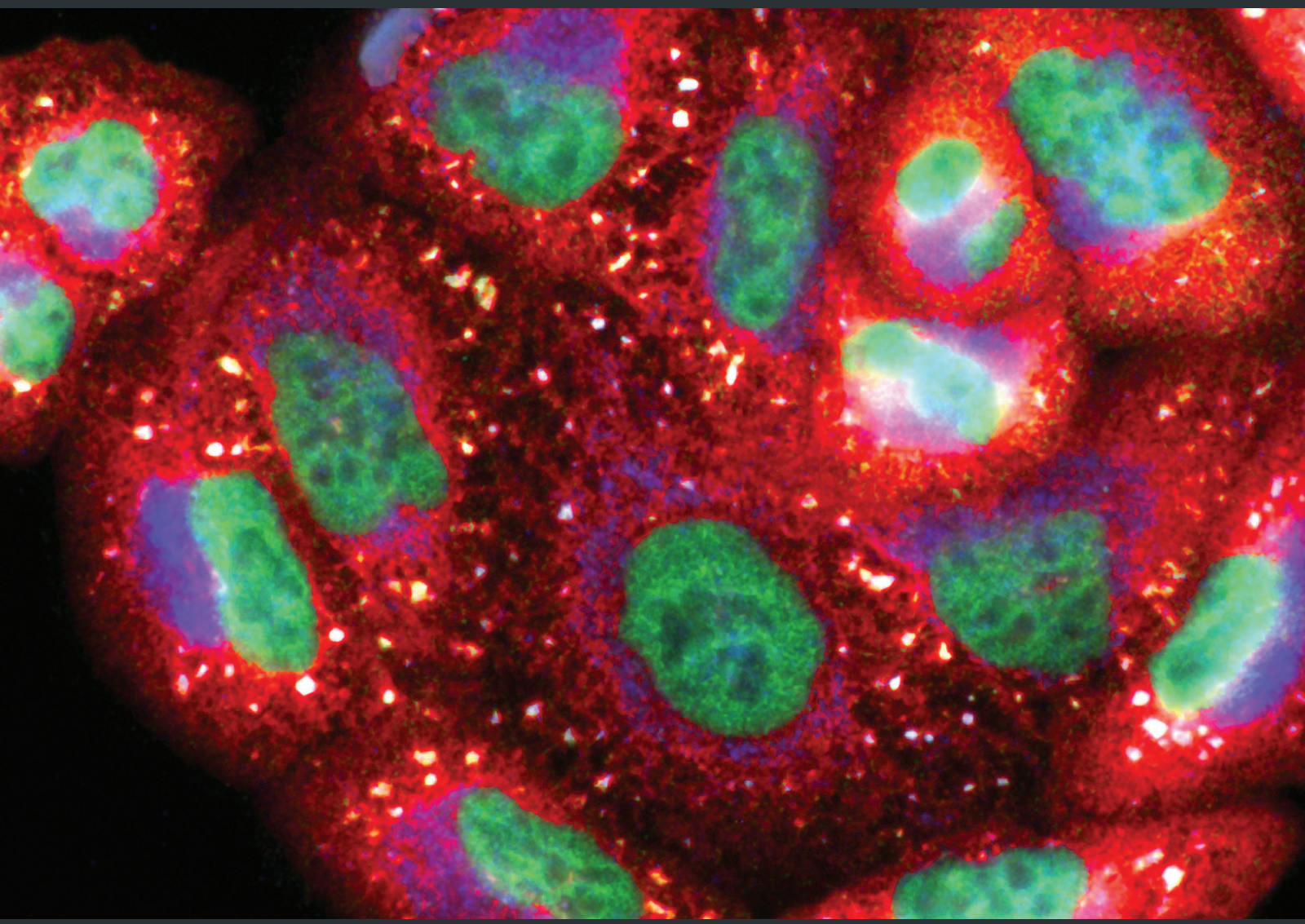
Oxidative Medicine and Cellular Longevity

Redox-Activated Signal Transduction Pathways Mediating Cellular Functions in Inflammation, Differentiation, Degeneration, Transformation, and Death

Guest Editors: Mikko O. Laukkanen, Michael Courtney, and Tetsuro Kamiya



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Editorial Board

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Editorial

Redox-Activated Signal Transduction Pathways Mediating Cellular Functions in Inflammation, Differentiation, Degeneration, Transformation, and Death

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The early atmosphere of the earth contained low concentration of oxygen, which then slowly initiated to increase approximately 2.5 billion years ago after development of photosynthetic organisms and reached life-supporting levels 500–1000 million years ago. According to the oxygenic theory of evolution even the most primitive organisms used oxidative-antioxidative mechanisms in energy production, hence underlining the importance of reactive oxygen species (ROS) in normal cellular functions [1]. In reduction-oxidation (redox) biology the balance of oxidants and antioxidants determines the normal cellular homeostasis that is altered in tissue injuries and pathological conditions. Therefore, ROS, or unbalanced production of ROS, are often a consequence of injury or pathological condition with corresponding impact on cellular signal transduction and damage to macromolecules. The focus of this special issue is to characterize signaling pathways involved in tissue pathogenesis. Such studies potentially result in drug targets that supplement targets observed in currently used screenings [2].

ROS, especially hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$), are important second messengers able to activate or inhibit cellular signaling. It is noteworthy that the cellular response to ROS directly correlates to their concentration: low concentration often supports growth and survival, whereas high concentration is involved in aberrant cellular behavior, such as growth arrest and transformation. Ras oncogene induced proliferative burst of primary cells, consequent cancer barrier forming senescence of the cells, and escape from premature senescence through immortalization and transformation of primary cells is a classical example of ROS involvement in aberrant cellular functions [3]. Epigenetics, a topic discussed in the special issue by T. Kamiya et al., is essentially involved in carcinogenesis. The role of ROS in epigenetic regulation of gene expression through DNA methylation, histone methylation, and histone acetylation has received attention in the research society emphasizing the significant liaison of ROS with carcinogenesis. Part of the epigenetic control in carcinogenesis is derived from tumor stroma cells. Tumor stroma develops in several phases responding to the needs of the epithelial cancer cells. Thus, the continuously growing tumor contains all developmental phases of stroma [4]. Mesenchymal stem cells (MSC) are a rich source of ROS (reviewed in the current issue by R. A. Denu et al.) being able to regulate tumor cells growth and stromal inflammatory cell immunomodulatory properties by, for example, affecting macrophage maturation.

Infections and inflammation are frequently connected to aberrant ROS production. In the current issue D. Limongi and S. Baldelli review the role of infections in neurogenerative diseases. Increased ROS levels with consequent increased inflammatory cytokine expression via activation of Toll like receptor 4 (TLR4) signaling have been suggested to sensitize cells to amyotrophic lateral sclerosis (ALS) development in patients who carry mutations in cupper zinc superoxide

dismutase (SOD1) gene in astrocytes and in microglia. Wild type SOD1 binds small GTPase RAC1 subunit of NADPH oxidase complex in a redox sensitive manner responding to increasing concentration of H_2O_2 by disassociating from RAC1. Mutant SOD1 lacks the redox sensitivity in RAC1 binding, therefore causing continuous accumulation of H_2O_2, which then stimulates TLR4 signal transduction and downstream inflammatory cytokine expression. Although the function of inflammatory cytokines in ALS pathogenesis is not completely understood, both increased ROS and inflammatory cytokine concentrations have been demonstrated to be risk factors in ALS development. Extracellular superoxide dismutase (SOD3) previously characterized in atherosclerotic diseases [5] has been shown to attenuate nonbacterial inflammation, mainly macrophage migration, in ischemic injuries by reducing inflammatory cytokine and adhesion molecule expression [6]. Hence, these data demonstrate that dismutase reaction by SOD1 and SOD3, a catalysis of $O_2^{\bullet-}$ to H_2O_2, has an opposite effect on inflammatory cytokine expression correlating to cellular location of the SOD enzymes.

Although the role of ROS and redox genes is well characterized in several pathological conditions redox balance is a sum of various actions in cellular homeostasis, therefore being challenging to manipulate. Recently a number of studies have pointed out a single transcription factor in redox system, NF-E2-related factor-2 (NRF2), which coordinates oxidant-antioxidant balance in collaboration with Keap1 (reviewed in the current issue by D. I. Kim et al. and A. Sparaneo et al.). Although these factors are attractive drug targets as they are involved in the development of severe pathological conditions, such as malignant cancer cell chemoresistance, the overall development of antioxidative drugs has faced several obstacles. A number of antioxidative molecules tested in cell models in vitro, in preclinical models in vivo, and in clinical trials have demonstrated the diversity of redox balance, even suggesting unexpected negative outcomes. N-Acetyl-L-cystein (NAC), a precursor of glutathione, represents one of the most thoroughly investigated molecules, which has been tested in several clinical trials related to oxidative stress derived diseases, such as chronic inflammation, brain injury, lung diseases, acute liver diseases, cardiovascular diseases, and fibrotic diseases. NAC primarily neutralizes $O_2^{\bullet-}$, although it has been reported to reduce H_2O_2 concentrations and therefore recognized as an antioxidative molecule. Although NAC potentially attenuates the oxidative damage in cardiovascular and liver injuries in short-term treatment, it has less prominent effect on long-term survival of liver injury patients [7, 8]. A more sophisticated approach in the control of redox balance is to focus on signaling routes that mediate the effect of ROS in tissue injuries or cancer, as suggested by P. A. N. Thi et al. in the current issue article, which reports the use of MEK inhibitor (PD98059) to protect brain and cardiac tissue in cardiac arrest remote conditioning model.

Despite impressive resources utilized in characterization of ROS, their function, and manipulation of production in disease progression, we still do not quite understand the connection between ROS production and cellular signal transduction. Further analysis in characterization of ROS regulated signaling routes could reveal critical molecules needed for ROS production, such as small GTPase RAC1 that is involved in NADPH oxidase function and in SOD3 signaling [9, 10].

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Review Article

Nrf2 and Notch Signaling in Lung Cancer: Near the Crossroad

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The transcription factor Nrf2 (NF-E2 related factor 2) is a master regulator of the cell antioxidant response associated with tumor growth and resistance to cytotoxic treatments. In particular, Nrf2 induces upregulation of cytoprotective genes by interacting with the closely situated AREs (Antioxidant Response Elements) in response to endogenous or exogenous stress stimuli and takes part to several oncogenic signaling pathways. Among these, the crosstalk with Notch pathway has been shown to enhance cytoprotection and maintenance of cellular homeostasis, tissue organization by modulating cell proliferation kinetics, and stem cell self-renewal in several organs. The role of Notch and Nrf2 related pathways in tumorigenesis is highly variable and when they are both abnormally activated they can synergistically cause neoplastic proliferation by promoting cell survival, differentiation, invasion, and metastases. *NFE2L2*, *KEAP1*, and *NOTCH* genes family appear in the list of significantly mutated genes in tumors in both combined and individual sets, supporting the crucial role that the aberrant Nrf2-Notch crosstalk might have in cancerogenesis. In this review, we summarize current knowledge about the alterations of Nrf2 and Notch pathways and their reciprocal transcriptional regulation throughout tumorigenesis and progression of lung tumors, supporting the potentiality of putative biomarkers and therapeutic targets.

1. Introduction

Notch receptors (Notch1–Notch4) are a family of transmembrane proteins which interact with ligands of the Delta and/or Jagged/Serrate family. These receptors play a key role in the normal development of tissues and cell types, through diverse effects on differentiation, survival, and proliferation [1–3]. In tumors, Notch signaling has been observed to exert either oncogenic or antiproliferative effects within the mechanisms of cell invasion and metastases development.

The Nrf2 is a key regulator of the cell adaptive response to reactive oxygen species (ROS) and xenobiotics through the interaction with its master negative regulator, the Keap1 protein. Currently, the dark side of Nrf2 has emerged and growing evidences suggest that constitutive upregulation of Nrf2 is linked to cancer development and progression and contributes to chemo- and radioresistance.

Notch and Nrf2 are both transcription factors and their related pathways were discovered independently [4]. However, recent data have demonstrated the existence of a Nrf2-Notch crosstalk which supports cytoprotection and improves maintenance of cellular homeostasis and tissue organization. This review will mainly focus on the available scientific data which underlie the biological relevance of Nrf2 and Notch pathways and their crosstalk in lung tumors and suggest the potentiality of molecularly targeted agent combinations to overcome resistance to therapies.

2. Notch Signaling

NOTCH genes encode for highly conserved cell membrane receptors from *Drosophila* to humans that orchestrate a complex signaling pathway involving a number of ligands, negative and positive modifiers, and transcription factors [5]. In mammals, four Notch receptors (Notch1 to Notch4) and two families of Notch ligands (Jagged1 and Jagged2 and Delta-like-1, Delta-like-3, and Delta-like-4) have been described to play a critical role in the cell-contact-dependent cellular communications [2, 3, 6].

Although the overall structure of Notch receptors is similar, there are significant differences in the protein domains. The Notch1–4 receptors share an extracellular portion which

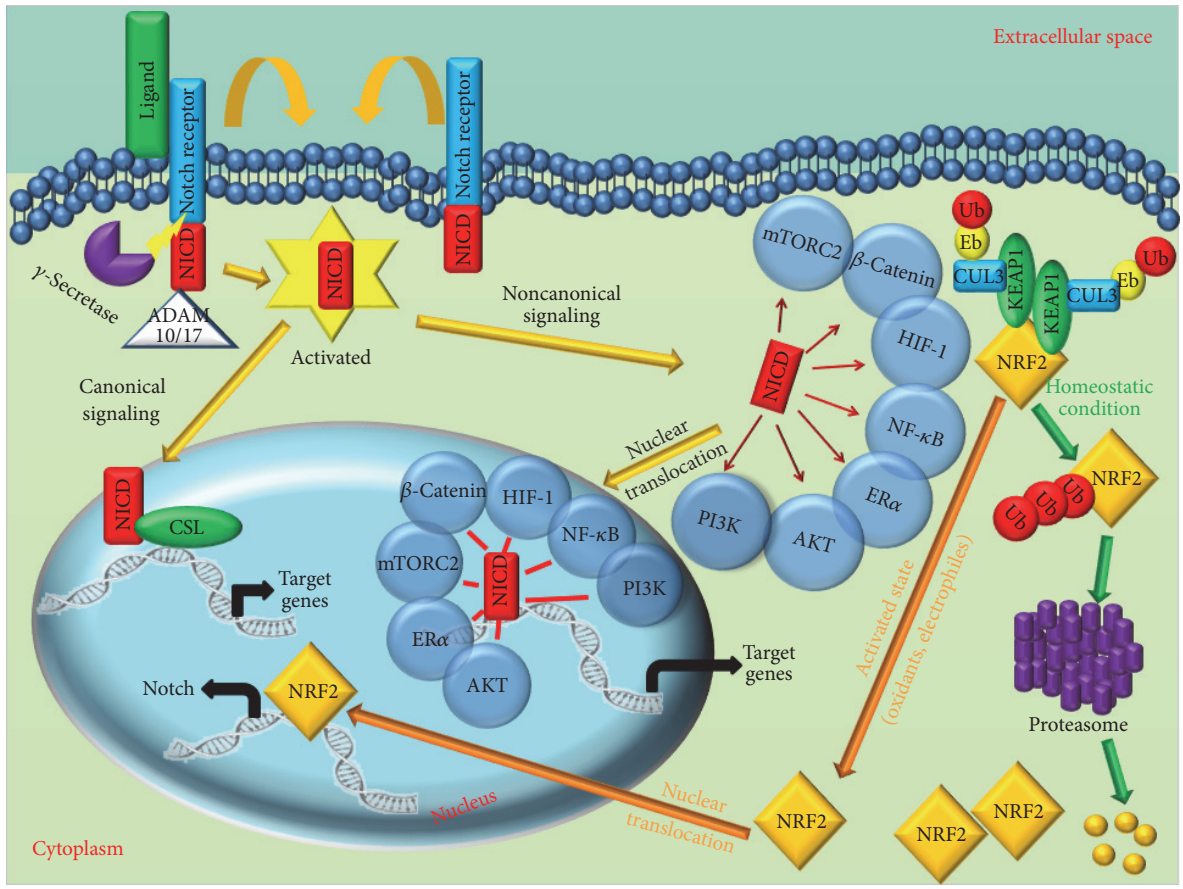

Figure 1: Canonical and noncanonical Notch signaling pathways. Notch signaling has a pleiotropic effect and is involved in cell survival, cell proliferation, cell metabolism, and differentiation. Canonical Notch pathway is primed by interaction of the Notch protein with a cell bound ligand. Upon interaction, Notch results cleaved, firstly by ADAM 10/17 protease and then by cleavage by the γ- secretase. Furthermore, Notch activated (NICD) translocates into nucleus and interacts with CSL protein, where, upon interaction, the proteins complex is converted into a transcriptional activator of targets genes. Noncanonical Notch pathways may be activated either dependently or independently of ligand interaction and may be γ-secretase dependent or independent. Noncanonical Notch signaling interacts with mTORC2, AKT, Wnt, HIF-1α, NFκB, and PI3K pathways at either the cytoplasmic or nuclear levels. The gene regulatory region of the major Notch1 transcript has been described to possess a functional ARE through which Nrf2 can regulate Notch1 gene expression. In the activated state (orange arrow, transient upon stress stimuli or constitutive due to mutations in tumor cells), *de novo* synthesized Nrf2 protein accumulates into the nucleus, where it activates the transcription of several ARE-genes, including *NOTCH1*. In the basal state (green arrow), Keap1 binds Nrf2 and induces its ubiquitination. Upon ubiquitination, Nrf2 is degraded by proteasome complex.

contains a variable number of epidermal growth factor- (EGF-) like repeats: the Notch1 and Notch2 receptors contain 36 EGF repeats, whereas Notch3 contains 34 repeats and Notch4 contains 29 repeats. The other difference is in the transactivation domain (TAD). Notch1 and Notch2 contain a strong and a weak TAD, respectively, Notch3 has a potent but specific TAD best suited to the activation of the HES-5 promoter. In contrast, Notch4 does not contain a TAD. These structural differences may offer clues to the functional divergence among mammalian Notch paralogs [7].

The EGF-like repeats of extracellular portion of Notch are essential for ligand binding. The bond between ligands and extracellular Notch domains activates the intracellular portion and promotes intracellular sequential proteolytic cleavages by a metalloproteases of ADAM's family. Then the Notch intracellular domain (NICD) is released from the cytoplasmic membrane and translocates as active form into the nucleus, where it enhances the expression of several target genes encoding for Hairy Enhancer of Split (HES) family proteins, HES-related proteins (HEY), and p21cip1/waf1, cyclin D1 and 3, c-myc, and Her2, in a cell-context-dependent manner [3, 8, 9].

Beside this canonical pathway activation, additional non-canonical Notch signaling pathways have been described. These additional pathways are independent from CSL (CBF1, Suppressor of Hairless, and Lag-1) transcription factor and related to other different transcription factors, such as beta-catenin, HIF-1a (hypoxia-inducible factor-1a), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), and estrogen receptor ERα (Figure 1) [10–13].

The Notch transcriptional machinery and signaling pathway are conserved among species, but in mammals this

system shows the peculiarity to induce several, even opposite, biological effects depending on specific tissue types [4, 14]. Notch signaling networks can regulate a wide range of events in embryonic and postnatal development, including proliferation, apoptosis, border formation, and cell fate decisions. Aberrant expression of Notch receptors and Notch target genes have been reported in different human malignancies, including lung, skin, pancreas, breast, and colon cancers [15–20]. In lung tumors, depending on the subtype or specific molecular profiles, Notch family activity is often deregulated and activates several oncogenic pathways via direct or indirect induction [21, 22].

In a transgenic mouse model, Notch1 was overexpressed in the alveolar epithelium and induced alveolar hyperplasia and pulmonary adenomas through regulating type II lung epithelial cells. Moreover, the concomitant expression of MYC led to a progression to adenocarcinoma and metastases, indicating a synergistic effect between Notch1 and other oncogenes [23]. It has also been reported that Notch1 signaling plays a central role in the negative modulation of cell growth in lung adenocarcinoma through the ADAM metalloproteases and promotes apoptosis escape through a negative modulation of the p53 stability at protein level. These findings might explain the correlation between Notch1 activation and poor prognosis in NSCLC patients without *TP53* mutations [24–28]. Few data have been provided so far concerning the roles of Notch1 in lung adenocarcinoma harboring mutations in other lung cancer driver genes, such as *PIK3CA* or *EGFR* (Epidermal Growth Factor Receptor). In NSCLC cell lines, it has been preliminary observed that the expression of the active form of Notch1 (NICD1) leads to increased proliferation activity, malignant transformation, and tumor growth in presence of EGF (Epidermal Growth Factor), suggesting that EGFR activation may be essential for Notch-mediated malignant transformation and tumor growth [25].

Notch1 signaling has been shown to also act either as a negative or as a positive regulator of Phosphatase and Tensin Homologue gene (*PTEN*) transcription [29]. *PTEN* downregulation is modulated by Notch1 through the activation of the transcription factor hair and enhancer of SPLIT (HES1), whereas *PTEN* upregulation derives from the inhibition of the binding protein suppressor of hairless (RBPJ), also known as CBF-1 [30–32]. In NSCLC and malignant mesothelioma cells, the activation of *PTEN* transcription by Notch1 upregulation has been observed to lead the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway [29–32].

In contrast with the role of Notch1 in promoting tumor initiation and progression, Notch2 shows a tumor suppressive activity by mediating cell differentiation in lung carcinogenesis. This evidence was also supported by immunohistochemical analysis of human NSCLC samples showing the loss or downregulation of Notch2 compared with normal lung tissues [33, 34]. In malignant mesothelioma (MM) cells Notch2 also appears to be diminished with a consequent decrease of toxic effects and a general benefit for cells [35]. Finally, the effect of Notch3 in lung carcinoma has been observed to be strongly dependent on cell type being a tumor suppressor in Small Cell Lung Cancer (SCLC) and a tumor promoting in NSCLC by differentially modulating cell adhesion, Epithelial Mesenchymal Transition (EMT), and cell motility [36].

Various studies have assessed the clinicopathological and prognostic value of Notch1 and Notch3 expression in NSCLC, but the results remain controversial. NSCLC tissues have significantly higher Notch1 protein levels compared to lung normal tissues, with strong variations in different studies and even within the same histotypes. Overall, Notch signaling can be suggested as a valuable biomarker to predict tumor progression in NSCLC. Overexpression of Notch1 and Notch3 has been associated with increased risk of lymph node metastasis and advanced TNM (tumor size, lymph nodes, and metastases) stages. Notch1 also represents an independent prognostic factor in surgically resected adenocarcinoma patients with a major impact in combination with VEGF-A (Vascular Epidermal Growth Factor-Alpha) upregulation [37, 38]. Future investigations might clarify the usefulness of targeting Notch signaling in specific subpopulation of NSCLC patients [39].

A key role of Notch signaling has been recently highlighted in the context of SCLC growth and resistance to therapy. Stable expression of the active form of Nocth1 in SCLC cells inhibits cell proliferation and decreases the expression of several neuroendocrine markers [40]. Moreover, alteration of Notch-Ascl1-Rb-p53 axis has been recently described as major driver of secondary transition from NSCLC to neuroendocrine phenotype and SCLC [41]. These findings provide a novel cellular mechanism for lung histology transition [42] and suggest Notch signaling reactivation as a possible therapeutic strategy for SCLC patients [43].

