanced bone resorption and remodeling.
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Conflict of interest

The authors declare no conflicts of interest.

Figure Legends

Figure 1. Domain structure of Nrf2. The relative positions of the Nrf2 ECH homology (Neh) 1–7 domains are shown. The numbering of amino acids is based on the human protein. The DLG and ETGE motifs in the Neh2 domain that bind Keap1, and the DSGIS and DSAPGS motifs in the Neh6 domain that bind β -TrCP, are indicated. The Neh4 and Neh5 domains form the transactivation domain of Nrf2 that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXR α) binds to the Neh7 domain of Nrf2, leading to repression of the transcription factor. Neh1 mediates binding to DNA and to the heterodimeric partner of Nrf2, a small Maf transcription factor. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6).

Figure 2. Regulation of Nrf2 by ubiquitination and proteasomal degradation. Three known ubiquitin ligase systems mediate the ubiquitination of Nrf2: (i) Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for Cullin-3 (Cul3)-Rbx1/Roc1 ubiquitin ligase (i.e. CRL^{Keap1}), which binds through its Kelch domains to the DLG and ETGE motifs in the Neh2 domain of Nrf2. For substrate adaptor activity, the cysteine sensors of Keap1 are in reduced state; (ii) β -transducin repeat-containing protein (β -TrCP), a substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (i.e. SCF ^{β -TrCP}). Recognition by β -TrCP requires formation of a DSGIS motif-containing phosphodegron in the Neh6 domain of Nrf2 by glycogen synthase kinase 3 (GSK3), which in turn requires phosphorylation of Nrf2 by a priming kinase; and (iii) the E3 ubiquitin ligase Hrd1/synoviolin, an endoplasmic reticulum (ER) protein. Degradation by Hrd1 has been shown to occur during ER stress.

Figure 3. Role of ROS signaling in OA pathology. The low level of ROS can sustain cartilage and bone integrity as ROS regulate nuclear programming during osteoclasts and osteoblasts differentiation. At high level ROS can trigger oxidative stress with mitochondrial dysfunction and a failure in cells signaling and epigenetic regulation of gene expression. Oxidative stress causes inflammation, chondrocyte apoptosis with cartilage degradation and dysfunction in subchondral bone with imbalanced bone resorption and remodeling.

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3 **Figure 4. Role of Nrf2 in OA.** Bone resorption at early stage of OA is due to a favorable
4 osteoclastogenesis via low Nrf2/Keap ratio (as shown by Nrf2 deficiency). In advanced
5 stage of OA, intensive bone remodeling triggered as a compensatory mechanism to bone
6 resorption is more likely related with enhanced osteoblastogenesis via high Nrf2/Keap ratio
7 (as shown by Nrf2 overexpression). The therapy approaches in OA may involve a
8 normalization of bone/resorption and remodeling via interference with Nrf2 expression and
9 ROS signaling.
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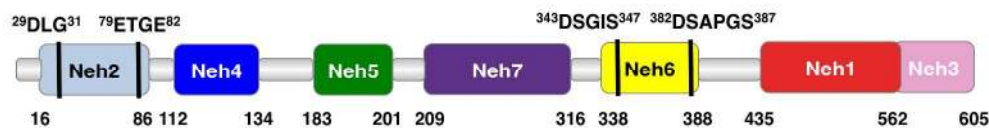


Figure 1. Domain structure of Nrf2. The relative positions of the Nrf2 ECH homology (Neh) 1–7 domains are shown. The numbering of amino acids is based on the human protein. The DLG and ETGE motifs in the Neh2 domain that bind Keap1, and the DSGIS and DSAPGS motifs in the Neh6 domain that bind β -TrCP, are indicated. The Neh4 and Neh5 domains form the transactivation domain of Nrf2 that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXR α) binds to the Neh7 domain of Nrf2, leading to repression of the transcription factor. Neh1 mediates binding to DNA and to the heterodimeric partner of Nrf2, a small Maf transcription factor. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6).