Finally, emerging evidences suggest that Notch signaling participates to the process of EMT, a highly coordinated process observed when epithelial cells lose some or most epithelial characteristics and acquire properties that are typical of mesenchymal cells. The transition of epithelial cells to mesenchymal cells is essential during embryogenesis and includes phenotypic changes such as loss of cell-cell adhesion, loss of cell polarity, and the acquisition of migratory and invasive properties. Accumulating evidences suggest that aberrant activation of the EMT developmental program contributes to tumor initiation, invasion, metastasis, and acquisition of therapeutic resistance [44, 45]. Notch, Wnt, Hedgehog (Hh), and TGF-b pathways induce well-differentiated epithelial cells to convert into motile mesenchymal cells in tumors via the activation of multiple EMT transcription factors, including Twist, Snail, Slug, and ZEB [46] and their deregulation correlates with poor clinical outcomes [47]. These findings corroborate the hypothesis that Notch1 and Notch3 may represent typical markers of stem-like cells indifferent solid tumors, including lung cancer [48].

3. Mechanisms of Notch Deregulation in Cancer

Notch receptors have been found deregulated in many tumors, and the prevalence and location of mutations within

each Notch receptor coding gene varied considerably according to the tumor type [49]. Many identified mutations are heterozygous and correlate with a haploinsufficiency in tissue pattering and suggest that loss of a single copy functionally impairs signaling and therefore induces tumorigenesis. In general, *NOTCH1* gene mutations are more frequently recognized than in the other *NOTCH* receptor genes. This was in part, but not entirely, due to the greater number of tumors with Notch1 sequencing data. For head and neck cancer (HNSCC) and lung and breast cancers, *NOTCH1* mutations were relatively recurrent (5–15%) and clustered at or near identified important domains.

In lung cancer, the deregulation of the Notch pathway is mainly correlated with activating missense mutations mostly affecting the ligand-binding domain (EGF repeats 11 and 12) or the ankyrin domains which lead to a ligand-independent activation [50]. *NOTCH1* activating mutations have been defined as a common event in human NSCLC [51] and have been correlated to poor prognosis and response to therapy in lung patients without p53 mutations [52]. To note, *NOTCH1* mutations in SqCC appeared to be more frequent than pulmonary adenocarcinoma, and their typical location in close proximity of the ligand-binding domain leads to the speculation that Notch1 is more likely to function as a tumor suppressor in SqCC than in the adenocarcinoma histology [49]. However, the real frequency of *NOTCH1* mutations in NSCLC remains to be determined. The limited size and intrinsic variations of the just reported studied cohorts, along with the differences in sequencing strategy, do not allow a definitive conclusion on the magnitude of this event [53].

By contrast, mutations affecting the *NOTCH* family genes have been widely assessed and described as one of the most mutated pathways driving neuroendocrine features and SCLC. Different missense changes affecting all *NOTCH1–NOTCH4* genes with different frequency in relation to the different histologies of lung neuroendocrine tumors have been reported [54]. In SCLC, frequent damaging mutations have been identified in the extracellular domain with an incidence of about 25%, suggesting that Notch may act a tumor suppressor [55], leading to growth inhibition and neuroendocrine markers reduction [56].

Mutations in *NOTCH1–NOTCH4* family genes (28%) have also been recently reported in Large Cell Neuroendocrine Cancer (LCNEC) by genomic analysis. Many mutations were located in the extracellular EGF-like domain and were mainly associated with NSCLC-like subgroup but differ from the typical mutation pattern of lung adenocarcinoma. This represents an additional, strong evidence of the crucial role of Notch in lung neuroendocrine development [57].

In addition to direct mutations of *NOTCH* genes, alternative mechanisms of Notch deregulation have been reported in lung cancers. Molecular profiling of alternative splicing variants in lung adenocarcinoma have revealed frequent alternative splicing events affecting the *NUMB* gene, similar to primary breast and colon cancers. These abnormal isoforms lack normal activity and aberrantly induce the reduction of Numb protein expression levels and activation of the Notch signaling pathway there by promoting cell proliferation [58].

Finally, recent evidences indicate that there is a significant crosstalk between Notch and microRNAs. As a key component of the Notch-mediated transcription complex, Notch can regulate expression of a number of microRNAs; at the same time, Notch ligands, Notch receptors, or Notch effectors are regulated by microRNAs [59]. Indeed, members of five different families of miRNAs (miR-2, miR-4, miR-7, miR-11, and miR-79) have been shown to negatively regulate Notch target genes by recognizing conserved binding motifs within their transcripts [60]. However, few evidences about the role of this epigenetic mechanism of expression regulation have been provided in lung cancer. Pharmacological induction of miR-34a decreased the expression of Notch1 and its downstream targets including HES-1, Cyclin D1, Survivin, and Bcl-2, impairing Notch signaling, cell proliferation, and invasion and inducing apoptosis in NSCLC cells [61].

4. Nrf2 Signaling

Nrf2 is a basic region-leucine zipper (bZIP) transcription factor that acts as a master modulator of cellular protection against carcinogens and oxidative damage in organisms. Although diverse mechanisms might be involved, it is speculated that the induction of phase II cytoprotective enzymes by Nrf2 chemical inducers occurs, at least in part, by modulating the activities of intracellular signaling kinases [62]. In the cellular basal state, the majority of *de novo* synthesized Nrf2 is repressed by physical interaction with Keap1, which is an adaptor protein to Cullin 3- (Cul3-) dependent ubiquitination and proteasomal degradation [63–67]. When cells are exposed to exogenous and endogenous toxic substances and to oxidative damage, a specific pattern of Keap1 cysteine modification arises [66]. By consequence, the Keap1 releases Nrf2 which translocates into the nucleus where it forms a heterodimer with small Maf proteins. This complex specifically recognizes enhancer sequences known as Antioxidant Response Elements (AREs), located in the regulatory regions of genes encoding for cellular defense enzymes, and activates their expression through the transcription machinery [68, 69]. Several Nrf2 target genes have been identified so far, and the number has increased through the recent technical advances [70]. Apart from the major cytoprotective functions of Nrf2 targeted genes, many of these genes also play in the context of oncogenesis, cell proliferation, apoptosis, and tumor cell growth in many cancer types (Figure 2). Recently, the involvement of Nrf2 has also been recognized in mitochondrial physiology as inductor of respiration substrates, membrane potential maintenance, integrity, and biogenesis [70–74].

Scientific findings in several neoplastic backgrounds underlined how the Nrf2 activity is clearly connected with oncogenic kinase pathways, structural proteins, hormonal regulation, other transcription factors, and epigenetic enzymes involved in the pathogenesis of tumors [75].

The large-scale genomic studies of NSCLC by The Cancer Genome Atlas (TCGA) consortium and others have supported that Nrf2 deregulation represents one of the major cancer driver pathways in the specific histotypes of SqCC

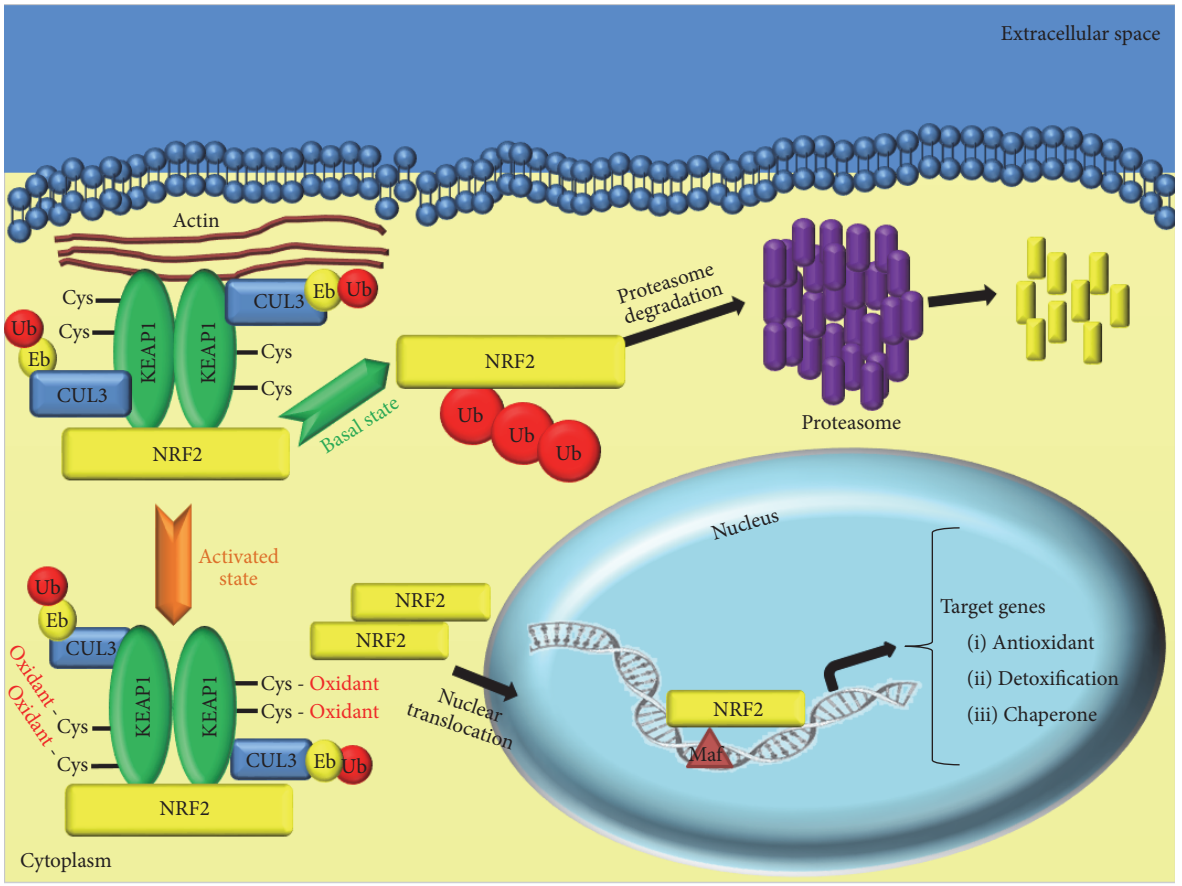

Figure 2: Keap1/Nrf2 axis in lung cancer. Under basal conditions (green arrow), Nrf2 is sequestered in the cytoplasm by the Keap1-Cul3 complex and rapidly degraded in the ubiquitin-proteasome dependent manner. This Keap1-mediated degradation activity requires two reactive cysteine residues of Keap1, located into the IVR domain. Upon stress stimuli (orange arrow), modification of these cysteine residues of Keap1 inhibits ubiquitin conjugation to Nrf2 by the Keap1-Cul3 complex, thereby provoking Nrf2-Keap1 impairment and resulting in the nuclear accumulation of *de novo* synthesized Nrf2 protein and enhancement of target genes transcription.

where cigarette exposure can activate the oxidant stress response [76] and LCNEC of the lung with Non-Small-Cell Carcinoma features [77, 78]. Several mechanistic studies proved opposite roles of Nrf2 during carcinogenesis, either protective or promoting malignant progression [79]. The latter is supported by many clinical observations showing that constitutive upregulation is strongly associated with cancer development, progression, and resistance to conventional chemotherapy and radiotherapy in NSCLC [79–82]. Measuring nuclear Nrf2 abundance in NSCLC patients might be a useful index to predict the efficacy of platinum-based treatments. Nuclear accumulation of Nrf2 correlated with worse NSCLC cancer-specific survival and worse progress-free survival in three independent datasets of SqCC patients treated with surgery only [83–85]. As the main negative regulator of Nrf2, Keap1 activity and impairment also correlated with NSCLC survival. Our group discovered that NSCLC patients harboring *KEAP1* alterations had worse progression-free survival compared with other patients [86]. Similarly, Takahashi et al. found that *KEAP1* mutations caused an increase of Nrf2 expression in NSCLC patients and were correlated with worse progression-free and overall survival [87].

Along with *KEAP1* mutations, the expression levels of two Nrf2 downstream transcripts expressions, Ho-1 [88, 89] and Nqo1 [90–92], were found significantly associated with tumor invasiveness and patients survival in NSCLC advanced stage. In this regard, a recent extensive meta-analysis of microarray data for 240 Nrf2-mediated genes expression signature identify a group of 50 genes (NFE2L2-associated molecular signature, NAMS) that predicts a worse clinical outcome in 60% of NSCLC cohorts analyzed. These data corroborate the idea that NAMS could represent a promising prognostic biomarker in human lung cancer [93]. Correlation of Nrf2 downstream transcripts expression with tumor invasiveness and patients survival in NSCLC advanced stage has been also reported for Ho-1 [88, 89] and Nqo1 [90–92].

Three major crosstalks between Nrf2 and other classical oncogenic signaling pathways such as phosphatidylinositol 3-kinase (PI3K) [94], Kirsten retrovirus-associated DNA sequence (K-ras), [95] and Notch [4] have been reported in lung cancer as having a strong impact on tumor resistance

outcome. The PI3K-Akt-mTOR pathway is commonly deregulated in several human malignancies including NSCLC [96], and activated PI3K signal increased accumulation of Nrf2 into the nucleus to enhance the transcription of enzymes involved in the pentose phosphate pathway [72]. Since radiotherapy agents can effectively induce apoptosis through generation of ROS [97] it was observed that specific PI3K inhibitor such as NVP-BKM120 can be used in SqCC to decrease Nrf2 protein levels and sensitize *NFE2L2* or *KEAP1*-mutant cells to radiation [94]. *KRAS* gene mutations occur approximately in 20–30% of NSCLCs and confer to cancer cells resistance and survival [98, 99]. Promoter analysis showed that a TPA response element (TRE) located in exon1 of *NFE2L2* gene was activated by Kras. Thus, oncogenic Kras confers in NSCLC chemoresistance by upregulating Nrf2, enhancing the antitumor efficacy of cisplatin and providing a strong preclinical rationale to improve the management of lung tumors harboring *KRAS* mutations with Nrf2 pathway inhibitors [79, 95, 100].

5. Mechanisms of Nrf2 Deregulation in Cancer

Firstly described in NSCLC cell lines and tissues by Singh et al. in 2006, molecular impairment of Keap1/Nrf2 axis has been then extensively investigated in lung with different mutation clusters found to be related to specific histological subtypes. The overexpression of nuclear Nrf2 and the subsequent increase in the antioxidant defense in lung cancer cells are mainly related to genetic and epigenetic alterations of the *KEAP1* and *NFE2L2* genes [101]. Somatic mutations of the *KEAP1* gene frequently affect the DC domain and produce a decrease in Keap1-promoted Nrf2 ubiquitination by Cul3 or the impairment of nuclear export of Nrf2 by Keap1/Cul3 complexes. In both cases, under cellular stress condition, Nrf2 escapes degradation and translocates into the nucleus to induce the expression of its target genes [102–104]. Mutations in *NFE2L2* gene were also widely described in lung tumors, suggesting a strong link between molecular perturbations of the Nrf2 pathway and tissue exposure to ROS [105]. *NFE2L2* mutations should determine a constitutive activation and have been found to mainly cluster within the DLG and ETGE motifs, which are hotspot sites for Nrf2 binding to the Keap1 DC binding domain. In particular, the ETGE mutant proteins are not ubiquitinated and concentrate in the nucleus, whereas mutations in the DLG resulting in the stabilization of Nrf2 increased its nuclear translocation and Nrf2 *de novo* molecules synthesis [106, 107].

Mutations and copy number alterations of *NFE2L2* and *KEAP1* and/or deletion or mutation of *CUL3* were observed in 25–34% of SqCC among the classical alterations associated with this smoking-related histology subtype of lung cancer [77, 84]. Instead, a low incidence of *KEAP1* mutations has been reported in advanced stage ADC patients with different ethnicity (3–19%) and a lower incidence of *EGFR* mutations [108, 109], except for papillary adenocarcinoma tumors subtypes (60%) [110]. In addition, TCGA analysis of lung adenocarcinomas has shown that the odds of a tumor carrying a *KEAP1* mutation increased more than sixfold among tumors with *LKB1* loss. *LKB1*-deficient tumors are susceptible to oxidative stress because they are unable to produce the appropriate adaptive responses in metabolism and biosynthesis. The high level of overlap in loss of function of *KEAP1* and *LKB1* genes may suggest that selective pressure exists for the activation of Nrf2 as a secondary protective mechanism to compensate for *LKB1* loss [111].

More recently, new experimental evidences have demonstrated a mutual regulation between Nrf2 and microRNAs, especially in the mechanisms of tumor chemoresistance. Indeed, several miRNAs have been validated to target Nrf2 and thus affect its signaling pathway, although only few data have been collected in lung tumor [112–115]. On the other hand, Nrf2 has been demonstrated to regulate the expression of different miRNAs. For instance, functional studies in human lung fibroblasts reported as Nrf2/miR-140 signaling confers radioprotection by inducing Nrf2 nuclear translocation and subsequent activation of miR-140 transcription [116]. Moreover, miR-200a reactivation by histone deacetylation has been reported to destabilize Keap1 transcript in resistant lung tumor cell lines [117], whereas Nrf2-dependent regulation of miR-1 and miR-206 has been described to crucially promote non-small-cell lung proliferation and tumorigenesis by modulating the pentose phosphate pathway [118].

Lately, *KEAP1* alterations have emerged as an important molecular feature of neuroendocrine tumors of the lung. By performing genome/exome and transcriptome sequencing Fernandez-Cuesta et al. have demonstrated that it is possible to distinguish an LCNEC SCLC-like group, carrying *MYCL1* amplifications and mutations in both *RB1* and *TP53* genes from an AD/SQ-like group, harboring *CDKN2A* deletions, *TTF1* amplifications, and frequent mutations in *KEAP1* and *STK11*. This represents a picture of an evolutionary trunk that can branch to SCLC or AD/SQ on the basis of a different genetic background [112]. These data have been confirmed by Rekhtman et al., who reported an incidence of 31% of *KEAP1* mutations in LCNEC NSCLC-like subset [78].

In addition to somatic mutations, other mechanisms affecting Nrf2 expression in lung tumors have been found, even though this field still remains mostly unexplored. For instance, there are compelling evidences that epigenetic regulation might play a key role in modulating Keap1/Nrf2 axis in lung cancer cells [119]. Hypermethylation of the *KEAP1* promoter region was firstly described by Wang et al. as a pivotal mechanism in the modulation of the KEAP1 mRNA expression in cell lines and primary lung tumors that could be restored by 5-Aza treatment [120]. A larger study from our group on a cohort of resected primary NSCLCs confirmed these results and further proposed the epigenetic inactivation of *KEAP1* by promoter hypermethylation as the main mechanism which leads to reduced or absent Keap1 protein expression previously reported in NSCLC. Genetic and epigenetic analyses on this cohort suggest *KEAP1* biallelic inactivation as molecular marker of worst prognosis [86]. It has been recently demonstrated by *in vitro* analysis that the methylation status of *KEAP1* can also predict the tumor cells sensitivity to radiation. Importantly, when radiation is combined with the angiogenesis inhibitor Genestein, there is an increase of ROS levels and cell apoptosis via overexpression

of Nrf2, GSS, and Ho-1 in lung adenocarcinoma cells [121]. A possible role of histone deacetylation/acetylation in the epigenetic regulation of the Keap1/Nrf2 pathway has been reported in human NSCLC, where hMOF-mediated acetylation of Nrf2 increased its nuclear retention and the transcription of its downstream genes, subsequently modulating tumor growth and drug resistance [122]. This new role of histone modification in the modulation of Nrf2 has been supported by Li et al., who showed that decreased Ezh2 expression significantly correlated with elevated expression of Nrf2 and its target genes, both in lung cancer tissues and in cell lines [113].

More recently, among the epigenetic mechanisms, new experimental evidences have demonstrated that miRNAs may crucially modulate the Nrf2 expression and affect its signaling pathway in a chemoresistance context [114–116, 119]. Nevertheless, most of the data have been reported in epithelial tumors such as breast and colon, whereas only few have been provided in lung cancer. On the other hand, functional studies on human lung fibroblasts reported as Nrf2/miR-140 signaling confer radioprotection by inducing Nrf2 nuclear translocation and subsequent activation of miR-140 transcription [117]. MiR-200a reactivation by histone deacetylation has been reported to destabilize Keap1 transcript in resistant lung tumor cell lines [118], whereas Nrf2-dependent regulation of miR-1 and miR-206 has described as crucial in non-small-cell lung proliferation and tumorigenesis through the modulation of the pentose phosphate pathway [123].

6. Nrf2-Notch Pathways Crosstalk in Lung Cancer

Nrf2, Keap1, and Notch1 rank among the first frequently mutated genes in tumors and were deemed to be significant both in the combined sets of tumors and in individual tumor types. This observation leads to the speculative general notion that the outcome from aberrant Nrf2-Notch crosstalk by molecular impairment in these genes might enhance tumorigenesis and progression to cancer [4], especially in the stem cell (SC) context.

A number of experimental models have been employed to demonstrate that Nrf2 is involved in the maintenance of the stem cell phenotype. ROS have more recently been found to have useful roles in SC proliferation and differentiation [129]. However, the functional significance of the ROS status in different types of SCs, the downstream signaling events, and the role of ROS in SC self-renewal for repair and homeostasis is controversial [130–132]. In *Drosophila* intestinal stem cells, loss of the CncCbZIP-CNC (cap-n-collar subfamily of basic leucine zipper) transcription factor has been reported to increase ROS levels and cell proliferation rates, suggesting that CncC is required to keep the intestinal stem cells in a state of quiescence and to prevent them from entering the cell cycle [130]. In mouse hematopoietic stem cells, loss of Nrf2 has been shown to lead to an expansion of the progenitor pool of myeloid and lymphoid lineages, again suggesting that Nrf2 supports stem cell renewal and proliferative quiescence [133]. A reciprocal Nrf2-Notch transcriptional regulation has been described in hepatobiliary system, having a key role in liver development and in maintenance of hepatic function and its deregulation might be one of the main pathways for promoting cancer [134–136].

Less evidences have been provided for a clear Notch-Nrf2 crosstalk in lung cancer. The airway epithelium is constantly exposed to environmental oxidants and therefore serves as an interesting model system to study redox signaling. Cigarette smoke is known to cause oxidative stress-induced airway injury [137], diseases, and cancer airway-related through well-known mechanisms [138].

Additionally, Nrf2 has been implicated in the self-renewal of human airway basal stem cells, but in this case the flux of ROS levels appeared to be the critical factor. In the same context, Notch1 signaling pathway was implicated in helping dynamic changes in ROS levels [138] and has been noted to be essential in early lung development and in the regulation of stem cell self-renewal; thus, when abnormally activated these pathways can cause neoplastic proliferation, representing an early event in tumorigenesis [139]. Beside this, an inverse modulation of Notch by Nrf2 was observed. The gene regulatory region of the major Notch1 transcript has been described to possess a functional ARE region through which Nrf2 can directly regulate Notch1 gene expression, thus promoting airway basal stem cells' self-renewal [138, 140]. Finally, recent data have shown that Nrf2 strongly regulates Notch1 activity and promotes radiation-induced apoptosis through Nrf2 mediated Notch1 signaling in NSCLC cells. Thus, Notch signaling is an important determinant in radioresistance of lung cancer cells [141] (Figure 3).

An indirect suggestion of a functional interdependence in the Notch and Nrf2 pathways comes from recently published studies of genomic analysis in LCNECs. Genes mutation profiling revealed a high incidence of *NOTCH* genes family (33%) and *KEAP1-NFE2L2* (39%) alterations in specific subsets of LCNECs. In particular, *NOTCH* genes family alterations represent one of the most relevant differences in NSCLC-like LCNEC from classic lung adenocarcinoma and are of particular interest because they give a strong evidence for their crucial role of Notch receptors in neuroendocrine fate specifications in normal and tumor development [55, 57]. However, despite the overall similarity, the most relevant differences identified in SCLC-like LCNEC were an elevated rate of *KEAP1-NFE2L2* mutations that rarely occur in conventional SCLC but are frequent in SqCC, suggesting a stronger histogenetic relationship of some conventional SCLCs and SqCCs [78]. Finally, frequent cooccurring mutations *NFE2L2*, *KEAP1*, and *NOTCH1* in a study on more than four thousand human cancers support the notion that the outcome from aberrant Nrf2–Notch crosstalk by mutations in these genes might specifically enhance tumorigenesis and progression to cancer [142].

7. Therapeutic Targeting of Notch and Nrf2 Pathways

The central role of Notch signaling in cancer, cancer stem cell maintenance, and angiogenesis has significantly fostered

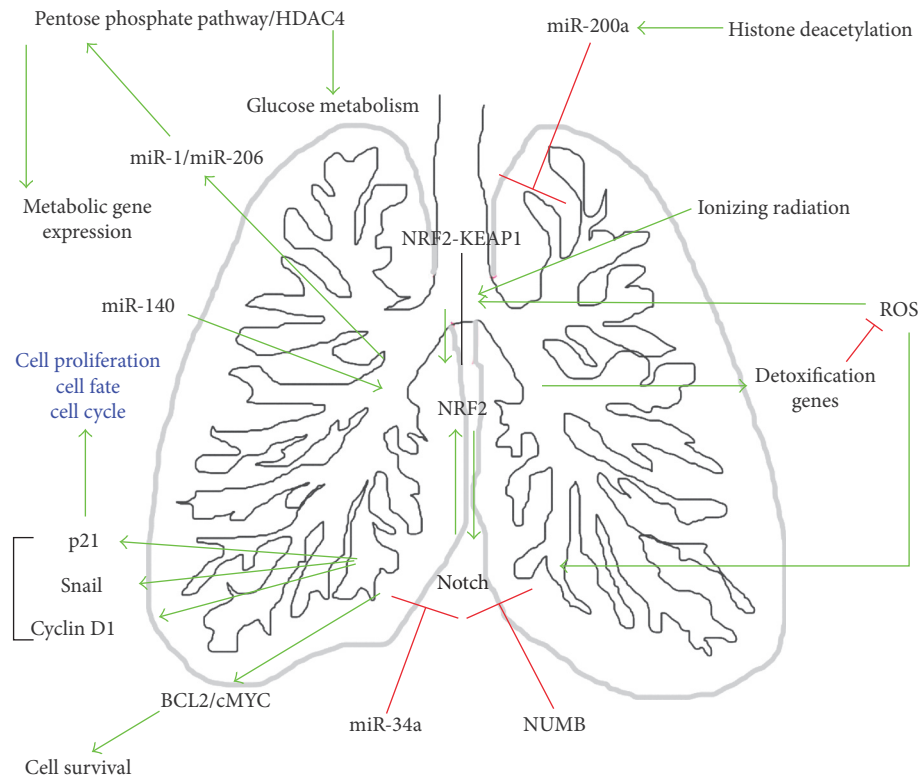

Figure 3: Integrated network of Nrf2-Notch crosstalk in lung. Links for biological events occurring in lung involving Nrf2 and Notch interaction with other signaling networks. Black lines indicate interaction, green arrows indicate activation, and red arrows indicate repression.

the transition from preclinical research into clinical application of alternative targeted compounds, including small molecule inhibitors and large mAbs (monoclonal antibodies) targeting Notch signaling [143]. At the same time, the adjuvant effect of these inhibitors in combination with current chemotherapeutics is still under evaluation in different clinical trials (Table 1) [144].

The interaction between Notch receptor(s) and ligand(s) takes place within a tight cell-to-cell compartment. Following ligand-receptor association, the sequential two-steps cleavage by the ADAM/TACE proteinase and γ-secretase, respectively, culminates in the functional activation of Notch signaling. Numerous preclinical models have documented so far the anticancer effects of different classes of compounds inhibiting Notch signaling activation such as siRNAs, GSIs, and mAbs [145], with encouraging results for clinical implementation in combination with either chemotherapy or targeted agents [146]. Among these, the oral GSI PF-0308414 showed clinical activity in a phase I study in patients with advanced stage solid tumors [147].

In this context, Notch pathway inhibition is currently under investigation as novel therapeutic option of SCLC. For instance, the fully human IgG2 antibody Tarextumab (TRXT, OMP59R5) combined with chemotherapy has been shown to significantly reduce tumor recurrence in patient-derived SCLC xenografts, by targeting Notch2/Notch3 [148]. On the strength of these results, a phase I/II study of Tarextumab in combination with six cycles of cisplatin and etoposide in ES-SCLC, followed by Tarextumab maintenance (PINNACLE, NCT01859741), is currently ongoing [149].

Alternative approaches for targeting Notch signaling in lung cancer may include several natural agents, such as curcumin (3,3′-diindolylmethane, DIM), resveratrol 3,5-bis (2,4-difluorobenzylidene)-4-piperidone (DiFiD), and epigallocatechin-3-gallate (EGCG) [150], whose anticancer activity has been demonstrated in both *in vitro* and *in vivo* models of other solid tumors [151, 152].

Similarly, the pharmacological inhibition of Nrf2 signaling may represent a further therapeutic strategy for cancer treatment, especially in those patients carrying increased levels of Nrf2 (Table 2). Indeed, recent reports have demonstrated that the high levels of Nrf2 are significantly associated to chemo- and radioresistance, rendering the development of novel Nrf2 inhibitors particularly intriguing [153]. For example, it has been demonstrated that all-trans retinoic acid (ATRA) and retinoic acid receptor-α (RARα) agonists can directly sequester Nrf2 and prevent its binding to the ARE, leading to the global downregulation of Nrf2-dependent gene expression [154]. Similar outcomes have been reported as a result of the physical blocking of Nrf2 operated by other nuclear receptors, such as peroxisome proliferator-activated receptor-γ (PPARγ), estrogen receptor-α (ERα), estrogen-related receptor-β (ERRβ), and glucocorticoid receptor (GR) [155].

Table 1: Details of selected trials with therapeutic Notch-targeting agents for lung cancer treatment.

Class	Mechanism	Drug	Target	Condition	Development phase	Trial status/ID
mAbs to Notch receptors or ligands (neutralizing antibody)	Interference with ligand-induced Notch subunit separation and Notch ligands	OMP21M18 + CP*	DLL4 (Delta-like ligand 4)	Nonsquamous NSCLC	Ib	Active, not recruiting/NCT01189968
		OMP-59R5 + EP*/CDDP* or CBDCA*	Notch2/Notch3	Stage IV Small Cell Lung Cancer (SCLC)	Ib/II	Recruiting/NCT01859741
mAbs to Wnt signaling pathway (monoclonal antibody)	Blocking canonical WNT/β-catenin signaling through binding of five FZD receptor	OMP-18R5 + DTX*	Wnt cancer stem cell pathway	Recurrent or advanced NSCLC	Ib	Recruiting/NCT01957007
γ-Secretase inhibitor (GSI)	Inhibition of final Notch cleavage by γ-secretase	RO4929097	Notch1, Notch2, Notch3, Notch4, Notch ligands	Recurrent NSCLC Stage IIIB NSCLC Stage IV NSCLC	II	Terminated/NCT01193868
		RO4929097 + OSI-774*		Recurrent NSCLC Stage IV NSCLC	I	Terminated/NCT01193881
		BMS-906024		Advanced/metastatic squamous NSCLC	I	Ongoing/NCT01292655
		BMS-906024 + chemotherapy regimens*		Advanced/metastatic squamous NSCLC	Ib	Ongoing/NCT01653470
		BMS-906024 + PTX*		NSCLC	I	Synergistic antitumor activity of the Notch gamma secretase inhibitor BMS-906024 and paclitaxel in the treatment of lung adenocarcinoma. Abstract 2535, AA
γ-Secretase inhibitor (GSI)/VEGFR tyrosine kinase inhibitor	Inhibit of final Notch cleavage by γ-secretase and stop the growth of tumor cells by blocking some of the enzymes needed for cell growth	RO4929097 + AZD2171		Recurrent NSCLC Stage IIIA NSCLC Stage IIIB NSCLC Stage IV NSCLC	I	Completed/NCT01131234

*Chemotherapy agent: CP (carboplatin plus paclitaxel); EP (etoposide), CDDP (cisplatin), CBDCA (carboplatin); DTX (docetaxel); OSI-774 (Erlotinib); chemoregimens: PTX (paclitaxel), 5-FU plus irinotecan (FOLFIRI), or CP (carboplatin plus paclitaxel).

Each status of development phases results from https://www.clinicaltrials.gov/.

Table 2: Details of selected trials or scientific reports on therapeutic Nrf2-inhibition for lung cancer treatment.

Class	Mechanism	Drug	Target	Condition	Development phase	Trial status/ID or scientific reports
Vitamin A metabolite	All-trans retinoic inhibits the basal and inducible activity of Nrf2	13-CRA + IFN-A^{+}	Retinoid X receptor alpha binding to Neh7 domain of Nrf2	Recurrent Squamous Cell Lung Cancer (SqCC)	II	Completed/NCT00002506
	RAR-alpha complex (with Nrf2) is not able to bind to ARE and decreases the Nrf2 ability to activate ARE-driven genes	ATRA + CDDP* + MTC* + NVB*		Stage IIIB or IV NSCLC	II	Unknown/NCT00005825
		13-CRA + IFN-A^{+} + PTX*		Recurrent Small Cell Lung Cancer (SCLC)	II	Completed/NCT00062010
		ATRA + PCB***		Stage IIIB or IV NSCLC**	II	Completed/NCT01048645
Quinoid diterpene	Inducing apoptosis by sensitizing A549/DDP cell and inhibiting Nrf2 pathway in chemoresistant lung carcinoma	CTS + CDDP*	Inhibitor of STAT3 and AChE	A549/DDP cell line	*In vitro* and *in vivo*	Xia et al., 2015 [124], Cell PhysiolBiochem
Flavonoid	Inhibiting ARE-driven gene expression redox-independently, leading to a dramatic decrease in Nrf2 protein levels with depletion of reduced glutathione	LUT	SRC tyrosine kinase	A549 adenocarcinoma cell line	*In vitro* and *in vivo*	Tang et al., 2011 [125], Free Radical Biology & Medicine
	Cell proliferation, the expression of Nrf2, and antioxidant enzyme were all reduced in tumor xenograft tissues after cotreatment and inhibiting tumor cell growth	LUT + CDDP*		A549 cell line in athymic nude mice	*In vitro* and *in vivo*	Chian et al., 2014 [126], Biochemical and Biophysical Research Communication
Glycopeptide antibiotic	Involveing suppression of Nrf2 activation, inhibiting the incorporation of thymidine into DNA strand, and causing cell cycle arrest in G2 and in mitosis	BLM + CDDP* + 5-FU* +	Synthesis of nucleic acid	A549 adenocarcinoma cell line LC-AI squamous cell line NCI-H292 mucoepidermoid cell line	*In vitro*	Homma et al., 2009 [127], Clin Cancer Res

Table 2: Continued.

Class	Mechanism	Drug	Target	Condition	Development phase	Trial status/ID or scientific reports
Quassinoids	Inhibiting the Nrf2-mediated protective response at subnanomolar concentration, increase ubiquitination, enhancing Nrf2 degradation, and reducing Nrf2 protein levels	Brusatol	Formation of the first peptide bond between puromycin and methionyl-transfer RNA	A549 cell line	*In vitro* and *in vivo*	Vartanian et al., 2016 [128], Molecular & Cellular Proteomics
	Cotreatment inhibits the Nrf2 protective mechanism, leads to decreases cell proliferation, enhances oxidative DNA damage, and reduces apoptosis	Brusatol + CDDP*		Cell culture and murine A549 xenograft models	*In vitro* and *in vivo*	Tao et al. [95], Cancer Res

AA panel of Nrf2 inhibitor cited in the table as follows: 13-CRA (13-*cis*-retinoic acid), ATRA (all-trans retinoic acid); CTS (cryptotanshinone); LUT (luteolin); BLM (bleomycin); brusatol.
+Biological agent: IFN-A (interferon alpha).
*Chemotherapy agent: CDDP (cisplatin), MTC (mitomycin C), NVB (vinorelbine tartrate); PTX (paclitaxel); 5-FU (fluorouracil).
** In this study patients that have already received paclitaxel and cisplatin (PC) were recruited.
*** PCB (placebo) means an innocuous medication given to the control group in experiments on the efficacy of a drug.
Each status of development phases results from https://www.clinicaltrials.gov/.

An increasing number of natural compounds are known to also exert a strong effect on Nrf2, thus corroborating the idea of a cross-link with Notch pathway. Among these, sulforaphane thereby induces an activation of the Nrf2/Keap1 cellular detoxification cascade by reacting with thiols of Keap1 DGR domain [156, 157], whereas benzo(a)pyrene(B(a)P) has been shown to inhibits carcinogenesis process in lung mouse model by promoting ROS-mediated apoptosis [158, 159]. Similar mechanism of action has been observed with Oltiplraz, known as a dithiolthione substitute able to induce phase II enzymes, which exhibited a chemoprevention effect in mouse lung adenocarcinoma [160, 161]. Resveratrol restored cigarette smoke exposure- (CSE-) depleted GSS (glutathione synthetase) levels by upregulating GCL (γ-glutamate cysteine ligase) by reducing CSE-mediated Nrf2 modifications [162]. Intriguingly, many studies has shown that curcumin, a natural phenolic compound, which is extracted from a member of the ginger family, has a dually role as inhibitor of Notch1 in osteosarcoma cells and a Nrf2 activator in normal tissues [163, 164].

In conclusion, the therapeutic Nrf2 targeting holds great promise for the treatment of lung cancers especially because it has documented a beneficial adjuvant effect in combination with any category of chemotherapeutics, both ROS generating and non-ROS generating agents. To date, one limitation is represented by the lack of few selective inhibitors for Nrf2 and related pathway. Another limitation is represented by the high risk of off-target toxic effects that most of the Nrf2-targeting drugs may generate due to unspecific interactions with other proteins by their electrophilic surface [165]. Nevertheless, the rational design of nonreactive small molecules directly targeting the Keap1-Nrf2 pathway appears to be the most promising strategy to limit the toxic effects often related to indirect inhibitors and increases stability and bioavailability, as compared with peptide inhibitors [166, 167].

8. Concluding Remarks

There are compelling evidences that developmental pathways, including Notch, act in concert with other pathways such as Nrf2 pathway involved in resistance to therapy, rather than as a simple on-off switch. Notch can play as tumor suppressor or oncogene depending on the cell type [168] and interestingly, Nrf2 likely functions in a similar fashion [169]. Nrf2 acts as a prosurvival factor through the expression of its cytoprotective target genes, and molecular deregulation of either Nrf2 or Keap1 is widely described in lung cancer, such as Notch family impairment. In tumors, Nrf2 and Notch signaling pathways appear to mutually regulate each other in which Notch1 is an Nrf2 target gene and Nrf2 is a RBPjk target gene. The roles of the Nrf2-Notch bidirectional interaction in driving or impeding a tumor lung phenotype are still

unclear mainly in the context of stem cell renewal and cell proliferation and differentiation. Pharmacological interventions based on these transcription factors collaboration are demanded close with a further explorations of the regulation of this crosstalk in cellular and lung tissue context.

Competing Interests

The authors declare that they have no competing financial interests.

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Research Article

Identification of Redox and Glucose-Dependent Txnip Protein Interactions

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Thioredoxin-interacting protein (Txnip) acts as a negative regulator of thioredoxin function and is a critical modulator of several diseases including, but not limited to, diabetes, ischemia-reperfusion cardiac injury, and carcinogenesis. Therefore, Txnip has become an attractive therapeutic target to alleviate disease pathologies. Although Txnip has been implicated with numerous cellular processes such as proliferation, fatty acid and glucose metabolism, inflammation, and apoptosis, the molecular mechanisms underlying these processes are largely unknown. The objective of these studies was to identify Txnip interacting proteins using the proximity-based labeling method, BioID, to understand differential regulation of pleiotropic Txnip cellular functions. The BioID transgene fused to Txnip expressed in HEK293 identified 31 interacting proteins. Many protein interactions were redox-dependent and were disrupted through mutation of a previously described reactive cysteine (C247S). Furthermore, we demonstrate that this model can be used to identify dynamic Txnip interactions due to known physiological regulators such as hyperglycemia. These data identify novel Txnip protein interactions and demonstrate dynamic interactions dependent on redox and glucose perturbations, providing clarification to the pleiotropic cellular functions of Txnip.

1. Introduction

Thioredoxin-interacting protein (Txnip/VDUP1/TBP-2) was originally discovered as a vitamin D3-inducible gene [1] but has gained recent interest for being involved in diabetes, hyperlipidemia, carcinogenesis, cardiac function, angiogenesis, and inflammation [2]. Structurally designated as part of the α-arrestin family, Txnip contains two aminoterminal SH3-binding domains while the carboxyl-terminus contains two PPxY motifs and three SH3 domains [3]. Txnip is involved in several prominent biological processes including proliferation, fatty acid and glucose metabolism, inflammation, and apoptosis [2]. A nonsense mutation in *Txnip* results in lipid and cholesterol accumulation due to reduced TCA cycle activity in the HcB-19 mouse strain [4]. Txnip also plays a major role in glucose homeostasis. The Txnip promoter contains several carbohydrate response elements (ChoRE) and Txnip is one of the most highly upregulated genes in pancreatic β-cells in response to hyperglycemia [5, 6]. As part of a negative-feedback loop, Txnip inhibits glucose uptake but also promotes caspase-3 cleavage, contributing to glucose-dependent β-cell death [7]. In addition, Txnip also regulates proinflammatory gene expression by inflammasome activation via NLRP3 binding [8]. Although we do not understand precise mechanisms governing differential Txnip signaling, it is clear that several of these pathways are linked by alterations in redox homeostasis.

Txnip is a unique target for redox perturbations since it is the only α-arrestin with a thioredoxin-binding domain [9]. Thioredoxins have vicinal thiols in their catalytic sites (CXXC) to facilitate redox signaling by regulating reversible cysteine oxidations of protein substrates [10]. In fact, genetic

deletion of either thioredoxin-1 (Trx1, predominantly cytosolic) or thioredoxin-2 (Trx2, mitochondrial) results in embryonic lethality [11, 12]. Txnip is the only known endogenous inhibitor of both Trx1 and Trx2 activity [7, 13, 14]. Txnip forms an intermolecular disulfide via C247 to sequester and inhibit thioredoxins. Although cellular redox status is an important mediator of Txnip signaling, there are also redox-independent mechanisms to consider. Txnip also participates in a negative-feedback loop inhibiting glucose uptake; however, this inhibitory activity is retained by a cysteine to serine mutation incapable of binding thioredoxins. In this case, $Txnip^{C247S}$ retains glucose uptake inhibitory activity in adipocytes and fibroblasts [15].

Because of its diverse array of functions, Txnip has been considered a novel candidate drug target for diabetes and cardiac ischemia-reperfusion injury. In fact, oral administration of verapamil, a calcium channel blocker, reduced murine Txnip expression and glucose-mediated apoptosis in β-cells [16]. Because of antiproliferative functions, Txnip silencing detected in numerous cancers is thought to be an important tumor-initiating event [17]. There is very little known about how impeding or reactivating Txnip expression influences downstream signaling pathways. To understand myocardial Txnip signaling during cardiac ischemia-reperfusion injury, Yoshioka et al. performed multiplex polony analysis of gene expression and proteomic profiling of *Txnip*-deficient mouse hearts to identify disrupted expression of genes functioning in mitochondrial metabolism [18]. However, since none of the reported mitochondrial enzymes were known to directly interact with Txnip, the molecular mechanisms underlying these cellular physiologies are not known.

To understand the pleiotropic cellular functions of Txnip, we used a proteomic approach to identify dynamic Txnip protein interactions in response to redox and glucose perturbations. Txnip fused to a promiscuous biotin ligase (BioID-Txnip) was expressed in HEK293 cells for biotinylation and affinity purification of interacting proteins [19, 20]. Subsequent proteomic analysis identified 31 Txnip protein interactions, only one of which was experimentally validated in a prior study. Many of the interactions were redox-dependent since protein binding was disrupted by mutating a critical cysteine (C247S) in Txnip known to facilitate binding to Trx1 [21]. Furthermore, many interactors were glucose-dependent and induced by hyperglycemic culture conditions. These data identify a large number of novel Txnip protein interactions which have the capacity to mediate differential signaling in response to changes in cellular redox and glucose concentration.

2. Materials and Methods

2.1. Cell Culture and Treatment. HEK293 cells (ATCC) were cultured in 5% CO_2 at 37°C in high glucose (25 mM) DMEM with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 20 μg/mL gentamycin. For glucose sensitivity experiments, cells were acclimated in DMEM containing low glucose (5.5 mM) for a minimum of three passages and were treated with media supplemented with 5.5, 10, 15, 20, or 25 mM glucose for 24 hours before processing.

2.2. Generation of Stable BioID-Txnip Cell Lines. Myc-$BirA^*$-Txnip was generated by first generating Myc-$BirA^*$ by PCR from the pcDNA3.1 mycBioID plasmid [20]. PCR products were digested and ligated into pIRES2-EGFP (Clontech). Next, pCR4-TOPO-Txnip (Thermo Fisher Scientific) was used as template to amplify Txnip which was ligated in frame with Myc-$BirA^*$ in pIRES2-EGFP. Subsequently, Myc-$BirA^*$-$Txnip^{C247S}$ was generated by site-directed mutagenesis (Agilent Technologies). Plasmids were linearized and transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were selected in 200 μg/mL hygromycin and stable clones were selected based on EGFP fluorescence visualized with an Olympus IX71 inverted epifluorescent microscope.

2.3. SDS-PAGE and Immunoblot. As previously described [22], cell lysates were diluted in Laemmli buffer, separated by polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. Membranes were blocked in 5% nonfat dry milk before incubating overnight at 4°C in rabbit anti-Txnip (1 : 1,000, Thermo Fisher Scientific), rabbit anti-myc (1 : 10,000, Abcam), or rabbit anti-β-actin (1 : 1,000, Sigma Aldrich). Blots were incubated with HRP-conjugated anti-rabbit secondary antibodies (1 : 5,000, Southern Biotech) or HRP-conjugated streptavidin (1 : 40,000, Invitrogen) for 1 hr at 25°C. Immune complexes were detected by chemiluminescence and images were captured and analyzed using a UVP bioimaging system.