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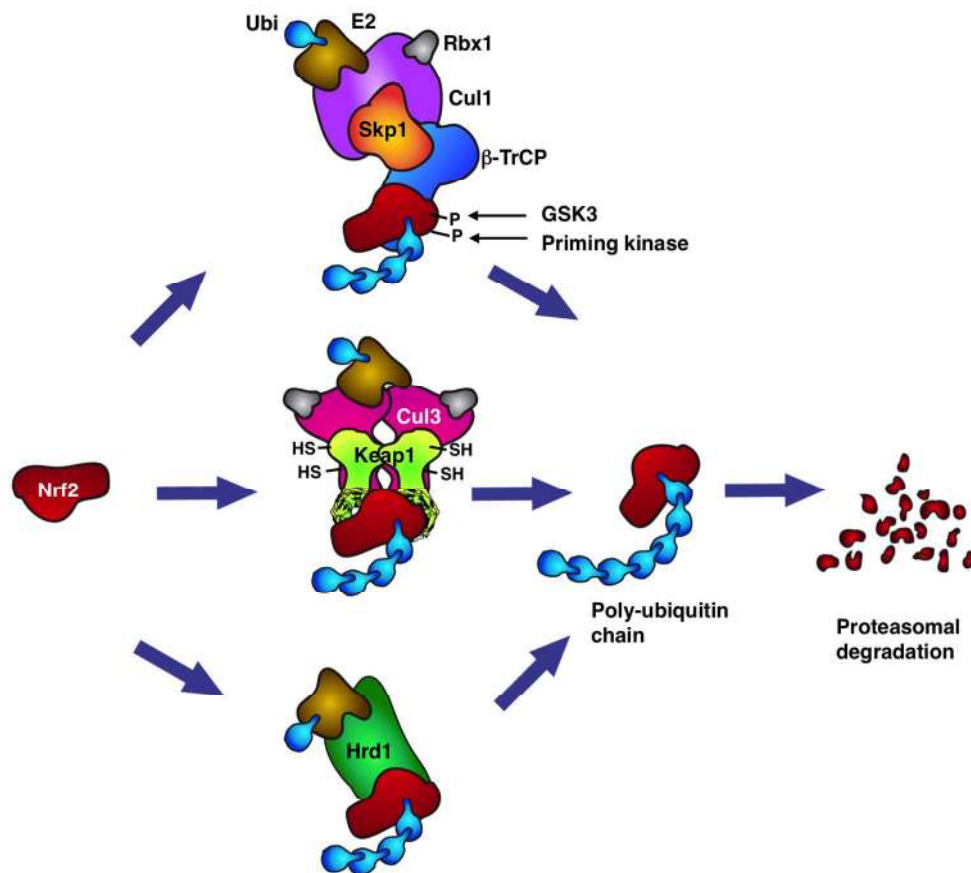


Figure 2. Regulation of Nrf2 by ubiquitination and proteasomal degradation. Three known ubiquitin ligase systems mediate the ubiquitination of Nrf2: (i) Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for Cullin-3 (Cul3)-Rbx1/Roc1 ubiquitin ligase (i.e. CRLKeap1), which binds through its Kelch domains to the DLG and ETGE motifs in the Neh2 domain of Nrf2. For substrate adaptor activity, the cysteine sensors of Keap1 are in reduced state; (ii) β -transducin repeat-containing protein (β -TrCP), a substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (i.e. SCF β -TrCP). Recognition by β -TrCP requires formation of a DSGIS motif-containing phosphodegron in the Neh6 domain of Nrf2 by glycogen synthase kinase 3 (GSK3), which in turn requires phosphorylation of Nrf2 by a priming kinase; and (iii) the E3 ubiquitin ligase Hrd1/synoviolin, an endoplasmic reticulum (ER) protein. Degradation by Hrd1 has been shown to occur during ER stress.