2.4. Immunocytochemistry. Cells were cultured on 15 mm coverslips coated with poly-D-lysine in 12-well plates. Cells were washed with 1x PBS and fixed for 20 min with 3% paraformaldehyde (0.2 M phosphate buffer pH 7.3, 11% sucrose, and 0.1% Triton X-100). Cells were incubated for 30 min in blocking buffer (5% goat serum, 15 μM BSA, 0.5% Triton X-100, and 0.05% sodium azide in 1x PBS) before overnight incubation with primary antibodies (1 : 1,000) at 4°C. Alexa Fluor-conjugated anti-secondary antibodies or streptavidin was incubated (1 : 2,000, Thermo Fisher Scientific) for 1 hr at 25°C and nuclei were counterstained with 0.5 μg/mL DAPI in 1x PBS. Coverslips were mounted on slides and cells were visualized using a Nikon Eclipse 90i fluorescent microscope.

2.5. Affinity Capture of Biotinylated Proteins. Cells were cultured for 24 hrs in DMEM containing either 5.5 mM or 25 mM glucose supplemented with 50 μM biotin. After washing three times with 1x PBS, cells were lysed at room temperature in 1 mL lysis buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 0.4% SDS, 1 mM DTT, and 1x complete protease inhibitor [Roche]). Triton X-100 was supplemented to a 2% final concentration and sonicated two times using the Branson Sonifier 250 at 30% duty cycle and an output level of 3 for 1 min. An equal volume of 4°C 50 mM Tris (pH 7.4) was added before additional sonication and centrifugation at 16,000 rpm at 4°C. Supernatants were incubated with 600 μL Dynabeads (MyOne Streptavidin C1, Invitrogen) overnight at 4°C with an end-over-end rotator. Beads were collected and

washed two times for 8 min at 25°C in 1 mL wash buffer 1 (2% SDS in ddH_2O). This was repeated once with wash buffer 2 (0.1% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM Hepes, pH 7.5), once with wash buffer 3 (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1), and two times with wash buffer 4 (50 mM Tris, pH 7.4, and 50 mM NaCl). After the final wash, 10% of the sample was reserved for immunoblot analysis. To this end, 50 μL of Laemmli SDS-sample buffer saturated with biotin was added to the 10% saved sample and heated at 98°C. For the larger scale mass-spectrometry analysis, 90% of the sample was reserved in in 50 mM NH_4HCO_3.

2.6. BioID, On-Bead Protein Digestion, and Identification by 1D LC-MS/MS. Large-scale BioID pull-downs for MS analysis were performed using the 90% of sample resulting from affinity capture with streptavidin-conjugated magnetic beads. Sample volume was adjusted to 200 μL with 50 mM ammonium bicarbonate. 4 μL of 0.5 M tris(2-carboxyethyl)phosphine was added to 200 μL of the beads-proteins suspension mix, and proteins were reduced at 40°C for 30 min. Next, 8 μL of 0.5 M iodoacetamide was added, and proteins were alkylated at room temperature for 30 min, in the dark. MS-grade trypsin (Promega) was added (1 : 20 ratio) for overnight digestion at 37°C using an Eppendorf Thermomixer at 700 rpm. Peptides were separated from magnetic beads by centrifugation and a GE Healthcare MagRack and were transferred to a new tube. Formic acid was added to the peptide solution to 2% final concentration, followed by desalting by Microtrap (Thermo Fisher Scientific) and then online analysis of peptides by high-resolution, high-mass accuracy liquid chromatography tandem MS (LC-MS/MS) consisting of a Michrom HPLC, a 15 cm Michrom Magic C18 column, a low-flow ADVANCED Michrom MS source, and a LTQ-Orbitrap XL (Thermo Fisher Scientific). A 120 min gradient of 10–30% B (0.1% formic acid, 100% acetonitrile) was used to separate the peptides. The total LC time was 140 min. The LTQ-Orbitrap XL was set to scan precursors in the Orbitrap followed by data-dependent MS/MS of the top 10 precursors. Raw LC-MS/MS data were submitted to Sorcerer Enterprise (Sage-N Research Inc.) for protein identification against the ipi.HUMAN.vs.3.73 protein database. Differential search included 16 Da for methionine oxidation, 57 Da for cysteines to account for carboxyamidomethylation, and 226 Da for biotinylation of lysine. Search results were sorted, filtered, statically analyzed, and displayed using PeptideProphet and ProteinProphet (Institute for Systems Biology). The minimum Trans-Proteomic Pipeline (TPP) probability score for proteins was set to 0.95 to ensure a TPP error rate lower than 0.01. The relative abundance of each of the identified proteins in different samples was analyzed by QTools, an open-source tool developed in-house for automated differential peptide/protein spectral count analysis. Proteins from the HEK293 control sample and common BioID background proteins were eliminated from the results to minimize noise.

2.7. Bioinformatics Analysis. Proteins with less than three spectral counts or common mass-spec background proteins including keratins, histones, and ribosomal proteins were removed due to the lack of confidence. Proteins identified from parental HEK293 cells were used to remove false positive candidates from BioID-Txnip samples. However, proteins whose relative percentage of total spectral counts was threefold more than in the HEK293 parental controls were also considered candidates. Primary subcellular localization of proteins was determined based on information from UniProt and the Human Protein Atlas. Functional categorization was based on information from UniProt and NCBI:Gene reports. Proteomic data was analyzed for predicted molecular and cellular functions and referenced against experimentally observed protein-protein networks by Ingenuity Pathway Analysis (IPA) software.

3. Results

We utilized the novel BioID system as an unbiased proteomic approach to identify Txnip protein interactions [20]. A promiscuous biotin ligase from *E. coli* (BirA*) with an aminoterminal myc epitope was fused to human Txnip (henceforth called BirA*-Txnip). We chose to generate an aminoterminal fusion protein because BirA* is similarly sized to green fluorescence protein (GFP, ~35 and 27 kDa, resp.) and ectopic expression of GFP-Txnip retained proper localization [23] as well as apoptotic function [24]. By mapping biotinylation of known nucleoporin complexes, it is suggested that BirA* has a labeling radius ~10 nm [19]. As such, this system was used to biotinylate only those proteins within close enough physical proximity highly likely to facilitate protein-protein interactions.

HEK293 cells stably expressing BirA*-Txnip or a cysteine mutant, BirA*-Txnip^{C247S}, which is incapable of binding thioredoxins [21], were cultured in excess biotin to label endogenous proximal proteins (Figure 1). Cells had very similar expression levels and biotinylation signals with either BirA*-Txnip or BirA*-Txnip^{C247S} (Figures 2(a) and 2(b)). Consistent with prior reports for endogenous Txnip, the BirA*-Txnip transgene localized primarily to the nucleus with faint cytosolic staining (Figure 2(c)) [23, 25]. BirA*-Txnip activity is noted due to colocalization of the transgene (anti-myc) with biotinylated proteins (streptavidin). A similar localization pattern was detected with BirA*-Txnip^{C247S} (data not shown).

To identify Txnip interacting proteins, cells were cultured in media supplemented with 50 μM biotin and subsequently lysed under stringent conditions to solubilize proteins and disrupt protein-protein interactions. Biotinylated proteins were affinity-purified with streptavidin-conjugated paramagnetic beads from lysates of HEK293 parental (control), BirA*-Txnip, and BirA*-Txnip^{C247S} cells (Figure 2(b)). After extensive washing, biotinylated proteins that bound the streptavidin beads were digested and analyzed by 1D LC-MS/MS. In total, we detected 31 proteins unique to the BirA*-Txnip pull-down that were identified in at least 2 of 3 independent BioID trials (Table 1). From this list, 30 of the proteins were novel Txnip interactions not previously identified through experimental validation. ITCH (E3 ubiquitin-protein ligase Itchy homolog), a known Txnip interactor, was identified

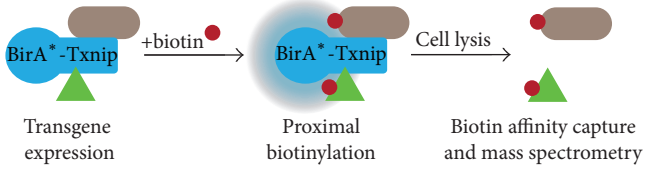


Figure 1: BioID system for identification of Txnip interacting proteins. Myc-BirA* was fused to the aminoterminus of Txnip (BirA*-Txnip) to screen for protein interactions. BirA* catalyzes a two-step reaction: first, generation of reactive biotinyl-AMP from biotin and ATP, and second, the attachment of that biotinyl-AMP to a specific lysine on an interacting/proximal protein. Streptavidin beads are used to affinity-purify biotinylated proteins, which then are analyzed by mass spectrometry.

as an E3 ubiquitin ligase for Txnip. This interaction was previously shown to be governed by the PPxY motifs of Txnip with WW domains of ITCH [3]. Consistent with BirA*-Txnip localization, the majority of proteins identified (19 of 31) primarily localized to the nucleus. However, proteins known to localize to the cytoplasm, plasma membrane, ER, and vesicles were also detected. Txnip protein interactors had a diverse array of molecular functions, with a majority serving as chaperones or in the regulation of chromatin structure and gene expression (Table 1).

Proteins identified by BirA*-Txnip were analyzed via pathway analysis using IPA software. Txnip function is already associated with alterations in cell proliferation and lipid metabolism, two of the IPA predicted molecular and cellular functional categories (Table 2). After filtering out nonexperimentally validated interactions, two protein networks aligned with our proteomic data set: (i) drug metabolism, endocrine system development and function, and lipid metabolism (Figure 3) and (ii) cell death and survival, cellular development, and embryonic development.

Since Txnip^{C247} was previously shown to be critical for thioredoxin binding, we performed a comparative proteomic analysis between cells expressing BirA*-Txnip and BirA*-Txnip^{C247S} to identify redox-sensitive protein interactions. 17 of the 31 Txnip protein interactions were lost following the cysteine to serine mutation (Table 1). Interestingly, interactions with all 6 chaperones (HSP90AB1, HSP1A/1B, HSPA13, DNAJC7, HSPA8, and STIP1) were lost in BirA*-Txnip^{C247S}. However, this is not surprising as heat shock proteins are known to harbor highly reactive cysteines [26, 27]. It is also not surprising that many of the protein interactions were sustained and are likely to occur through the multiple PPxY and/or SH3 domains in Txnip. For example, ITCH interacts through a PPxY domain near the Txnip carboxyterminus and was detected in BirA*-Txnip^{C247S} [3]. Together, these data demonstrate the utility of BioID to identify novel Txnip protein interactions, many of which are dependent on C247 thiol reactivity.

Txnip gene expression is upregulated in response to glucose [5]; therefore, we hypothesized that Txnip protein interactions may also be glucose-dependent. All prior experiments were performed using media supplemented with 25 mM glucose. To investigate Txnip glucose-inducibility, HEK293 parental cells were acclimated to low glucose media (5.5 mM) for at least three passages. Endogenous Txnip expression was increased in cells cultured in hyperglycemic media (Figure 4(a)). Augmented Txnip protein expression in HEK293 cells was robust with a 7.1-fold increase following culture in media supplemented to 25 mM glucose. Maximal Txnip expression was detected after culturing cells for 24 hrs in 25 mM glucose (data not shown). Cells expressing BirA*-Txnip were similarly acclimated to low glucose conditions and were pulsed with biotin following a switch to media supplemented with high glucose (25 mM). There were no gross changes in the biotinylation signal due to glucose culture conditions via antistreptavidin immunoblot (Figure 4(b)). However, mass spectrometry of affinity purified lysates identified Txnip interactions that were independent of glucose concentration (identified in both conditions) or dependent on increased glucose in the media (only identified in 25 mM glucose culture) (Figure 4(c)). These data suggest that the list of Txnip interactions is dynamic and depends upon the glucose concentration in the culture media.

4. Discussion

While there is much interest in the physiological relevance of Txnip, there is limited knowledge of the underlying molecular interactions supporting Txnip-dependent changes in cellular function. We sought to identify novel Txnip protein interactions which may govern differential signaling pathways responsible for pleiotropic cellular functions. Using BioID as an approach to identify protein interactions is advantageous over common methods such as a yeast-2-hybrid screen (not typically performed in human cells) and endogenous immunoprecipitation (only detecting static interactions). An alternative proximity-based labeling system, APEX or APEX2, is not amenable for identification of redox-dependent pathways since it requires cell treatment with hydrogen peroxide to generate biotin-phenoxyl radicals and appears limited to compartmental proteomics [28, 29]. There are several considerations using HEK293 cells for proteomic analysis. Use of an immortalized tumor cell line may not mirror physiological conditions due to imbalances in redox homeostasis [30] and altered metabolic preferences [31]. This is more critical given known roles of Txnip in response to redox perturbations and glucose and lipid metabolism. Txnip protein interactions may be cell-specific based on unique expression or regulatory mechanisms in specialized cells such as pancreatic β-cells [5] or cardiomyocytes [18]. However, Txnip expression is relatively ubiquitous with the exception of the central nervous system; therefore, use of HEK293 cells as a prototypical cell conduit for these untargeted proteomic studies was justified.

Our data support that dynamic protein interactions may facilitate unique signaling pathways, accounting for the pleiotropic cellular functions of Txnip. We propose two likely mechanisms that influence protein binding partners: (i) subcellular trafficking and localization and (ii) differential posttranslational modifications. As expected, Txnip-BirA* was predominantly localized in the nucleus with faint

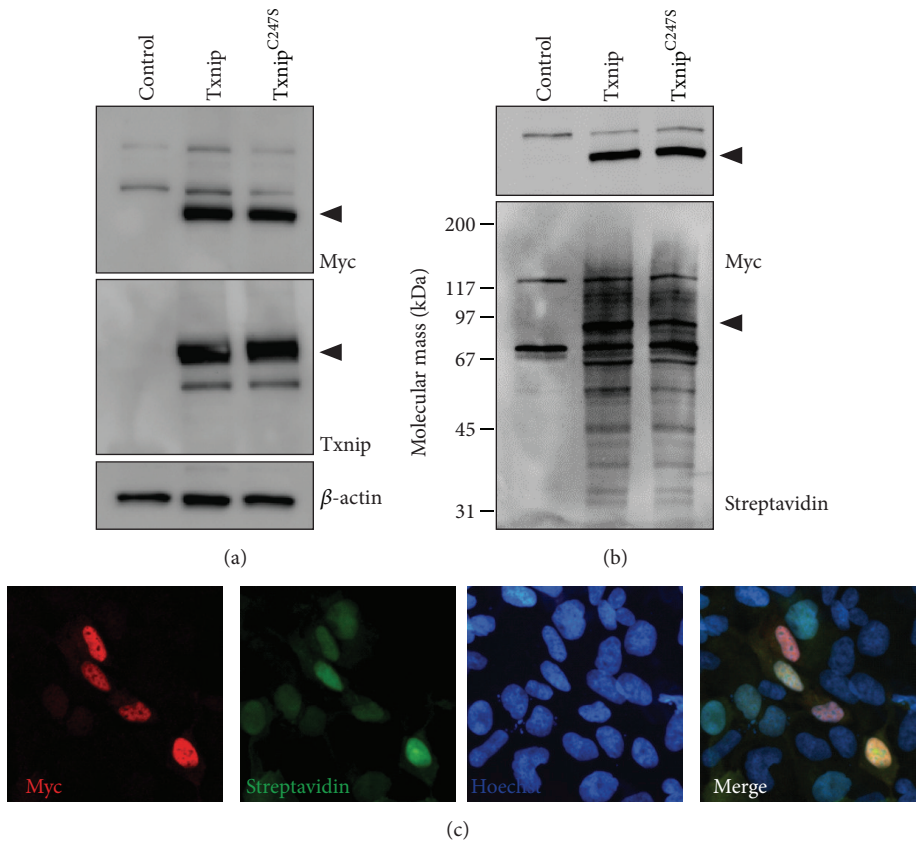


Figure 2: BirA*-Txnip expression, activity, and localization. (a) Protein lysates from HEK293 parental (control) or cells stably expressing either BirA*-Txnip or BirA*-Txnip^{C247S} were separated by SDS-PAGE and analyzed via immunoblot for transgene expression using myc and Txnip with β-actin as a loading control. Arrowheads indicate the expected size of the BioID transgenes. Cells were pulsed with biotin for 24 hrs prior to lysis and biotinylated proteins were detected by (b) affinity purification and SDS-PAGE/immunoblot and (c) immunocytochemistry. The BioID transgene was detected with myc and biotinylated proteins were detected with streptavidin. Images are representative of 3 biological replicates.

detection of cytosolic staining (Figure 2(c)). Depending on the oxidative stimulus, Txnip has been demonstrated to traffic from the nucleus. For example, Txnip accumulates in the cytosol and complexes with the NLRP3 inflammasome following hydrogen peroxide treatment [8]. Upon glucose stimulation, Txnip shuttles to the mitochondria and relieves Trx2 inhibitory binding to apoptosis signal-regulating kinase (ASK1), promoting glucose-dependent β-cell apoptosis [7]. Although hyperglycemic treatment increased Txnip expression (Figure 4(a)), neither endogenous Txnip nor the BioID transgene (or any biotinylated proteins) was detected in mitochondria (data not shown). While it is already known that thiol oxidation of C247 facilitates Trx1 and Trx2 binding, Liu et al. recently described a novel tyrosine phosphorylation of the PPxY motifs that influences ITCH binding [32]. Txnip PPxY phosphorylation decreased ITCH binding and promoted PTPN11 (tyrosine-protein phosphatase nonreceptor type 11) interactions, resulting in c-Src tyrosine-protein kinase activation. Further understanding of the relationship between Txnip localization, posttranslational modifications, differential protein interactions, signaling pathways, and functional consequences is necessary to refine therapeutic strategies targeting Txnip [16].

Currently, 15 proteins have been validated to directly interact with Txnip through experimental means. This includes Trx1 [13], NLRP3 [8], HDAC1 [33], and p53 [34]. We identified 31 proteins biotinylated by BirA*-Txnip but only one, ITCH, was previously described as a Txnip binding partner [3]. While it is possible that many of these interactions exhibit cell specificity, there are technical and functional differences likely accounting for dissimilarities. Since BirA* has a labeling radius of ~10 nm [19], it is important to consider that spatial constrictions may exclude protein biotinylation in addition to physical interactions restricted by BirA* fusion. For example, Txnip is mainly comprised of β-strands that form an elongated S-shaped domain divided into amino- and carboxyterminal domains [9]. Trx1 C35 interacts exclusively with Txnip C247 of the carboxyterminal domain, which may be spatially restricted to aminoterminal BirA*. However, 15 of 31 Txnip interactions were not detected in BirA*-Txnip^{C247S}. It is difficult to speculate if these dynamic interactions were a direct consequence of loss of binding at C247 or if this

Table 1: List of proteins identified by BirA*-Txnip.

Protein name[1]	Gene ID	UniProt	Localization[2]	Molecular function[3]	C247S
Src substrate cortactin	CTTN	Q14247	Plasma membrane	Actin polymerization	Yes
Filamin-A, isoform 2	FLNA	Q60FE6	Cytoplasm	Actin polymerization	No
4F2 cell-surface antigen heavy chain, isoform 2	SLC3A2	P08195	Plasma membrane	Amino acid transport	Yes
Heat shock protein HSP 90-beta	HSP90AB1	P08238	Cytoplasm	Chaperone	No
Heat shock 70 kDa protein 1A/1B	HSPA1A/1B	P0DMV8	Cytoplasm	Chaperone	Yes
Heat shock 70 kDa protein 13	HSPA13	P48723	ER	Chaperone	Yes
DnaJ homolog subfamily C member 7	DNAJC7	Q99615	Cytoplasm	Chaperone	Yes
Heat shock cognate 71 kDa protein	HSPA8	P11142	Cytoplasm, nucleus	Chaperone	Yes
Stress-induced phosphoprotein 1	STIP1	P31948	Cytoplasm, nucleus	Chaperone	Yes
JmjC domain-containing histone demethylation protein 2C	JMJD1C	B7ZLC8	Nucleus	Chromatin structure	No
TOX high mobility group box family member 4	TOX4	O94842	Nucleus	Chromatin structure	Yes
YEATS domain-containing protein 2	YEATS2	Q9ULM3	Nucleus	Chromatin structure	Yes
Double-strand break repair protein MRE11A	MRE11A	B3KTC7	Nucleus	DNA repair	No
Poly ADP-ribose polymerase 1	PARP1	P09874	Nucleus	DNA repair	No
Epidermal growth factor receptor substrate 15-like 1	EPS15L1	A5PKY0	Vesicles	Metal binding	No
Nuclear mitotic apparatus protein 1	NUMA1	Q14980	Nucleus	Mitotic spindle formation	Yes
TPR nucleoprotein	TPR	P12270	Nucleus	Nuclear transport	No
E3 ubiquitin-protein ligase Itchy homolog	ITCH	Q96J02	Cytoplasm	Proteolysis	No
E2 ubiquitin-conjugating enzyme	UBE2O	Q9C0C9	Nucleus	Proteolysis	Yes
Thioredoxin-like protein 1	TXNL1	O43396	Cytoplasm	Redox	Yes
Neuroblast differentiation-associated protein AHNAK	AHNAK	Q09666	Nucleus	RNA binding	No
Nucleolar protein 58	NOP58	Q9Y2X3	Nucleus	RNA binding	No
U4/U6.U5 tri-snRNP-associated protein 1	SART1	O43290	Nucleus	RNA binding	Yes
Activity-dependent neuroprotector homeobox protein	ADNP	Q9H2P0	Nucleus	Transcription	Yes
SAP30-binding protein	SAP30BP	Q9UHR5	Nucleus	Transcription	No
Sex comb on midleg-like protein 2	SCML2	H0Y6S1	Nucleus	Transcription	No
Transcription intermediary factor 1-beta	TRIM28	Q13263	Nucleus	Transcription	Yes
Transcriptional regulator Kaiso	ZBTB33	Q86T24	Nucleus	Transcription	No
Eukaryotic translation initiation factor 5	EIF5	P55010	Cytoplasm	Translation	Yes
cDNA FLJ56180 fis	N/A	Q6ZNN8	Unknown	Unknown	No
UPF0428 protein CXorf56	CXorf56	Q9H5V9	Nucleus	Unknown	No

[1]Proteins were identified on at least 2 of 3 biological replicates.
[2]Primary localization based on information from UniProt and The Human Protein Atlas.
[3]Based on information from UniProt and NCBI:Gene reports.

was a result of Txnip structural alterations in disulfide bond switching [9]. A crystal structure including the C-terminal PPxY motifs (amino acid positions 331 and 375) has not been determined although our proteomic data identifying ITCH suggest that the Txnip carboxyterminal tail is flexible and accessible to aminoterminal BirA*.

It was not surprising that IPA analysis of Txnip protein interactions identified cellular growth and proliferation as a major predicted functional pathway based on known interactions (Table 2). *Txnip*-deficient fibroblasts proliferate more rapidly than wild-type counterparts [35] and the HcB-19 strain (spontaneous Txnip mutation) has increased incidence

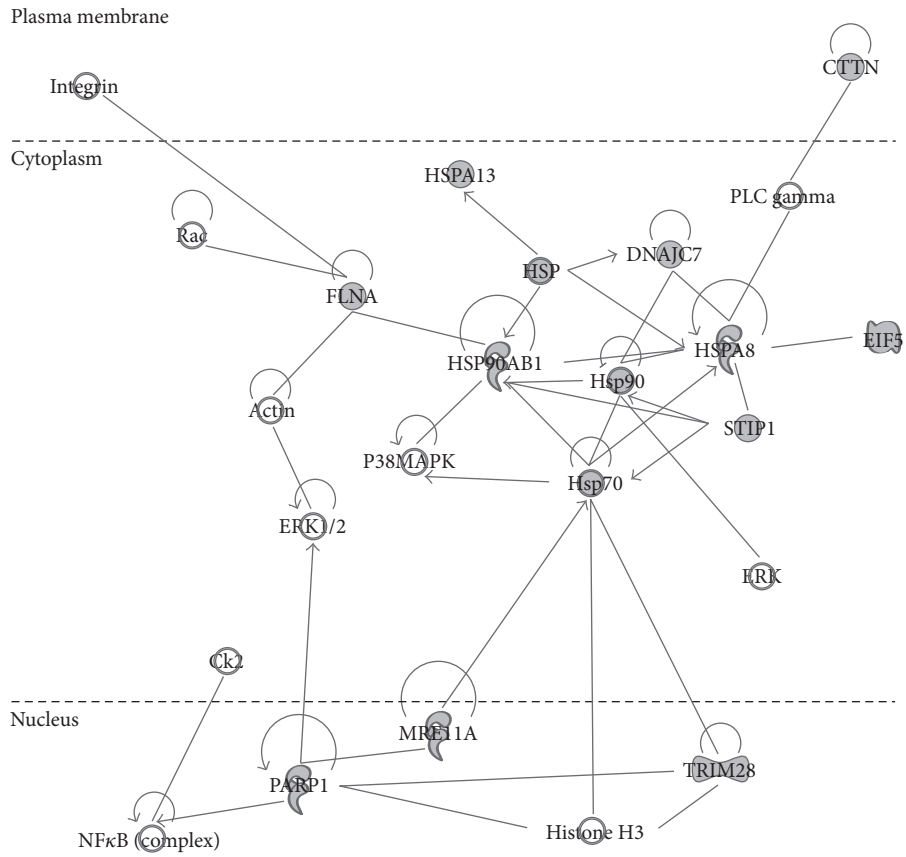


Figure 3: IPA network analysis of Txnip proteins identified by BirA* -Txnip. BirA* -Txnip interactions analyzed by IPA identifying 14 proteins (shaded in gray) included the drug metabolism, endocrine system development and function, and lipid metabolism network. IPA analysis was restricted to only include experimentally observed protein interactions.

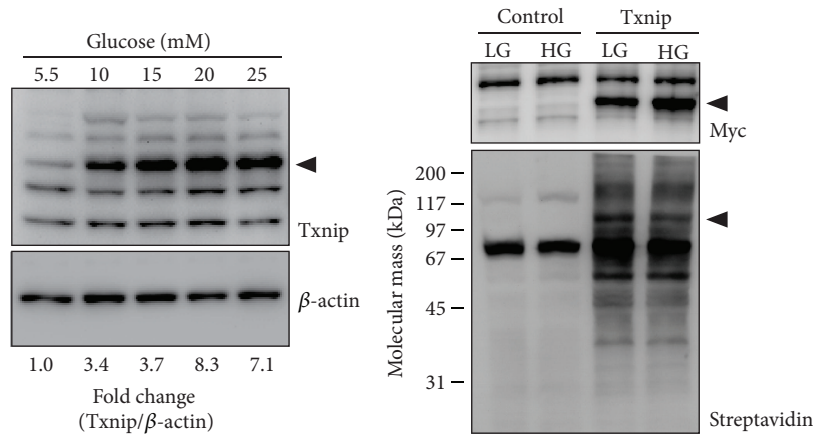
Table 2: Predicted molecular and cellular functions of BirA* -Txnip interacting proteins.

Molecular and cellular function	p value range	# molecules
Cellular growth and proliferation	$3.12e^{-2}$–$1.70e^{-5}$	19
Drug metabolism	$8.12e^{-3}$–$6.36e^{-5}$	5
Lipid metabolism	$8.12e^{-3}$–$6.36e^{-5}$	3
Small molecular biochemistry	$3.60e^{-2}$–$6.36e^{-5}$	5
Cellular assembly and organization	$3.73e^{-2}$–$1.37e^{-4}$	9

of hepatocellular carcinomas [36]. Txnip has been considered a tumor suppressor because of suppressed expression in a variety of cancers (reviewed by [17]). Therefore, there is high therapeutic interest in using small molecules to reactivate Txnip expression as an anticancer strategy [37]. One speculative growth pathway based on our proteomic data involves Poly ADP-ribose polymerase 1 (PARP1). Reduced expression of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, correlated with loss of Txnip [35]. The Txnip:PARP1 axis (Table 1) may regulate $p27^{Kip1}$ since PARP1 represses FOXO1-mediated $p27^{Kip1}$ expression [38].

Another speculative pathway that may be linked to pleiotropic effects of Txnip involves heat shock proteins (HSP). HSP90 and HSPA1A/B (also known as HSP70) were exclusively detected during culture in high glucose (Figure 4(c)). This suggests that these chaperones play a signaling role during hyperglycemia and are not caused by supraphysiological exogenous gene expression. The HSP90:HSP70 chaperone machinery may regulate Txnip trafficking and/or turnover due to oxidative damage [39]. This hypothesis is supported by glucose-dependent interactions with the E3 ubiquitin ligase, ITCH, a likely negative-feedback response to promote Txnip proteasomal degradation following hyperglycemic gene induction. Conversely, Txnip interactions with HSP90:HSP70 may directly facilitate signaling pathways linked to pleiotropic cellular functions ascribed to Txnip. For example, HSP90 and HSP70 have cyclic function to regulate and enhance glucocorticoid receptor activation and function [40].

In conclusion, these studies identified novel Txnip protein interactions in response to redox and glucose perturbations which may have relevance for several cellular functions and pathologies. Since BioID relies on affinity purification of biotinylated targets, as opposed to organelle or bait purification, we demonstrate how this approach is advantageous for



Static interactions	
SLC3A2	TPR
EIF5	
FLNA	
HSPA13	
AHNAK	
EPS15L1	
MRE11A	

High glucose interactions	
HSP90AB1	STIP1
HSPA1A/1B	TOX4
CTTN	TRIM28
ITCH	ZBTB33
HSPA8	cDNA FJ56180 fis
NOP58	CXorf56
SAP30BP	

(c)

Figure 4: Glucose-dependent Txnip expression and protein interactions. HEK293 parental cells acclimated to culture in low glucose media (5 mM) were treated for 24 hrs with increasing glucose concentrations (5, 10, 15, 20, and 25 mM) and protein lysates were analyzed by SDS-PAGE/immunoblot for Txnip with β-actin as a loading control. The arrowhead indicates the expected size of Txnip and densitometry values were determined by the pixel density ratio for Txnip/β-actin. (b) HEK293 parental (control) or cells stably expressing BirA*-Txnip were cultured in low glucose (5.5 mM, LG) or high glucose (25 mM, HG) for 24 hrs and then pulsed with biotin. Biotinylated proteins were detected by SDS-PAGE/immunoblot and affinity purification. (c) Affinity purified biotinylated proteins were digested and analyzed by mass spectrometry. BirA*-Txnip interactors were classified as static (identified in both LG and HG) or high glucose (only identified in HG). Images are representative of 3 biological replicates.

detection of dynamic, redox-dependent Txnip interactions occurring across different cellular compartments [41].

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Peter F. Vitiello conceived and coordinated the study. Benjamin J. Forred, Dae In Kim, Kyle J. Roux, and Peter F. Vitiello designed the experiments. Benjamin J. Forred, Skyla Neuharth, Dae In Kim, Michael W. Amolins, and Khatereh Motamedchaboki provided technical assistance. Benjamin J. Forred, Dae In Kim, Kyle J. Roux, Khatereh Motamedchaboki, and Peter F. Vitiello analyzed the data. All authors reviewed the results and approved the final version of the paper.

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Review Article

Redox Homeostasis and Cellular Antioxidant Systems: Crucial Players in Cancer Growth and Therapy

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Reactive oxygen species (ROS) and their products are components of cell signaling pathways and play important roles in cellular physiology and pathophysiology. Under physiological conditions, cells control ROS levels by the use of scavenging systems such as superoxide dismutases, peroxiredoxins, and glutathione that balance ROS generation and elimination. Under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids, and DNA, leading to cell damage that may contribute to carcinogenesis. Several studies have shown that cancer cells display an adaptive response to oxidative stress by increasing expression of antioxidant enzymes and molecules. As a double-edged sword, ROS influence signaling pathways determining beneficial or detrimental outcomes in cancer therapy. In this review, we address the role of redox homeostasis in cancer growth and therapy and examine the current literature regarding the redox regulatory systems that become upregulated in cancer and their role in promoting tumor progression and resistance to chemotherapy.

1. Pathophysiology of Reactive Oxygen Species and Antioxidant Defenses

Reactive oxygen species (ROS) are highly reactive molecules that are principally derived from the oxygen that is consumed in various metabolic reactions occurring mainly in the mitochondria, peroxisomes, and the endoplasmic reticulum. ROS include the superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$) as well as nonradical molecules such as hydrogen peroxide (H_2O_2) [1]. H_2O_2 is the more stable and diffusible form of ROS, it is selectively reactive towards cysteine residues on proteins, and, in the low nanomolar range, it can control cellular signaling (Figure 1).

ROS are mainly produced by the mitochondrial respiratory chain and also by enzyme-catalyzed reactions involving NADPH oxidase (NOX), xanthine oxidase, nitric oxide synthase (NOS), arachidonic acid, and metabolizing enzymes such as the cytochrome P450 enzymes, lipoxygenase, and cyclooxygenase [2] (Figure 1).

The modulation of intracellular ROS levels is crucial for cellular homeostasis, and different ROS levels can induce different biological responses. At low and moderate levels ROS can act as signaling molecules that sustain cellular proliferation and differentiation and activate stress-responsive survival pathways [3]. For instance, ROS can stimulate the phosphorylation of protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK)1/2, phosphoinositide 3-kinase/serine-threonine kinase (PI3K/Akt), protein kinase B (PKB), and JUN N-terminal kinase (JNK) [4–6]. ROS are also involved in the increased expression of antioxidant genes related to the activation of transcription factors such as the nuclear factor erythroid 2-related factor 2 (Nrf2), activator protein 1 (AP-1), nuclear factor κB (NF-κB), hypoxia-inducible transcription factor 1a (HIF-1a), and p53 [7–9].

At high levels, ROS promote severe cell damage and death. Cancer cells display elevated ROS compared to normal counterparts as the result of the accumulation of intrinsic

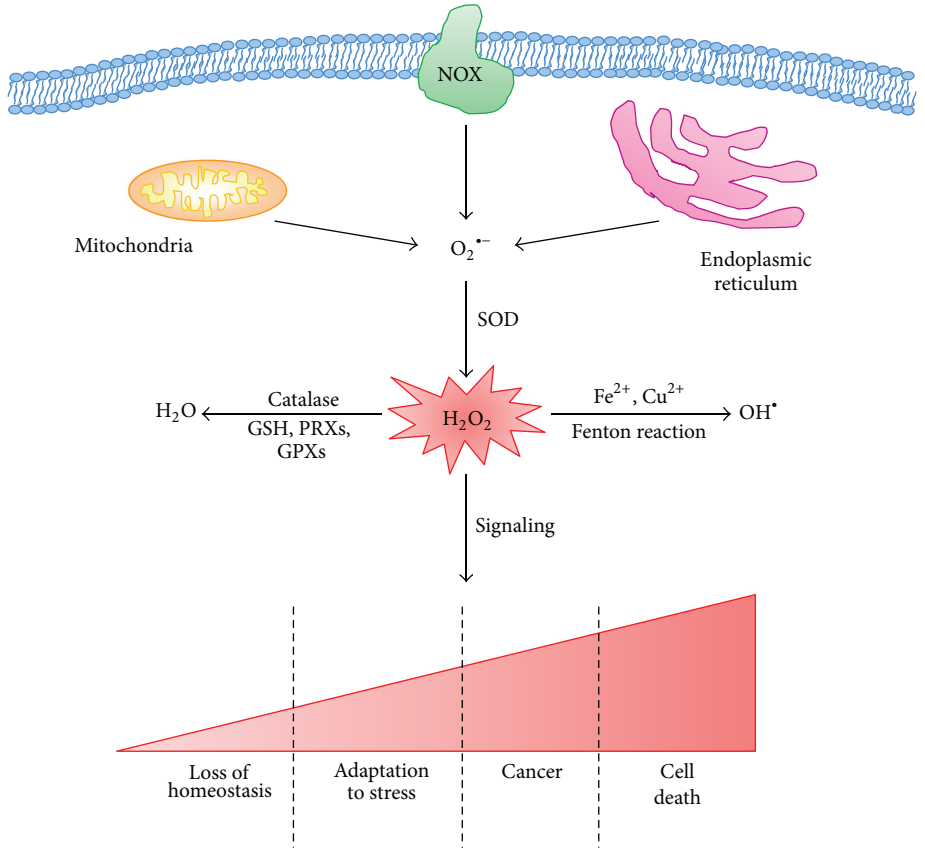

Figure 1: Redox homeostasis is a balance of ROS generation and elimination. Mitochondria, NAPH oxidase (NOX), and endoplasmic reticulum are the three major intracellular sources of ROS. Anion superoxide ($O_2^{\bullet -}$) is the principal form of ROS and can be rapidly converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 can be catalyzed to hydroxyl radical ($OH^{\bullet}$) in the presence of Fe^{2+} or Cu^{2+} ions or be converted to H_2O by catalase. The amount of H_2O_2 is decisive for the cell fate: low and intermediate levels of the peroxide stimulate loss of cell homeostasis and increased adaptation to stress leading to neoplastic transformation while high levels induce cell death.

and/or environmental factors. The more relevant factors include hypoxia, enhanced cellular metabolic activity, mitochondrial dysfunction, oncogene activity, increased activity of oxidases, lipoxygenases and cyclooxygenases, and the cross talk between cancer cells and immune cells recruited to the tumor site. Recent research has revealed that conditions inducing oxidative stress lead the neoplastic cells to develop powerful antioxidant mechanisms.

Several types of antioxidants play important roles in ROS homeostasis, including dietary natural antioxidants (e.g., vitamins A, C, and E), endogenous antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and peroxiredoxins), and antioxidant molecules (e.g., glutathione, coenzyme Q, ferritin, and bilirubin).

Superoxide Dismutases. Superoxide dismutases (SOD) were the first characterized antioxidant enzymes [10] able to dismutate two $O_2^{\bullet -}$ anions into H_2O_2 and molecular oxygen. Three different types of SOD are expressed in human cells: copper-zinc SOD (CuZnSOD), which is present mainly in the cytoplasm, manganese SOD (MnSOD), located in the mitochondria, and extracellular SOD. It has been demonstrated that mice lacking MnSOD produce a massive oxidative stress and die perinatally [11] while CuZnSOD-deficient mice have persistent oxidative damage and develop hepatocellular carcinoma [12]. In addition, a variant allele of MnSOD has been associated with an elevated risk of prostate [13], lung [14], ovarian cancers [15], and non-Hodgkin's lymphoma [16].

Catalase. Catalase, a heme enzyme that catalyzes the reaction that converts two molecules of H_2O_2 to O_2 and two molecules of H_2O, is responsible for the detoxification of various phenols, alcohols, and hydrogen peroxide. Several epidemiologic studies have investigated the relationship between the mutations of catalase and human cancer but the results obtained are contradictory. In fact, a decreased catalase activity has been found both in blood samples and in tissues of breast cancer patients [17, 18] and in oral and pancreatic carcinomas [19, 20]. However, an increase in catalase levels has been reported in breast cancer tissue [21], malignant mesothelioma, and colorectal carcinoma [22, 23].

Peroxiredoxins. Peroxiredoxins (PRDXs) are a family of six isoenzymes able to reduce alkyl hydroperoxides and H_2O_2 to their corresponding alcohol or H_2O. PRDXs are considered to

be amongst the most important antioxidant enzymes, known to balance the production of cellular H_2O_2 which is essential for cell signaling and metabolism [24]. Under oxidative stress conditions, PRDXs are upregulated by Nrf2 activity and several studies have shown that the overexpression of PRDXs could either inhibit the development of cancer or promote growth of cancers [25].

In fact, PRDX1 interacts with the c-Myc oncogene and suppresses its transcriptional activity playing a tumor-suppressive role in breast cancer development [26, 27]. On the contrary, PRDX1 is associated with the promotion of oral, esophageal, lung, hepatocellular, and pancreatic carcinoma by upregulating heme oxygenase 1 and activating the NF-κB pathway [28–31]. Moreover, also PRDX2 promotes colorectal carcinoma through upregulation of Wnt/β catenin and prostate cancer through upregulation of androgen receptor activity [32, 33]. Furthermore, several studies have demonstrated that the overexpression of PRDX1, PRDX2, and PRDX3 has an important role in many cases of drug resistance and that the therapeutic agents targeting these PRDXs are frequently studied for the treatment of cancer [34]. While PRDX3, PRDX4, and PRDX6 play a tumor-promoting role in the progression of many cancers [35–37], PRDX5, similar to PRDX1, has an antitumor effect in breast cancer development [38, 39].

Thioredoxins. Thioredoxins (Trxs) protect cells from oxidative stress by means of their 2-cysteine active site that reacts with ROS and is able to reduce oxidized proteins. They also serve as hydrogen donors to the thioredoxin-dependent peroxide reductases. Trx1, expressed in the cytoplasm and the nucleus, and Trx2, expressed in the mitochondria, are indispensable for cell survival [40]. Nuclear Trx1 has been shown to be overexpressed in *in situ* breasttumors [41], in melanoma, lung, colon, cervix, gastric, liver, and pancreatic carcinomas [42–45].

Glutathione. Glutathione (GSH) is the major cellular thiol protein, consisting of three amino acids glutamine, cysteine, and glycine, and it participates in antioxidant defense, in the detoxification of xenobiotics, and in many metabolic processes such as the synthesis of proteins and nucleic acids [46]. It is synthesized from L-glutamate, L-cysteine, and glycine in two consecutive steps, catalyzed by glutamate-cysteine ligase (GCL) and glutathione synthase (GS) [47]. GCL is considered the rate-limiting enzyme of GSH synthesis. While GSH loss, or a decrease in glutathione/glutathione disulphide ratio (GSH/GSSG), leads to an increased susceptibility to oxidative stress and to carcinogenesis, elevated GSH levels increase the antioxidant capacity of many cancer cells enhancing their resistance to oxidative stress [48]. Remarkably, the inhibition of GSH and Trx dependent pathways induces a synergistic cancer cell death, demonstrating the importance of these two antioxidants in favoring tumor progression [49]. Glutathione peroxidases (GPx) are another group of enzymes capable of reducing hydroperoxides, including lipid hydroperoxides, using GSH as a substrate and generating GSSG which is, once again, reduced by the specific enzyme glutathione reductase (GR). A proline-leucine substitution at codon 198 of human GPx has been associated with the increased risk of breast [50, 51], lung [52], and bladder cancer [53].

Heme Oxygenase. Heme oxygenase (HO)-1 is the first rate-limiting enzyme in the degradation of heme into biliverdin/bilirubin, carbon monoxide (CO), and free iron [54]. Normally expressed at low levels in most of the mammalian tissues, HO-1 expression is efficiently upregulated by the availability of its substrate heme and by different stress stimuli such as heavy metals, UV irradiation, ROS, nitric oxide, and inflammatory cytokines [55]. By increasing the availability of bilirubin, ferritin, and CO, with antioxidant and antiapoptotic properties, HO-1 is recognized as a key player in the maintenance of cellular homeostasis and in the adaptive response to cellular stressors [56]. For this reason, HO-1 activity is crucial in the protection of healthy cells, maintaining cell viability and counteracting ROS-mediated carcinogenesis as well [57]. However, the involvement of HO-1 in cancer cell biology has been proven [58] and the upregulation of HO-1 has been widely related to cancer cell metastatic and proangiogenetic potential and poor prognosis [59–61]. Nevertheless, the role of HO-1 seems to be strongly dependent on the types of tumor considered. For instance, in breast cancer cells, HO-1 activity reduces cell proliferation and favors the efficacy of certain drugs [62, 63]. Thus, it is important to note that the metabolic status of cancer cells may influence HO-1 expression that is dependent on different signaling pathways and transcription factors, suggesting a possible, but not completely understood, regulation of HO-1 [64]. In addition, it has been recently demonstrated that the response of myeloma cells to bortezomib could be due to the noncanonical functions of HO-1 which translocates to the nucleus where it plays a role in genetic instability, favoring cancer progression independently of its enzymatic activity [65]. Within this context, the nuclear localization of HO-1 has also been demonstrated to be involved in the gain of resistance to other chemotherapeutic agents such as imatinib in chronic myeloid leukemia [66]. As a whole, these findings open up a new scenario of the role of HO-1 in cancer cell biology.

2. Redox-Signaling Pathways Involved in Tumorigenesis and in Tumor Progression

In many tumors dysregulation of proliferation, apoptosis, and autophagy depends on the constitutive activation of redox-sensitive targets such as protein kinase C (PKC), protein kinase B (Akt), mitogen-activated protein kinases (MAPK), and ataxia telangiectasia mutated (ATM) kinase [135].

2.1. Protein Kinase C. Among redox-modulated signaling molecules playing a role in cancer, PKC may be activated by oxidative modifications of its enzymatic structure [136–138]. In this regard, *in vivo* and *in vitro* studies have demonstrated that high doses of prooxidant compounds cause PKC inactivation and proteolytic degradation while low doses induce the stimulation of the kinase activity [139–142].

For most PKC isoenzymes there is conflicting evidence as to whether they act as oncogenes or as tumor suppressors [143]. For example, the overexpression of PKCα has been

demonstrated in prostate, endometrial, and high-grade urinary bladder carcinoma [144] while downregulation of PKCα has been described in basal cell carcinoma and colon cancers [145, 146]. Also PKCβ overexpression is an early event in colon cancer development [147] and the transgenic overexpression of PKCβII induces hyperproliferation and invasiveness of intestine epithelial cells [148]. It has been reported that PKCβ isoenzyme is responsible for the activation/phosphorylation of p66/shc, which can bind to cytochrome c and stimulate the generation of ROS [149]. Recent findings have demonstrated that PKCα plays a critical role in hepatocarcinoma development by inducing DUOX (a member family of NOX) expression and ROS production [150]. Moreover, also PKCδ has been shown to be implicated in NOX activation that *via* alterations of redox state influence retinoic acid-induced differentiation of neuroblastoma cells [151]. Likewise, PKCδ can act as either a positive or a negative regulator of tumor progression [152, 153]. Specifically, PKCδ may be overexpressed in colon cancers and downregulated in malignant gliomas, bladder carcinomas, and endometrial tumors [154]. Moreover, while the upregulation of PKCδ, in breast cancer patients, has been linked with the acquisition of resistance to tamoxifen [155] the overexpression of PKCδ in neuroblastoma cells induces apoptosis by sensitizing cells to etoposide [156].