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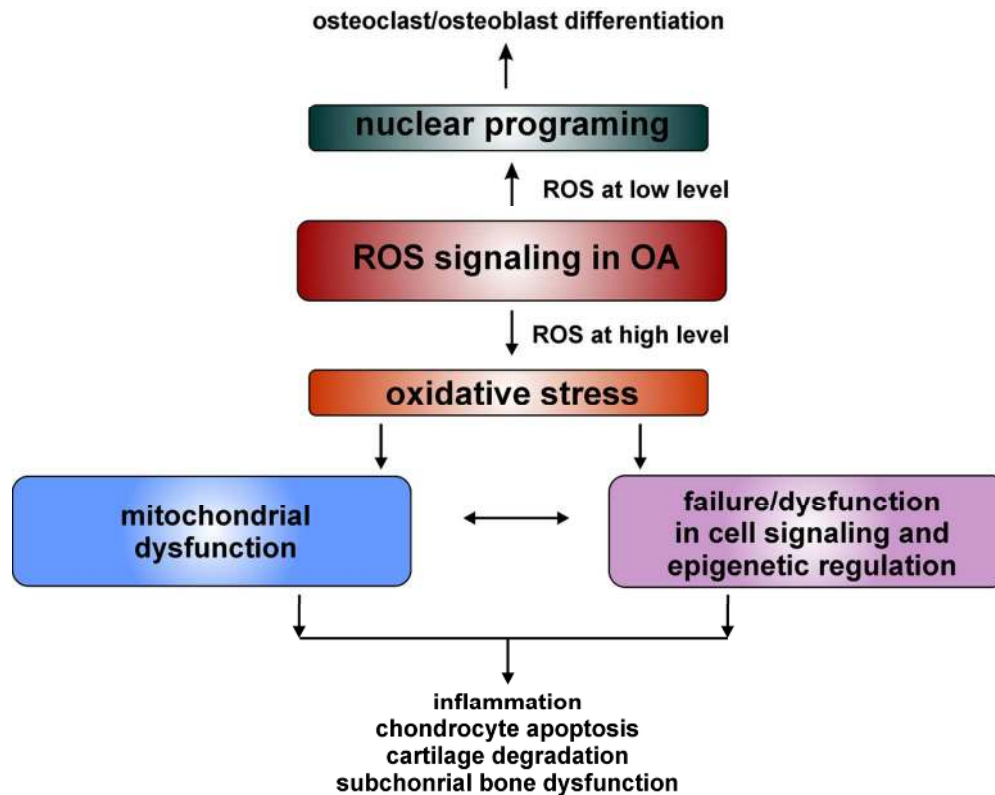
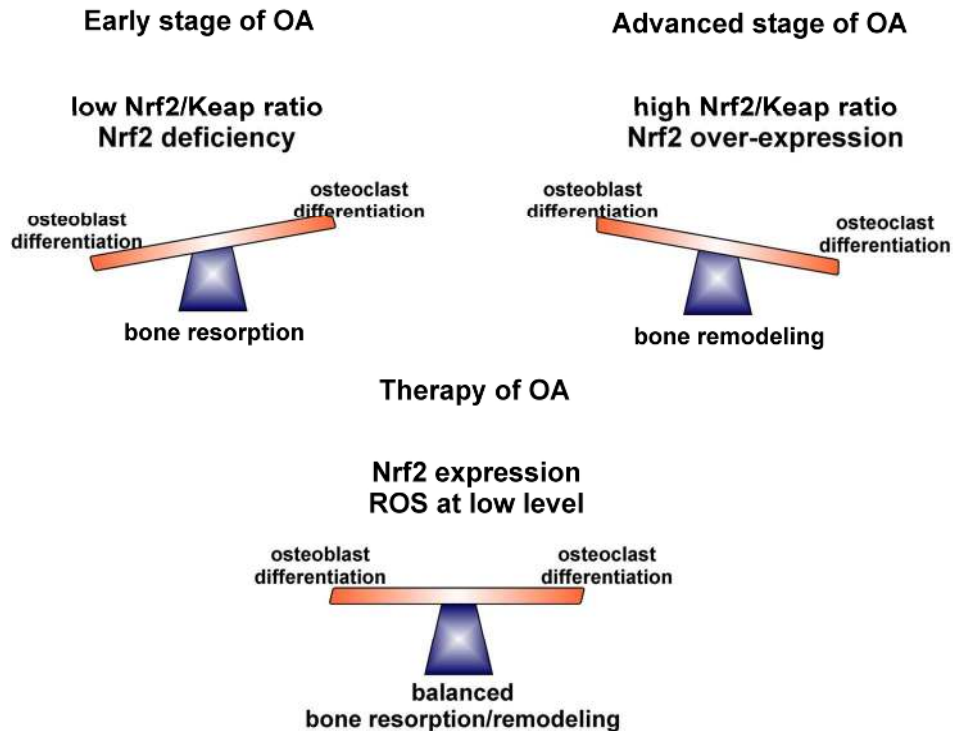


Figure 3. Role of ROS signaling in OA pathology. The low level of ROS can sustain cartilage and bone integrity as ROS regulate nuclear programming during osteoclasts and osteoblasts differentiation. At high level ROS can trigger oxidative stress with mitochondrial dysfunction and a failure in cells signaling and epigenetic regulation of gene expression. Oxidative stress causes inflammation, chondrocyte apoptosis with cartilage degradation and dysfunction in subchondral bone with imbalanced bone resorption and remodeling.

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32 Figure 4. Role of Nrf2 in OA. Bone resorption at early stage of OA is due to a favorable osteoclastogenesis
33 via low Nrf2/Keap ratio (as shown by Nrf2 deficiency). In advanced stage of OA, intensive bone remodeling
34 triggered as a compensatory mechanism to bone resorption is more likely related with enhanced
35 osteoblastogenesis via high Nrf2/Keap ratio (as shown by Nrf2 overexpression). The therapy approaches in
36 OA may involve a normalization of bone/resorption and remodeling via interference with Nrf2 expression and
37 ROS signaling.

38 131x97mm (300 x 300 DPI)