2.2. PI3K/AKT. PI3K/AKT signaling contributes to tumorigenesis and to the expression of different cancer hallmarks. It facilitates the invasion and metastasis of cancer cells by promoting matrix metalloproteinase-9 (MMP-9) secretion [157] and by inducing the epithelial mesenchymal transition (EMT) [158] while it also increases telomerase activity and replication by activating telomerase reverse transcriptase (TERT) [159].

Furthermore, the PI3K/AKT signaling pathway has been found to activate NOX with production of ROS that on one hand may increase the genomic instability of cancer cells [160] and on the other hand may render cancer cells more sensitive to chemotherapy [161]. In addition, the upregulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor gene frequently deleted or mutated in many human cancers, has been demonstrated to reduce ROS generation by regulating the PI3K/AKT pathway [162]. ROS-dependent PTEN inactivation shifts the kinase-phosphatase balance in favor of tumorigenic tyrosine kinase receptor signaling through Akt, which inhibits apoptosis by phosphorylating and inactivating several targets, including Bad, forkhead transcription factors, and c-Raf and caspase-9 [163].

2.3. Apoptosis Signal-Regulating Kinase 1 (ASK1) and p38 MAPK. Apoptosis signal-regulating kinase 1 (ASK1) has been shown to act as a redox sensor by mediating the sustained activation of JNK and p38MAPK [164] resulting in apoptosis upon oxidative stress conditions [165]. In its inactive state, ASK1 is coupled to the reduced form of Trx 1 that induces its ubiquitination and degradation [166].

As above reported, p38 MAPK is able to inhibit tumor initiation by inducing apoptosis, by regulating cell cycle progression, and/or by inducing premature senescence of primary cells [167] This protein kinase contains four active cysteine residues that can be potentially oxidized. Although the activation of p38α is normally associated with antiproliferative functions [168, 169], several studies indicate that p38α can positively modulate cancer progression [170] as observed in malignant hematopoietic cells [171] and in other tumor cell lines [172]. Consistent with the prooncogenic role of p38MAPK, the inhibition of p38MAPK activity has been found to impair the proliferation and anchorage-independent growth of neuroblastoma cells [173].

2.4. Ataxia Telangiectasia Mutated (ATM) Kinase. A critical enzyme in maintaining genome stability is ATM, which can regulate DNA damage repair [174]. In fact, ATM upregulates the glucose-6-phosphate dehydrogenase to promote NADPH production and thus reduces ROS levels [175]. In cancer stem cells (CSCs), the ATM signaling pathway is highly active. In CD44+/CD24− stem-like cells, compared with other cell populations from breast cancer, the expression of ATM was significantly increased [176] and the employment of an ATM inhibitor reversed their resistance to radiotherapy, suggesting the importance of ATM signaling in CSC formation [176].

3. Role of Transcription Factors as ROS Modulators in Carcinogenesis and Cancer Progression

Many transcription factors are key players in regulating several pathways involved in carcinogenesis and cancer progression. Through their binding to the gene promoter regions, they can transactivate or repress the expression of antioxidant genes leading to the alteration in redox state and changes in proliferation, growth suppression, differentiation, and senescence.

3.1. p53. *p53* functions as a transcription factor able to activate or repress a large number of target genes that are involved in cell cycle control, DNA repair, apoptosis, and cellular stress responses [177]. It is kept at low levels by several E3 ubiquitin ligases, such as Mdm2, responsible for its degradation [178], and it is stabilized by posttranslational modifications such as phosphorylation, acetylation, and methylation [179, 180].

p53 has a controversial role in ROS regulation as it can promote both pro- and antioxidant responses [174].

Stress-induced p53 activation leads to the upregulation of several genes encoding ROS-generating enzymes, such as NQO1 (quinone oxidoreductase) [181] and proline oxidase (POX) [182], and redox-active proteins, including Bax and Puma. In particular, p53-induced ROS overproduction may be due to the overexpression of Puma, a critical mediator of mitochondrial membrane impairment [183], to the transcriptional activation of p67phox, a component of NADPH oxidase responsible for $O_2^{\bullet-}$ production [184] and to the action of p66Shc which oxidizes cytochrome c and affects mitochondrial permeability [149].

Moreover, the prooxidant activity of p53 has been found to be modulated by several genes named PIG1–13 (p53-inducible genes 1–13) which are able to encode redox-active

proteins [181]. In particular, PIG1, a member of the galectin family, is involved in superoxide production; PIG3, homolog of NADPH-quinone oxidoreductase, is a potent ROS generator and PIG8, a human homolog of mouse E-24 gene, is a quinone able to regulate ROS [181].

In contrast, p53 is also able to transactivate different genes controlling antioxidant response in order to maintain ROS production at nontoxic levels [185]. In fact, p53 has been found to activate MnSOD expression *via* the direct recognition of the MnSOD human gene promoter [186] and to induce the expression of heme-oxygenase-1 (HO-1) by directly binding to the HO-1 promoter, favoring cell survival [187].

Another important antioxidant target of p53 is Tp53-induced glycolysis and apoptosis regulator (TIGAR) [188]. TIGAR encodes a protein that is similar to the glycolytic enzyme fructose-2,6-bisphosphatase, which degrades fructose-2,6-bisphosphate [189]. A decrease in fructose-2,6-bisphosphate levels inhibits the activity of the rate-limiting enzyme phosphofructokinase I (PFK1), thereby blocking glycolysis and promoting the shuttling of metabolites to the pentose phosphate pathway (PPP). By upregulating TIGAR, p53 amplifies PPP-mediated NADPH production that is required by glutathione reductase in order to convert GSSG to GSH. A third important antioxidant target of p53 is glutaminase 2 (GLS2) that converts glutamine to glutamate which is subsequently converted to GSH *via* GCLC and GCLM [190].

3.2. Nrf2. *Nrf2* is a transcription factor that controls not only the expression of antioxidants as well as phase I and phase II drug metabolizing systems, but also multidrug-resistance-associated protein transporters [58]. In a resting state, Nrf2 is sequestered in the cytoplasm through the binding with Keap1, responsible for Nrf2 ubiquitination and proteasomal degradation *via* Cul3. Oxidative/electrophilic stress causes a conformational change in Keap1-Cul3 by acting on specific residues in Keap1, leading to Nrf2 dissociation. Thus, Nrf2 translocates to the nucleus where it dimerizes with a small Maf protein and binds to the antioxidant response element (ARE) sequence within regulatory regions of a wide variety of target genes [191, 192]. In fact, Nrf2 is essential for the expression of stress-responsive or cytoprotective enzymes such as NQO1, SODs, HO-1, catalase, and Trx. In addition, Nrf2 activation regulates GSH levels and metabolism by inducing the expression of GCL, GS, GSH S-transferases (GSTs), GR, and GPx [193, 194].

Several mechanisms have been shown to be involved in the constitutive activation of Nrf2 in cancer cells, mainly gain-of-function mutations in Nrf2 and loss-of-function mutations in Keap1 [195–198]. Shibata et al. [199] have reported that Keap1 and Nrf2 mutations, in lung cancer, are responsible for the upregulation of ARE-modulated genes, which favor cancer promotion and/or progression [58]. Recently, these alterations of Keap1/Nrf2 pathway have been considered among the potential novel targets for the treatment of lung adenocarcinoma [200].

Among Nrf2 target genes glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, transketolase, and transaldolase I are responsible for NADPH and purine regeneration and then accelerate cancer cell proliferation [201]. Moreover, Nrf2 is directly involved in the basal expression of the p53 inhibitor Mdm2, through the binding to the ARE sequence located in the first intron of this gene, and inhibits cell death [202]. Cancer cells with high levels of Nrf2 have been shown to be less sensitive to etoposide, cisplatin, and doxorubicin [203] and our studies demonstrated that activation of Nrf2 and of its target genes plays a key role in the resistance of neuroblastoma cells to GSH depletion or proteasome inhibition [85, 204].

3.3. NF-κB. The transcriptionfactor *NF-κB* plays a critical role in cell survival, proliferation, immunity, and inflammation [205]. In stimulated cells, I-κB, an endogenous inhibitor able to retain NF-κB in the cytoplasm, is phosphorylated by I-κB kinase (IKK) which leads to I-κB ubiquitination and proteasomal degradation and induces NF-κB translocation to the nucleus where it can modulate the transcription of its target genes [206]. Morgan and Liu showed that ROS may regulate NF-κB activation to express antioxidant genes coding MnSOD, Cu,Zn-SOD, catalase, Trx, GST-pi, HO-1, and GPx [207]. NF-κB is also involved in the regulation of some enzymes catalyzing ROS production such as NOX2, xanthine oxidoreductase, NOS, and COX-2 [208].

NF-κB activation leads to the development and/or progression of cancer by upregulating several genes involved in cell transformation, proliferation, and angiogenesis [209]. In this regard, it has been found that NF-κB activation and ROS production promote the progression of hepatocellular carcinoma [210] and the initiation of colorectal cancer [211]. Moreover, as observed in high-risk myelodysplastic syndrome and in AML patients, NF-κB activation, due to the constitutive activation of ATM [212], is critical for the survival of human leukemia cells [213] by increasing MnSOD activity, reducing ROS levels and inhibiting oxidative cell death.

3.4. HIF-1. Hypoxia-inducible factor (*HIF-1*) is a heterodimeric transcription factor composed of an α-subunit (HIF-1α) and a β-subunit (HIF-1β) [214]. The expression of HIF-1α is mainly regulated at the posttranslational level in an oxygen-dependent manner and is largely responsible for the regulation of HIF-1 activity [215].

It has been demonstrated that HIF-1α interacts with the HIF-1β and acts as a transcription factor able to induce the expression of genes involved in metabolic adaptation, such as hexokinase II (HK II) and pyruvate dehydrogenase kinase 1 (PDK1) [216], and the expression of genes involved in improving oxygen availability [217, 218] and shifting the glucose metabolism from mitochondrial oxidative phosphorylation to anaerobic glycolysis [219].

In addition, it has been demonstrated that ROS, *via* the modulation of PI3K/AKT and ERK pathways, are able to activate HIF-1 in hypoxic tumors [220]. In fact, HIF-1 overexpression correlates with poor outcomes in patients with head, neck, nasopharyngeal, colorectal, pancreatic, breast, cervical, bone, endometrial, ovarian, bladder, glial, and gastric cancers [9] and it is associated with refractiveness to conventional therapies [221].

Table 1: ROS modulating drugs undergoing clinical trials in oncology.

Drug	Mechanism of action	Cancer type	Outcome	Ref.
L-Buthionine-sulfoximine	Inhibits GSH synthesis; activates PKCδ	Neuroblastoma Melanoma	Efficacious *in vitro*	[67–73]
Menadione	Depletes GSH; activates ERK1/2 and p38MAPK	Gastrointestinal and lung cancer	Under clinical trial	[74–77]
Imexon	Depletes intracellular thiols; increases AP-1 and Nrf2-DNA binding activity	Advanced breast cancer; NSCLC; prostate and pancreatic tumors	Efficacious	[78–82]
Disulfiram	Oxidizes GSH and inhibits proteasome; activates JNK; inhibits Nrf2 and NF-κB	Metastatic melanoma; liver cancer	Under clinical trial	[68, 83, 84]
Bortezomib	Inhibits proteasome activity; activates NF-κB; activates Nrf2 and upregulates HO-1	Myeloma, leukemia, AML, myelodysplastic syndrome, neuroblastoma, prostate cancer	Under clinical trial	[85–90]
NOV-002	Oxidizes GSH and induces S-glutathionylation	NSCLC; breast and ovarian cancer	Efficacious	[91–93]
Ezatiostat	Inhibits GST-P1 and activates JNK/ERK	Myelodysplastic syndrome	Under clinical trial	[94]
PX-12	Inactivates Trx-1	Advanced solid tumors	Efficacious	[95–97]
Dimesna	Targets Trx and Grx	Ovarian carcinoma, NSCLC	Efficacious	[95, 98, 99]
Motexafin gadolinium	Inhibits Trx	Pancreatic, biliary and haematological cancer, renal carcinoma	Under clinical trial	[97, 100–102]
Arsenic trioxide	Oxidizes GSH and thiol enzymes	APL, melanoma	Efficacious	[68]

4. ROS-Modulating Agents Undergoing Clinical Trials in Oncology

Several anticancer drugs are able to produce high levels of ROS leading to DNA damage and apoptosis [222, 223] that can be further stimulated by depleting cancer cell of GSH. The following compounds alter the intracellular redox state and induce cell death; for this reason some of them have been employed to improve the cytotoxic effects of conventional drugs (Table 1).

L-Buthionine-S,R-sulfoximine (BSO) induces oxidative stress by inhibiting GSH biosynthesis [67] and it synergizes with cytotoxic chemotherapeutic agents, including arsenic trioxide, cisplatin, doxorubicin, and melphalan [68]. Our studies have demonstrated that BSO-induced ROS overproduction and apoptosis of neuroblastoma cells is mediated by PKCδ activation [69–72] which is crucial for the sensitization of cancer cells to BSO and to etoposide [156]. In this context, BSO plus melphalan is currently undergoing clinical evaluation in children with neuroblastoma and in patients with persistent or recurrent stage III malignant melanoma [73].

Menadione (also known as vitamin K3) is a synthetic derivative of vitamins K1 and K2. The oxidative stress generated by menadione is dose-dependent and is due to GSH depletion capable of inducing cell death [74]. Moreover, a recent study reported that menadione analogues at submicromolar concentrations activate apoptosis of myeloid leukemia cells *via* the activation of ERK 1/2 and p38MAPK [75]. *In vitro* investigations have led to the employment of menadione in different human trials in patients with gastrointestinal and lung cancer [76, 77].

Imexon is a prooxidant small molecule that depletes intracellular thiols generating oxidative stress and, subsequently, induces apoptosis [78]. Preclinical studies have demonstrated that imexon treatment increases nuclear Nrf2 levels and AP-1-DNA binding activity in myeloma cells and breast cancer cells [79]. These findings suggest that imexon leads to an adaptive response to oxidative stress involving upregulation of several antioxidant genes such as Nrf2 [79] and CuZnSOD [224]. The increased antioxidant gene expression and the enhancement of GSH levels in myeloma cell lines have been associated with the phenomenon of resistance to imexon [225].

Successful phase I trials have been completed in combination with cytotoxic chemotherapy in advanced breast, non-small cell lung cancer (NSCLC), prostate [80], and pancreatic [81] tumors. In addition, a phase II study has been carried out in patients with relapsed/refractory B-cell non-Hodgkin lymphoma [82].

Disulfiram is an acetaldehyde dehydrogenase inhibitor that induces apoptosis *via* GSH oxidation and proteasome inhibition [68, 83]. Preclinical studies have demonstrated that disulfiram-induced apoptosis of human melanoma cells [226] and of lymphoid malignant cells is mediated by JNK activation and Nrf2 and NF-κB inhibition [84]. A phase I/II trial with disulfiram has recently been completed in patients with metastatic melanoma and other early-phase studies are ongoing in NSCLC and treatment-refractory liver tumors [68].

Bortezomib is a proteasome inhibitor that blocks inducible I-κB degradation and consequently activates NF-κB [86, 87]. It induces cell cycle arrest and apoptosis by preventing the degradation of p21/waf1, p53, and Bax [227]. Bortezomib has been extensively studied either alone or in combination

with other agents for the treatment of multiple myeloma [86] and of chronic lymphocytic leukemia (CLL) [88]. In addition, bortezomib has been demonstrated to exert cytotoxicity by increasing ROS production [228] and, in this context, our recent studies have shown that bortezomib treatment of human neuroblastoma cells is less effective as a consequence of Nrf2-mediated HO-1 upregulation [85]. Moreover, it has been reported that bortezomib induces HO-1 activity in multiple myeloma *via* the endoplasmic reticulum stress pathway and that HO-1 nuclear translocation confers resistance to chemotherapy and induces genetic instability in cancer cells [65].

NOV-002 is a product containing oxidized glutathione that alters the GSH/GSSG ratio and induces S-glutathionylation [91]. NOV-002-induced S-glutathionylation has been shown to have inhibitory effects on proliferation, survival and invasion of myeloid cell lines and significantly increases the efficacy of cyclophosphamide chemotherapy in a murine model of colon cancer [229]. NOV-002 has been most extensively studied with a phase III trial (NCT00347412) completed in the treatment of advanced NSCLC [92] and data is available from phase II trials in breast and ovarian cancers [230]. In a randomized phase II trial, NOV-002 in combination with standard chemotherapy has shown promising effects in patients with stage IIIb/IV of NSCLC [231]. Positive results were also obtained from a phase II trial in patients with neo adjuvant breast cancer therapy [93].

Ezatiostat hydrochloride (TLK199) is a GSH analogue that inhibits GST P1-1 leading to JNK/ERK activation and inducing apoptosis of malignant cells [94]. Treatment of leukemia cell lines with ezatiostat has been demonstrated to induce myeloblast differentiation without affecting myelopoiesis [94]. Ezatiostat has been evaluated in multiple phase I and phase II clinical trials in myelodysplastic syndrome (MDS) characterized by ineffective hematopoiesis presenting with anemia and, in some cases, neutropenia and thrombocytopenia [94].

PX-12 (1-methylpropyl 2-imidazolyl disulfide) irreversibly inactivates Trx-1 which is overexpressed in many human cancers and it is associated with aggressive tumor growth and decreased patient survival [95]. Furthermore, the antitumor activity of PX-12 is also due to a reduction of VEGF in cancer patient plasma [95] and it can be synergistically enhanced after combination of PX-12 with 5-FU in HCC cells [96]. PX-12 has shown promising pharmacokinetics and pharmacodynamics in phase Ib trials in patients with advanced solid tumors refractory to chemotherapy [97].

Dimesna (BNP7787, disodium 2,2-dithio-bis-ethane sulfonate) is a novel chemoprotective disulfide compound that targets Trx and Grx which are overexpressed in many tumors [98, 99]. Dimesna has been employed in the treatment of various solid tumors, including ovarian carcinoma and NSCLC. In addition, it is currently undergoing phase III clinical trials (NCT00966914), in combination with first-line taxane and platinum chemotherapy, in patients with diagnosed or relapsed advanced (stage IIIB/IV) NSCLC adenocarcinoma.

Motexafin gadolinium (MGd) is a Trx inhibitor that reversibly accepts electrons from NADPH, NADH, GSH, and ascorbate, with subsequent electron transfer to molecular oxygen [232]. Preclinical studies have shown that MGd alone has a proapoptotic effect in multiple myeloma, non-Hodgkin lymphoma, and chronic lymphocytic leukemia [233]. MGd has been tested in a phase I trial in patients with locally advanced pancreatic or biliary cancers [97], and in a phase II trial in renal cell carcinoma [100] and in haematological malignancies [101].

Arsenic trioxide (As_2O_3) is an inorganic compound that has antiproliferative and apoptogenic effects on cancer cells by inducing oxidation of cysteine residues in GSH and thiol enzymes [68]. It has been approved by the European Medicines Agency and US Food and Drug Administration, for induction and consolidation of chemotherapy in adults with relapsed/refractory acute promyelocytic leukemia (APL). Moreover, As_2O_3, in combination with disulfiram, is being evaluated as a second-line therapy in phase I trials (NCT00571116) in patients with metastatic melanoma.

5. Conclusions

The modulation of oxidative stress is considered an important factor in the development of cancer and in the response of tumor cells to therapy [189]. As shown in this review, high ROS levels in cancer cells are a consequence of alterations in cellular metabolism and their overproduction is counteracted by elevated defense mechanisms (Figure 2).

Among antioxidants, GSH is essential for maintaining a correct redox balance, has a crucial role in the protection of cancer cells from oxidative stress, and ensures cell survival in both hypoxia and nutrient deprivation that are present in solid malignant tumors [48]. For this reason, combinations of GSH antagonists or other antioxidant inhibitors with radio or chemotherapy may be useful for killing cancer cells. This "epigenetic-genetic" therapeutic approach is in sharp contrast to the conventional strategy of targeting oncogenes and oncosuppressors, an approach that has turned out to be uneffective also for the frequent gene mutations.

As reported in this review, many of these genes are redox-sensitive transcription factors that are involved in proliferation, angiogenesis, and metastasis and are able to induce a common set of cell stress adaptive responses, thus providing a survival advantage.

Therefore, the redox-signaling pathways underlying these adaptations may represent the most critical weak point in many cancers and the signaling molecules that mediate these changes could be the next important targets for future anticancer drug discovery research.

Recently, as summarized in Table 2, many clinical trials with modulators of kinases or transcription factors associated with conventional therapy are ongoing. Although the results of some of these combined strategies seem to be promising, further studies are needed in order to identify specific markers for a more personalized therapy and to minimize the side toxic effects.

Table 2: Modulators of redox signaling pathways employed in combination with anticancer agents and their effects.

Drug	Mechanism of action	Cancer type	Outcome	Ref.
Trametinib	MEK inhibitor	Melanoma	Efficacious	[103]
Selumetinib	MEK inhibitor	Thyroid, ovarian cancer	Efficacious	[104–106]
Tamoxifen	PKC inhibitor	Gliomas, breast cancer	Efficacious	[107–111]
Perifosine	Akt, MAPK and JNK inhibitor	Haematologic tumors, myeloma	Efficacious	[112–116]
Sulfasalazine	NF-κB inhibitor	Colorectal cancer	Efficacious	[117, 118]
Nelvinavir	Decreases HIF-1α	Adenoid cystic carcinoma, pancreatic cancer, NSCLC	Efficacious	[119–122]
Topotecan	HIF-1 and Topoisomerase I inhibitor	Endometrial and cervical cancer	Efficacious	[123, 124]
Aprinocarsen	Antisense oligonucleotide against PKC-α	Lymphoma, breast cancer	Contrasting results	[125–127]
Midostaurin	Multitarget inhibitor of PKCs, VEGFR2, PDGFR	AML, melanoma	Contrasting results	[128, 129]
MK-2206	Akt and PI3K inhibitor	Gastric, pancreatic and breast cancer	Under clinical trial	[130]
Serdemetan	mdm2 inhibitor	Refractory solid tumors	Under clinical trial	[131]
PRIMA-1 and PRIMA-1^{MET}	Reverse the oncogenic properties of mutant p53	Ovarian cancer	Under clinical trial	[132, 133]
AMG 232	mdm2-p53 interactions inhibitor	Melanoma, myeloma, myeloid leukemia	Under clinical trial	[134]

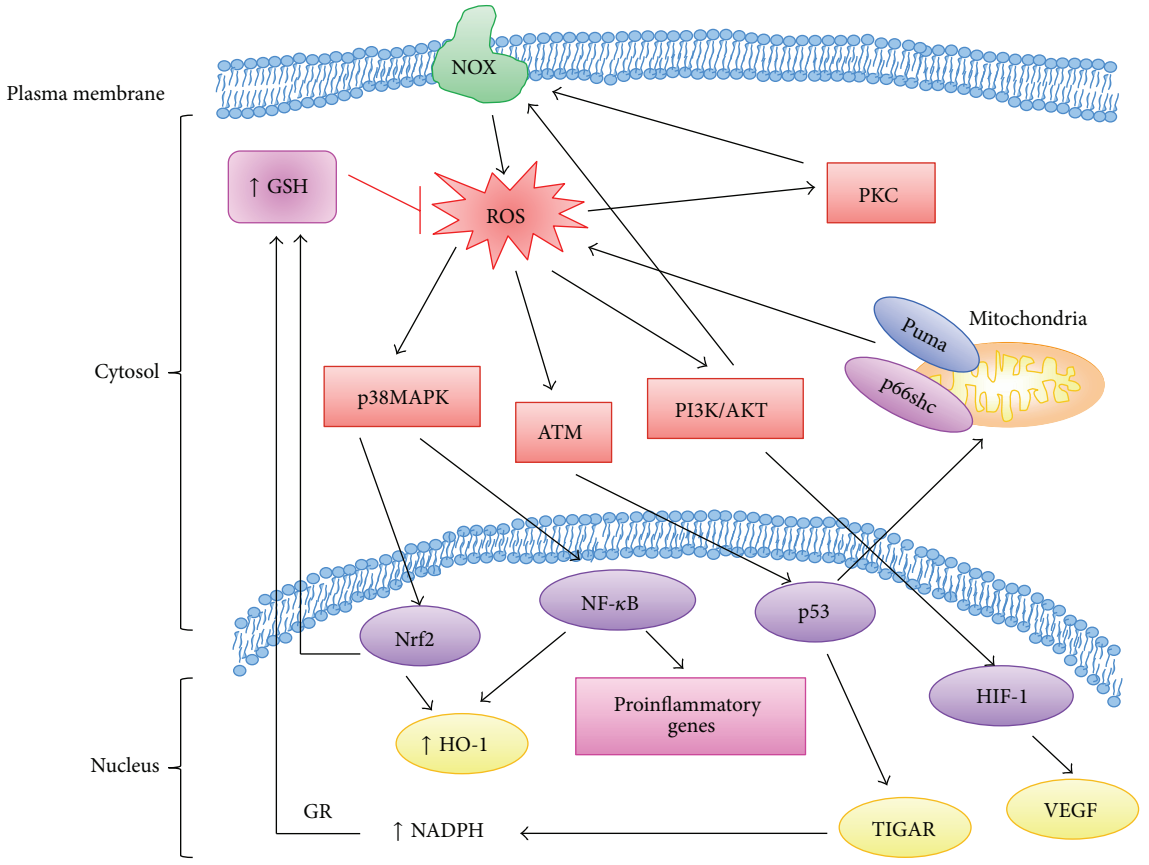

Figure 2: Redox-signaling pathways that are involved in cancer growth and progression. Cancer cells escape cell death and damage induced by high ROS levels by increasing their antioxidant defenses such as GSH that contribute to lower the amount of ROS. ROS are produced by NOX in the plasma membrane and by mitochondria, and at low levels they act as second messengers by activating many protein kinases (PI3/Akt, p38 MAPK, and ATM) and transcription factors (Nrf2, NF-κB, p53, and HIF-1) able to contribute to cancer cell survival by stimulating cell proliferation, inflammation, and angiogenesis. GR, glutathione reductase.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Effects of Oxidative Stress on Mesenchymal Stem Cell Biology

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Mesenchymal stromal/stem cells (MSCs) are multipotent stem cells present in most fetal and adult tissues. *Ex vivo* culture-expanded MSCs are being investigated for tissue repair and immune modulation, but their full clinical potential is far from realization. Here we review the role of oxidative stress in MSC biology, as their longevity and functions are affected by oxidative stress. In general, increased reactive oxygen species (ROS) inhibit MSC proliferation, increase senescence, enhance adipogenic but reduce osteogenic differentiation, and inhibit MSC immunomodulation. Furthermore, aging, senescence, and oxidative stress reduce their *ex vivo* expansion, which is critical for their clinical applications. Modulation of sirtuin expression and activity may represent a method to reduce oxidative stress in MSCs. These findings have important implications in the clinical utility of MSCs for degenerative and immunological based conditions. Further study of oxidative stress in MSCs is imperative in order to enhance MSC *ex vivo* expansion and *in vivo* engraftment, function, and longevity.

1. Introduction

Mesenchymal stromal/stem cells (MSCs) are multipotent cells characterized by their ability to differentiate into adipocytes, chondrocytes, and osteoblasts, their expression of surface markers CD73, CD90, and CD105, and their lack of hematopoietic lineage markers [1–4]. MSCs were initially studied for their ability to support hematopoietic stem cells in the bone marrow, but now they are being studied for their regenerative and immunomodulatory properties, as they home to injured tissues and contribute to tissue repair and suppression of inflammatory damage [5, 6]. MSCs have been isolated from a number of different tissues, including bone marrow, adipose, heart, vocal cord, and pancreatic islets [7–10]. They are also present in the tumor microenvironment, where they support the growth of tumor cells, activate mitogen and stress signaling, and increase resistance to cytotoxins [11–13].

MSCs have immunomodulatory properties and suppress the proliferation of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and NK cells, while they induce the proliferation of regulatory T cells (Tregs) [5, 6, 14–21]. In addition, MSCs alternatively activate macrophages and bias them toward an immunosuppressive M2 phenotype [22]. Further evidence of MSCs creating a more anti-inflammatory state includes the following actions: induction of type 1 dendritic cells to reduce TNFα secretion and type 2 dendritic cells to increase IL-10 secretion [16, 23, 24]; causing Th1 cells to decrease IFNγ secretion and Th2 cells to increase IL-4 secretion [16]; decreasing NK cell proliferation and IFNγ secretion [14]; and converting macrophages to an anti-inflammatory immunophenotype [22]. At the same time, MSCs express low levels of MHC class I and no MHC class 2 and costimulatory molecules CD40, CD80, and CD86, preventing alloreactive antibody production and destruction [25, 26]. Due to these multimodal properties, MSCs are being studied for their potential use in different modes

of therapy: (1) produce new tissues (e.g., cartilage repair); (2) assist with healing tissue damage (e.g., cardiovascular disease); (3) improve engraftment of other cells and tissues (e.g., hematopoietic cells and pancreatic islets); and (4) treat immune based pathologies (e.g., graft versus host disease, GVHD) [27–37].

MSCs have also been extensively studied because of their ability to differentiate into adipocytes, chondrocytes, and osteoblasts, which has significant potential in the field of regenerative medicine. However, MSCs are much farther from reaching clinical utility in regenerative medicine as compared to their utility in immunomodulation. Their chondrogenic ability has arguably gained the most attention [38] and could be utilized to aid in reconstitution of connective tissue loss in many joints, namely, the knee, which is crucial given the fact that chondrocytes are terminally differentiated, quiescent cells and do not regenerate damaged tissue.

While MSCs have been utilized with some success in the clinic, there is room for improvement in order for them to reach their full clinical potential. First, MSCs are rare cells *in situ* and must be expanded *ex vivo* in order to be utilized in the clinic. However, MSCs undergo replicative senescence, limiting the number of divisions [39–41]. Furthermore, this replicative senescence also compromises their immunomodulatory and differentiation functions and possibly their clinical activity against GVHD and other inflammatory pathologies [42, 43]. In addition, there is a lack of a well-defined and accepted potency assay to functionally assess MSC products [37, 44].

Another problem is the loss of transplanted MSCs at the site of graft, particularly after *ex vivo* culture [45, 46], which could possibly be due to loss of chemokine receptors [47]. Reactive oxygen species (ROS) and nonspecific inflammation generated at the ischemic site of injury have been hypothesized to lead to loss of transplanted MSCs from this site [48–50]. Therefore, there is great need to identify methods to manipulate MSCs to reduce ROS in both the MSCs themselves during their culture expansion production phase and in the injured tissue microenvironment in order to promote MSC engraftment and enhance tissue repair. First, this requires an understanding of the contributions of oxidative stress to MSC biology.

2. Oxidative Stress and MSC Differentiation

Oxidative stress is characterized by deregulated production and/or scavenging of reactive oxygen and nitrogen species (ROS and RNS, resp.). ROS are primarily generated from mitochondrial complexes I and III and NADPH oxidase isoform NOX4 during MSC differentiation [51]. The accumulation of free radicals can damage essentially all biomolecules, including DNA, protein, and lipids. High ROS levels cause cellular damage and dysfunction, but it is thought that a low basal level of ROS is necessary and advantageous in order to maintain cellular proliferation, differentiation, and survival [52–54]. Indeed, at baseline, MSCs have low levels of ROS and high levels of glutathione, the major cellular antioxidant [55]; however, other reports suggest that MSCs have low antioxidant activity and are more sensitive to oxidative stress compared to more differentiated cell types [56, 57]. In MSCs, excess ROS or exogenous addition of H_2O_2 can impair self-renewal, differentiation capacity, and proliferation [57–61]; concordantly, antioxidants stimulate MSC proliferation [62].

With regard to osteogenic differentiation, most studies suggest that ROS inhibit osteogenic differentiation [63]. Furthermore, addition of exogenous H_2O_2 reduces osteogenic differentiation in human and murine MSCs and osteoblast precursors [63–65]. In addition, MSCs from older donors demonstrate decreased osteogenic potential [66]. *In vitro* induction of osteogenesis in human MSCs is associated with an upregulation of mtDNA copy number, protein subunits of respiratory enzymes, superoxide dismutase 2 (SOD2, alias MnSOD), catalase oxygen consumption rate, and antioxidant enzymes, but a decrease in ROS [63]; undifferentiated MSCs showed higher levels of glycolytic enzymes and a higher lactate production rate, suggesting that MSCs rely more on glycolysis for energy supply in comparison with MSC-differentiated osteoblasts, which rely more on oxidative mitochondrial metabolism. These findings support the idea that ROS and oxidative stress must decrease to allow for osteogenic differentiation to proceed. However, it appears that at least a basal level of ROS may be required, as some reports show that ROS enhance calcification and osteogenesis [67]; one caveat is that this study investigated murine vascular smooth muscle cells, which could explain the difference. In summary, ROS and aging inhibit MSC osteogenesis.

With regard to adipogenesis, ROS increase as MSCs differentiate into adipocytes, but it is unclear whether this is a cause or consequence of adipogenesis. Antioxidant enzymes such as SOD, catalase, and GPX are upregulated during adipogenesis in human MSCs [68]. It has been reported that ROS and the addition of exogenous H_2O_2 induce adipogenesis in human and murine MSCs and adipocyte precursors [51, 68, 69], lending credence for the idea that ROS play a causal role in adipogenesis. Furthermore, this effect of H_2O_2 is dose-dependent, as higher doses of H_2O_2 increased adipogenesis [70]. Consistent with ROS stimulating adipogenesis, the ROS scavenger *N*-acetylcysteine (NAC) inhibited adipogenesis in the mouse MSC cell line 10T1/2 [71]. In addition, it has been demonstrated that ROS generated by mitochondrial complex III are imperative for the activation of adipogenic transcription factors [72]. Similar to osteogenic differentiation, mitochondrial biogenesis and oxygen consumption increase significantly during adipogenesis [73, 74]. Additionally, inhibiting mitochondrial respiration significantly suppresses adipogenic differentiation [73], which makes sense for two reasons: (1) mitochondrial biogenesis and metabolism are thought to be important for MSC differentiation [74] and (2) inhibiting mitochondrial metabolism reduces ROS, and ROS are thought to stimulate adipogenesis. There is an increase in SOD3 expression with the differentiation of human MSCs into adipocytes [75] and during the early stages of adipogenic differentiation in 3T3-L1 cells [54]. However, there are some reports that contradict the idea that ROS stimulate adipogenesis; these reports demonstrate that aging and senescence, which are often associated with higher oxidative stress, decrease adipogenic differentiation [41, 76–78].

Additionally, RNAi-mediated depletion of MnSOD, which results in higher ROS, reduces the expression of late adipogenesis markers such as adiponectin and fatty acid-binding protein 4 (FABP4) [79]. Nevertheless, the prevailing view is that ROS and aging enhance adipogenesis [54].

ROS generally increase during chondrogenesis, and ROS generated by NADPH oxidases 2 and 4 are necessary for chondrogenic differentiation of murine primary chondrocytes and the ATDC5 cell line [80]. Consistent with this, SOD3 levels were reduced upon chondrogenesis [75]; SOD3 is known to help reduce ROS in the extracellular matrix. Furthermore, ROS scavenging with NAC blocked chondrogenic differentiation [80]. Consistent with this, increasing ROS levels stimulated chondrocyte hypertrophy, and this effect was inhibited by NAC [81].

3. Oxidative Stress and MSC Immunomodulation

Evidence of the direct role of oxidative stress in MSC immunomodulation is lacking. However, we do know that as MSCs are expanded *ex vivo*, proliferation decreases, oxidative stress increases, the level of certain surface antigens decreases (e.g., CD13, CD29, and CD44), and the ability to suppress T cell proliferation diminishes [41, 82, 83]. Similarly, MSCs from older donors, which also likely have greater oxidative stress, have reduced capacity to inhibit T cell proliferation [84, 85]. In addition, MSCs from human patients with atherosclerosis and type 2 diabetes, two diseases associated with elevated oxidative stress, have reduced ability to inhibit T cell proliferation [85]. However, some studies conflict with the assertion that donor age negatively impacts MSC suppression of T cell proliferation [86, 87]; one of these studies analyzed 53 human donors ranging within 13–80 years demonstrated no significant correlation between age and T cell suppression capability [86].

As most of the clinical uses of MSCs are dependent on their immunomodulatory properties, it will be important to continue to elucidate how oxidative stress affects MSC immunomodulation and whether or not modulating ROS and oxidative stress can enhance MSC *ex vivo* expansion, immunomodulation, and clinical utility.

4. Sirtuins, Oxidative Stress, and *Ex Vivo* Expansion of MSCs

Oxidative stress also affects *ex vivo* culture expansion and longevity of MSCs, which has implications for cell therapy. As MSCs are continuously passaged and grown *ex vivo*, they undergo replicative senescence, and proliferation decreases [39–42, 88, 89]. Aging and senescence are associated with greater oxidative stress, which limit the number of times that MSCs can be passaged and the quality of the cells [90, 91]. Therefore, there is great need to identify methods to prevent oxidative stress and replicative senescence in MSCs.

One potential method to reduce oxidative stress in MSCs is by modulating sirtuin expression and/or activity. Sirtuins are protein deacetylases that are thought to play evolutionarily conserved roles in lifespan extension [92–94]. Humans have seven sirtuins (SIRT1–7) that localize to distinct subcellular compartments and serve very distinct functions [95, 96]. In general, sirtuins are protective against age-related pathologies such as hearing loss [97], neurodegeneration [98], metabolic disease [99, 100], and cancer [101, 102]. Their roles in MSCs have not been fully elucidated and represent an interesting avenue of future research.

SIRT1, SIRT6, and SIRT7 localize to the nucleus. SIRT1 deacetylates a number of protein substrates including p53, DNA methyltransferase 1 (DNMT1), NF-κB, forkhead transcription factors, PGC-1α, and histones [103–108]; an unbiased mass spectrometry-based acetylome analysis has revealed many more potential substrates [109]. SIRT1 knockdown decreases MSC proliferation and differentiation and increases senescence, and the opposite occurs with SIRT1 overexpression [110]. Consistently, SIRT1 activation with resveratrol enhances MSC osteogenesis [111]. SIRT1 is downregulated during human embryonic stem cell (ESC) differentiation at both mRNA and protein levels [112]. Therefore, SIRT1 is crucial for stem cell maintenance and differentiation. SIRT6 deacetylates histones H3K9Ac and H3K56Ac [113, 114] and is an imperative regulator of metabolism, transcription, telomere maintenance, and DNA repair in response to oxidative stress [115, 116]. It ADP-ribosylates and thereby activates PARP1, allowing for efficient double-strand break repair in the face of oxidative stress [116]. Furthermore, SIRT6 rescues the decline of base excision repair and homologous recombination repair during replicative senescence in primary human fibroblast strains [117, 118]. A study of human dermal fibroblasts from older subjects demonstrated that reprogramming into induced pluripotent stem cells (iPSCs) with Yamanaka factors was less efficient than in fibroblasts from older subjects, but that adding SIRT6 improved the efficiency of reprogramming [119]. Specific to MSCs, knockdown of SIRT6 inhibited while overexpression enhanced osteogenesis in rat MSCs [120]. Recent studies have demonstrated the possibility of activating SIRT6 with long-chain fatty acids [121], which may represent a way to modulate MSC function and longevity. The final nuclear sirtuin, SIRT7, has been less well characterized but localizes to the nucleolus and regulates rDNA transcription [122]. This is dependent on the deacetylation of U3-55k, a component of the U3 snoRNP complex, and this deacetylation enhances rRNA transcription and processing [123]. It is also important for proliferation and inhibition of apoptosis [122], perhaps via deacetylation of p53 [124]. In addition, SIRT7 has been recently shown to promote the regenerate capacity of aged hematopoietic stem cells, as inactivation increased mitochondrial protein folding stress and reduced regenerative capacity [125].

SIRT3, SIRT4, and SIRT5 localize to the mitochondria, where approximately 90% of ROS are produced in mitochondria [126]. SIRT3 is the major mitochondrial deacetylase and reprograms mitochondrial metabolism away from carbohydrate metabolism in favor of more efficient electron transport, which is thought to result in reduced ROS production [127–130]. SIRT3 deacetylates and thereby activates isocitrate dehydrogenase 2 (IDH2), an enzyme that catalyzes the TCA cycle redox conversion of isocitrate to α-ketoglutarate and

serves as a major source of NADPH production [97, 131, 132]. SIRT3 also deacetylates and thereby activates superoxide dismutase 2 (SOD2, alias MnSOD), which also neutralizes ROS [133–135]. One of the only studies of SIRT3 on stem cell function demonstrated that SIRT3 is not required for hematopoietic stem cell (HSC) maintenance and tissue homeostasis at a young age in mice; however, SIRT3 is imperative in HSCs at an older age and under stress [136]. Importantly, SIRT3 expression decreases with aging, and this is accompanied by a concomitant decrease in SOD2 activity; overexpressing SIRT3 in these aged HSCs reduces oxidative stress and improves their regenerative capacity [136]. SIRT3 overexpression can also protect against low-oxygen and low-glucose stresses [137]. Similarly, SOD2 acetylation, a target of SIRT3 deacetylation, increases with age in rats and humans, which can be restored *in vitro* by adding recombinant SIRT3 [138]. The next mitochondrial sirtuin, SIRT4, mono-ADP-ribosylates and thereby inhibits glutamate dehydrogenase, which slows the conversion of glutamate to α-ketoglutarate [139–141]. SIRT4 is also a lipoamidase that hydrolyzes the lipoamide cofactors from the E2 component of the pyruvate dehydrogenase (PDH) complex, which reduces the activity of the complex [142]. Defects in the PDH complex have been shown to increase ROS and oxidative stress [143]. Similarly, SIRT4 and ROS are upregulated during replicative senescence and in response to DNA damage [144, 145]; however, one study shows that SIRT4 depletion reduces ROS [144] and therefore suggests that inhibiting SIRT4 may be a strategy to prevent oxidative stress in MSCs, while another believes that SIRT4 is required for appropriate recovery from cellular stresses [145]. Further study of SIRT4 in MSCs is warranted. The final mitochondrial sirtuin, SIRT5, is the major mitochondrial desuccinylase [146, 147]. It desuccinylates and activates SOD1 to facilitate the elimination of ROS [148]. SIRT5 has not been studied in stem cells, but based on these known functions, we hypothesize that SIRT5 helps maintain ROS at low levels to preserve stem cell function and longevity.

SIRT2 is a cytoplasmic sirtuin that deacetylates and therefore destabilizes α-tubulin [149]. Furthermore, SIRT2 deacetylates p300, a histone acetyltransferase crucial for many biological processes including cellular proliferation and differentiation, which increases the affinity of p300 for preinitation complexes [150]; in this way, SIRT2 may help control transcription of genes needed for MSC differentiation. SIRT2 gene expression increases with differentiation of mouse ESCs [151]. In stem cells, SIRT2 has been shown to inhibit the expression of keratin 19, which is a stem cell marker [152]. These two studies suggest that SIRT2 is more important for differentiation. However, it may be specific to the type of differentiation being discussed; downregulation of SIRT2 promotes 3T3L1 adipocyte differentiation [153], and SIRT2 knockdown in mouse ESCs promotes differentiation into mesodermal and endodermal tissues and diminishes differentiation into ectodermal tissues [151]. Lastly, SIRT2 may play a role in autophagy, the catabolic process that allows the cell to recycle damaged proteins and organelles and has been shown to be important for stem cell function, particularly in the face of oxidative stress [154]; however, more work needs to be done here, as the exact role of autophagy in MSC biology is unclear. Nevertheless, most reports suggest that SIRT2 inhibits autophagy. One report shows that depletion of SIRT2 activates autophagy [155]. Consistent with this, another study shows that the FoxO1 transcription factor is required for autophagy caused by oxidative stress, and that dissociation from SIRT2 increases FoxO1 acetylation and induction of autophagy [156]; this suggests that SIRT2 inhibits autophagy. Therefore, modulation of SIRT2 expression and activity is worth further pursuing.

5. Conclusions

MSCs have immense therapeutic potential; yet this potential has not been reached for a number of reasons. Perhaps one of these reasons is the effect of oxidative stress on MSC *ex vivo* expansion, leading to problems with *in vivo* function and engraftment. Therefore, there is great need to identify novel methods to optimize ROS levels in MSCs to enhance their immunomodulatory and regenerative abilities so that their full therapeutic potential can be realized. The sirtuins represent a potential way to achieve this and warrant further study in MSCs. Many of the sirtuins may help enhance our ability to expand MSCs *ex vivo* for eventual clinical use.

Abbreviations

ESC: Embryonic stem cell
IFNγ: Interferon gamma
iPSC: Induced pluripotent stem cell
MSC: Mesenchymal stromal/stem cell
GVHD: Graft versus host disease
NAC: *N*-Acetylcysteine
PPARγ: Peroxisome proliferator-activated receptor gamma
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SIRT: Sirtuin
SOD: Superoxide dismutase.

Disclosure

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Competing Interests

The authors declare that they have no competing interests.

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Review Article

NRF2, a Key Regulator of Antioxidants with Two Faces towards Cancer

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While reactive oxygen species (ROS) is generally considered harmful, a relevant amount of ROS is necessary for a number of cellular functions, including the intracellular signal transduction. In order to deal with an excessive amount of ROS, organisms are equipped with a sufficient amount of antioxidants together with NF-E2-related factor-2 (NRF2), a transcription factor that plays a key role in the protection of organisms against environmental or intracellular stresses. While the NRF2 activity has been generally viewed as beneficial to preserve the integrity of organisms, recent studies have demonstrated that cancer cells hijack the NRF2 activity to survive under the oxidative stress and, therefore, a close check must be kept on the NRF2 activity in cancer. In the present review, we briefly highlight important progresses in understanding the molecular mechanism, structure, and function of KEAP1 and NRF2 interaction. In addition, we provide general perspectives that justify conflicting views on the NRF2 activity in cancer.

1. Introduction

A growing body of evidence indicates that oxidative stress is responsible for the development of chronic diseases, such as cancer, diabetes, atherosclerosis, neurodegeneration, and aging [1, 2]. Oxidative stress results from a perturbation between the production and removal of reactive oxygen species (ROS). ROS refers to free radical and non-free-radical oxygenated molecules, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). The majority of exogenous ROS is generated in organisms after exposure to oxidants and electrophiles, such as pollutants, tobacco, smoke, drugs, and xenobiotics [3]. Ionizing radiation also generates ROS through the direct activation of water, a process termed radiolysis [4]. On the other hand, intracellular ROS can be generated from many sources: cytosolic NAPDH oxidases (NOXs) take part in the regulated generation of ROS, while ROS is generated as by-product of the oxidative phosphorylation in mitochondria [5, 6]. Other significant sources of cellular ROS production include xanthine oxidase [7]. Oxidative metabolic process in peroxisomes cannot be negligible as well [8]. It is known that low levels of intracellular ROS are necessary to carry out a number of important physiological functions, such as intracellular signal transduction and host defense against microorganisms. However, high levels of intracellular ROS are considered detrimental because they impart significant oxidative damage on cellular macromolecules, such as nucleotides, lipid, and proteins [9].

In order to fight against the oxidative stress, organisms create a highly reducing intracellular environment by maintaining a large amount of antioxidant molecules, such as reduced glutathione (GSH) and soluble vitamins (vitamin C and vitamin E) [10, 11]. During evolution, organisms have also developed a variety of cellular defensive enzymes, such as alcohol dehydrogenase and aldehyde dehydrogenase to ATP binding cassette (ABC) transporters that mediate the adaptive responses to survive under the oxidative environment and xenobiotic assault. The first defense metabolism, for example, phase I reaction, is carried out by cytochrome P450 enzymes that catalyze the monooxygenation reaction of substrates [12], for example, the insertion of one atom of oxygen into the aliphatic

position of an organic substrate with the other oxygen atom reduced to water. A group of enzymes, including uridine 5′-diphospho-glucuronosyltransferases (UGT), glutathione S-transferases (GST), or sulfotransferases, carry out the subsequent reaction, referred to as phase II reaction, in which the hydroxylated metabolites are further conjugated with soluble molecules, such as glutathione, sulfate, glycine, and glucuronic acid [13]. Finally, the addition of these large anionic groups produces metabolites completely soluble in cells, which can be actively transported out, a process referred to as phase III reaction [14].

2. The Triad of ROS: Superoxide (O_2^-), Hydrogen Peroxide (H_2O_2), and Hydroxyl Radical (OH^-) and Their Biological Targets for Signaling

The first type of ROS, superoxide (O_2^-), is generated by the one-electron reduction of O_2 through the electron transport chain in mitochondria. Superoxide can also be produced by a family of NADPH oxidases (NOXs), using oxygen and NADPH as substrates [15], in which superoxide is rapidly disposed. The second type of ROS, hydrogen peroxide (H_2O_2), is rapidly formed in the cytoplasm, from O_2^- by superoxide dismutase 1 (SOD1), while extracellular SOD (SOD3) produces H_2O_2 outside the cell. Superoxide produced in the matrix of mitochondria is converted into H_2O_2 by superoxide dismutase 2 (SOD2) [16]. In addition, H_2O_2 can be produced as a by-product during β-oxidation of fatty acids in the peroxisome or by a wide array of cellular enzymes, including cytochrome P450s [17]. Finally, H_2O_2 is converted into harmless water and O_2 by various cellular antioxidant enzymes, such as peroxiredoxins (PRXs), glutathione peroxidases (GPXs), and catalases (CAT). While PRXs and GPXs are present in most cell compartments, catalase is confined to the peroxisome. In addition, PRXs are among the most abundant enzymes and have been suspected of degrading most of hydrogen peroxide with a slow rate whereas GPXs seem to be less abundant but have higher rate constants [18].

It is noteworthy that H_2O_2 is a bona fide signaling molecule. H_2O_2 is stable and readily diffuses across the membrane, thereby oxidizing cysteine residues of redox-sensitive proteins. Susceptible cysteine residues in redox-sensitive proteins exist as a thiolate anion in a physiological pH and they can be reversibly oxidized by hydrogen peroxide to yield sulfenic acid (SO^-). When hydrogen peroxide level is sufficiently high, sulfenic acid can undergo a further hyperoxidation into sulfinic (SO^{2-}) and sulfonic (SO^{3-}) acids, in which the formation of sulfonic acid is considered as an irreversible oxidative modification [19, 20]. Alternatively, the Fenton reaction can produce the third type of ROS, hydroxyl radical (OH^-), from H_2O_2 by accepting an electron from free cations (Fe^{2+} or Cu^+). Although recent studies provide some evidence that O_2^- and OH^- can participate in transmitting the signal transduction, the detailed molecular mechanisms are still largely unclear [21]. Together, it is likely that the type and local concentration of ROS determine whether redox signaling is transmitted or the oxidative-stress induced damage occurs in cells.

Mitogens, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), promote the rate of cell growth and proliferation by activating membrane-bound receptor tyrosine kinases (RTKs) via the autophosphorylation of specific tyrosine residues on the cytoplasmic tails [22]. This event results in the recruitment of multiple adaptors to RTKs and promotes subsequent activation of downstream signal transduction cascades. On the other hand, protein tyrosine phosphatases (PTPs) carry out tyrosine dephosphorylation of these receptors, thereby switching off the signal transduction cascades [23]. Interestingly, previous studies have demonstrated that the oxidation of catalytic cysteine residues in PTPs contributes to the inactivation and sustained promotion of cell growth and proliferation. For example, EGF treatment can generate intracellular H_2O_2 and promote the inactivation of protein tyrosine phosphatase 1B (PTP1B) by oxidizing the catalytic cysteine residues into sulfenic acid [24]. Likewise, PDGF treatment led to the generation of intracellular H_2O_2 and caused oxidation of cysteine residues of the PDGFR-associated phosphatase, SHP-2 [25]. Moreover, H_2O_2 can promote the cysteine oxidation of PTEN, a PTP that removes the phosphate from phosphatidylinositol and serves as a critical regulatory molecule of PI3K/Akt signaling cascade [26, 27]. Together, these results suggest that oxidizing cysteine residues in PTPs by H_2O_2 is an important switch to assist in the cell growth or proliferation by growth factors. In addition, it is also possible to speculate that the oxidation of cysteine residues in unknown redox-sensitive proteins other than PTPs might contribute to the signal transduction by hydrogen peroxide.

3. Structural Insights into NRF2 and KEAP1 Regulation

While a moderate amount of ROS can affect the cellular signaling activity by modifying cysteine residues in redox-sensitive proteins, an excessive amount of ROS is toxic and must be eradicated. The removal of intracellular ROS is carried out, at least in part, by a number of phase II cytoprotective enzymes, including heme oxygenase-1 (HO-1), NAD[P]H:quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST), and γ-glutamylcysteine ligase (γ-GCS) (Figure 1) [28, 29]. It is widely accepted that transcription of these enzymes is regulated by the antioxidant response element (ARE), a *cis*-acting DNA sequence that exists in the 5′-upstream promoter of these genes [30, 31]. NF-E2-related factor-2 (NRF2) is a transcriptional factor that binds to and mediates the ARE-dependent gene activation. Under a basal condition, NRF2 is sequestered in the cytoplasm and its expression is maintained to be low due to constant polyubiquitination. In response to a variety of stresses, NRF2 is significantly induced and translocates into the nucleus, where it activates the ARE-dependent gene expression in association with small Maf proteins and other coactivators. Detailed domain analyses have revealed that NRF2 comprises six conserved NRF2-ECH (Neh) domains. The Neh1 domain contains a basic leucine zipper motif (bZIP) and behaves

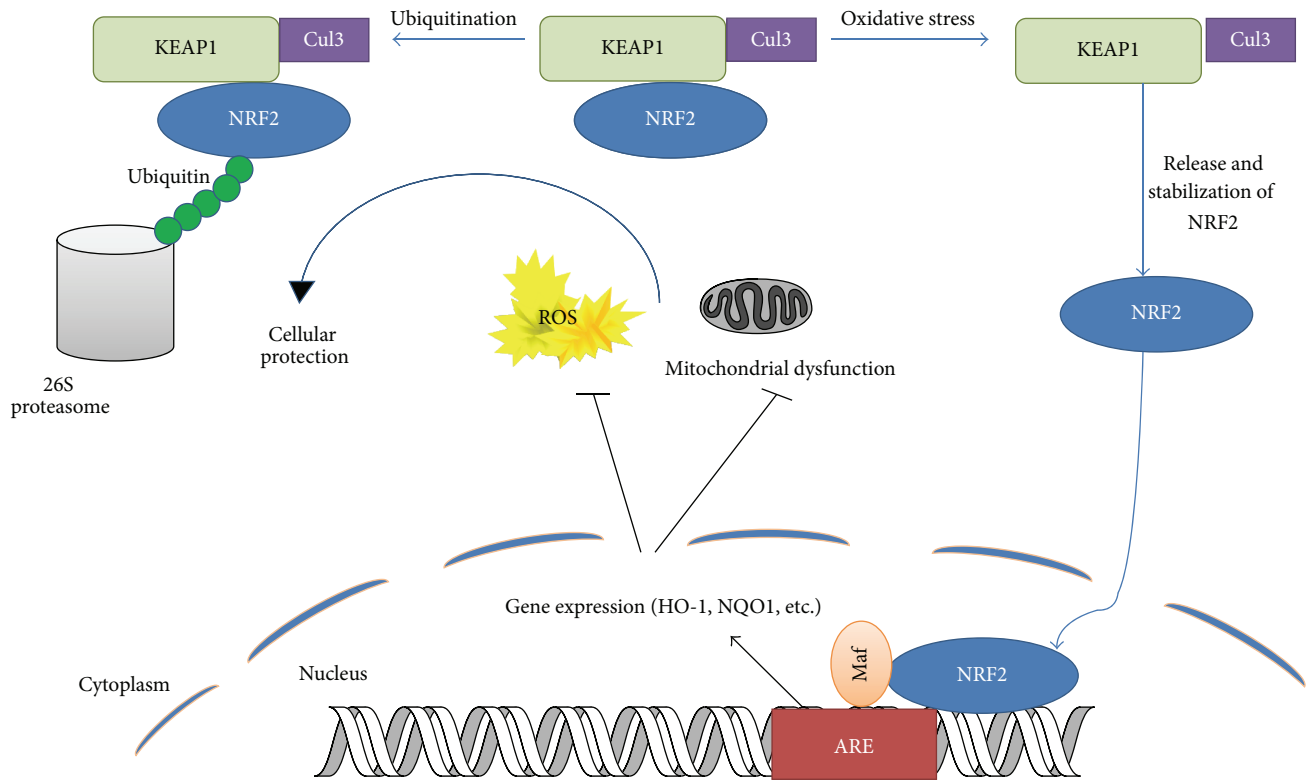

Figure 1: Regulation of NRF2 stability by KEAP1. NRF2 is constantly degraded by KEAP1-mediated ubiquitination in the cytoplasm. Oxidative stress will halt degradation of NRF2 and lead it to bind to ARE to activate transcription of oxidant and detoxifying enzymes.

as a platform for binding to the ARE. The Neh2 domain is located in the most N-terminal region and acts as a negative regulatory domain. The Neh3 domain is located in the most C-terminal region and plays a permissive role for NRF2 transactivation. The Neh4 and Neh5 domains seem to be essential for NRF2 transactivation and the Neh6 domain is required for NRF2 protein degradation [32, 33]. However, the detailed studies elucidating the in-depth function of individual domains of NRF2 are not available and required to fully characterize the exact molecular functions of individual domains.

Kelch-like ECH-associated protein 1 (KEAP1) was initially identified by yeast 2-hybrid assay as a novel binding partner of NRF2, using the Neh2 domain as bait [34]. Subsequent studies have identified that KEAP1 is a cytosolic protein that inhibits the NRF2 activity by acting as an adaptor for Cullin-3-based E3 ubiquitin ligase complex [35]. Due to the existence of a large number of cysteine residues, it has been proposed that KEAP1 is a sensor molecule for oxidative stress through Michael reaction and, based on this conjecture, the so-called cysteine code hypothesis was proposed, in which the structural changes of KEAP1 by thiol modifications of redox-sensitive cysteine residues alter and regulate the KEAP1 activity [36]. KEAP1 protein consists of 5 different domains: an amino-terminal region (NTR), a Broad Complex, Tramtrack, and Bric-a-Brac (BTB) domain, an intervening region (IVR), six Kelch/double glycine repeats (DGRs), and a carboxy-terminal region (CTR) (Figure 2) [37]. A number of biophysical and structural analyses have by far provided meaningful insights into how KEAP1 might control the NRF2 stability. Using NMR analysis, it was demonstrated that the peptide harboring the Neh2 domain assumes a rod-like structure and the regions flanked by the ETGE and DLG motifs form an α-helix [38]. Seven lysine residues located in the Neh2 domain are all potential polyubiquitination sites by KEAP1 and six of them are aligned on the same side of the α-helix. However, to the best of our knowledge, the crystal or NMR structure of full NRF2 protein is not available yet, possibly due to its intrinsic insolubility. On the other hand, biochemical studies have demonstrated that KEAP1 employs the DGR region to recognize two primary sequences on NRF2, for example, the ETGE and DLG motifs, both of which are located in the Neh2 domain of NRF2. Crystal structure studies revealed that KEAP1 DC (DGR + CTR) domain forms a barrel structure composed of six β-propellers and the ETGE or DLG peptides fit into the bottom of the DC barrel structure. Using a single particle electron microscopy, Tong and colleagues have demonstrated that the overall KEAP1 dimers assume a cherry-bob-like structure [39], in which two round globules are connected with a stem-like structure and each globular structure is a rounded cylinder with a narrow penetrating tunnel. Because the binding affinity of the ETGE motif to KEAP1 is much higher than that of the DLG motif to KEAP1 as demonstrated by the isothermal calorimetry (ITC), the so-called hinge and latch model was proposed [40], in which a strong interaction of KEAP1 with the ETGE

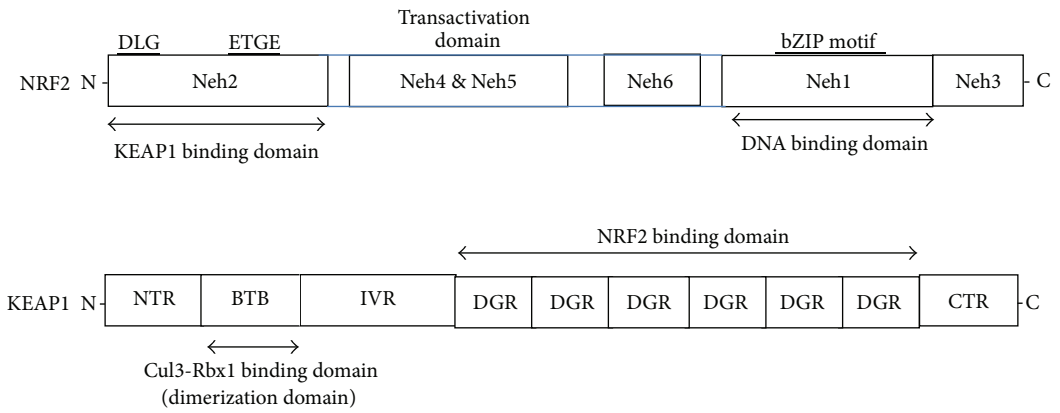

Figure 2: Domain structure of NRF2 and KEAP1 proteins. bZIP: basic leucine zipper, NTR: N-terminal region, BTB: Broad Complex, Tramtrack, and Bric-a-Brac, IVR: intervening region, DGR: double glycine repeat (=Kelch), and CTR: carboxyl terminal region.

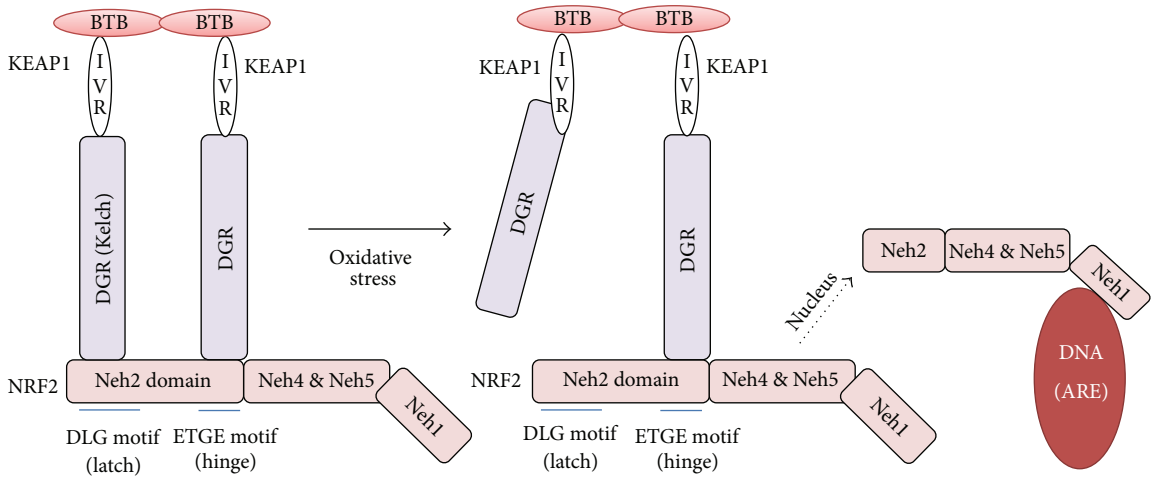

Figure 3: Interaction of NRF2 and KEAP1: hinge and latch model. KEAP1 proteins dimerize via BTB domains. The KEAP1 homodimer identifies the DLG (weak interaction) and ETGE (strong interaction) motifs in the NRF2. NRF2 tightly binds to KEAP1 homodimer in basal state. After stress, weaker DLG motif is detached, blocking ubiquitination of NRF2 and facilitating nuclear import and binding to ARE. BTB: Broad Complex, Tramtrack, and Bric-a-Brac, IVR: intervening region, DGR: double glycine repeat (=Kelch), and ARE: antioxidant response element.

acts as a hinge and a weak interaction of KEAP1 with DLG motif is regarded as a latch (Figure 3). While the "hinge and latch" model still holds as a primary model that accounts for the KEAP1 and NRF2 interaction, alternative or disruptive models explaining the NRF2 and KEAP1 interaction and the resulting activity were also provided by employing different experiment approaches [41].

4. The Janus Faces of NRF2: Good or Evil?

It is generally accepted that the induction of NRF2-dependent gene expression contributes to the detoxification of intracellular ROS, thereby alleviating the oxidative damage in organisms. This assumption is well supported by the observation that NRF2 knock-out mice were highly susceptible to oxidative stress-mediated injuries or carcinogenesis, compared with wild-type littermates [42]. Hence, it is plausible to assume that enhancing the activity of NRF2 would be beneficial to attenuate or block the progression of proinflammatory diseases. In line with this idea, a number of chemopreventive agents, including sulforaphane, curcumin, resveratrol, and a synthetic terpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me), unequivocally resulted in the attenuation of proinflammatory diseases through an induction of NRF2-dependent phase II cytoprotective enzymes in a variety of experimental animal models [43–46]. Notably, dimethyl fumarate (DMF), a strong inducer of NRF2, was recently approved by the Food and Drug Administration (FDA) with a brand name, Tecfidera, for treatment of recurrent multiple sclerosis (MS) patients [47]. This fact validates the feasibility of KEAP1/NRF2 signaling pathway as a drug target. Although diverse mechanisms might be involved, it is speculated that the induction of phase II cytoprotective enzymes by NRF2 chemical inducers occurs, at least in part, by modulating the activities of intracellular signaling kinases. This assumption is well supported by many previous experimental observations that genetic ablation or treatment of pharmacological kinase inhibitors significantly affected

the NRF2/ARE-dependent gene expression [48]. While it is certain that multiple intracellular signaling kinase cascades such as PKR-like endoplasmic reticulum kinase (PERK), phosphatidylinositol 3′-kinase (PI3K), and protein kinase C (PKC) are involved, the exact mechanisms underlying how these individual kinases are orchestrated to regulate the NRF2/ARE-dependent gene expression are relatively uncertain. Therefore, additional studies elucidating direct NRF2 kinases and their exact phosphorylation residues in NRF2 are necessary. By now, only two direct NRF2 kinases are reported: PKC delta (PKCδ) is known to phosphorylate NRF2 at serine 40 to activate the ARE-dependent gene expression, and Fyn kinase can phosphorylate NRF2 at tyrosine 568 to suppress the ARE-dependent gene expression. However, whether and, if so, how NRF2 phosphorylation contributes to the NRF2 stability, for example, polyubiquitination, are also unclear.

On the other hand, recent studies have indicated that cancer cells hijack the ability of NRF2 to survive under the oxidative or electrophilic conditions. This conjecture is supported by epidemiological observations that KEAP1 and NRF2 are abundantly mutated in various types of human cancer [49–51]. In addition, recent studies have established a role for NRF2 in modulating anabolic pathways to deal with metabolic demands of cancer cell growth and proliferation. Finally, an increased NRF2 activity is positively correlated with a poor prognosis and chemotherapeutic resistance [52]. It is known that multiple KEAP1 missense mutations occur in human lung adenocarcinoma and they are not limited in certain domains but widely distributed throughout KEAP1 [33]. No matter where KEAP1 mutations occur, they seem to promote the overall stability and/or nuclear translocation of NRF2, thereby contributing to the NRF2/ARE-dependent gene activation. On the other hand, NRF2 mutations were observed in patients in lung, esophagus, skin, and head and neck cancers [53, 54]. Unlike KEAP1, most NRF2 mutations were confined to the ETGD and DLG motifs, providing an indirect support for the hinge and latch hypothesis in the clinical setting. Another interesting aspect is that the occurrence of KEAP1 and NRF2 mutations is mutually exclusive in cancer patients, suggesting that targeting either KEAP1 or NRF2 is sufficient to activate ARE-dependent gene expression in cancer. In addition, recent studies have identified that some proteins bear analogous peptide sequences with the ETGE or DLG motif, which helps them to interfere with the molecular interaction between NRF2 and KEAP1. For example, Chen et al. have demonstrated that p21, a target of p53-mediated cell cycle and apoptosis, can associate with the DLG motif in NRF2 and increase the NRF2 level, resulting in the inhibition of KEAP1 and NRF2 interaction [55]. In addition, Komatsu et al. [56] have demonstrated that p62, a polyubiquitination binding protein that targets substrates for autophagy, contains the STGE motif and it stabilizes NRF2 by inhibiting the polyubiquitination of NRF2 by KEAP1. Together, the involvement of p21 and p62 in the regulation of KEAP1/NRF2 lends a good support for the assumption that modulating the NRF2/ARE signaling pathway is critical in executing the cell-cycle arrest or autophagy in cancer.

Cancer-preventive activity of NRF2 has been well demonstrated in experimental settings, not only by showing that enhanced NRF2 activity results in inhibition of carcinogenesis through its cytoprotective effects, but also by showing that impaired function of NRF2 through genetic deletion of NRF2 increased a susceptibility to cancer formation [57]. Consistent with this view a number of chemopreventive agents, such as sulforaphane, curcumin, CDDO-Me, and DMF, are effective in treating diverse proinflammatory diseases, via activation of NRF2 and a subsequent induction of antioxidative and cytoprotective enzymes.

On the other caveat, NRF2 is also considered as oncogenic and the results of several studies support this view. DeNicola et al. [58] showed that NRF2 might play a role in oncogenesis through elegant genetic animal studies. NRF2 can upregulate antiapoptotic proteins such as Bcl-2 and Bcl-xL [59] and the rate of glycolysis to promote cell proliferation, thereby contributing to cancer cell survival [60]. In the analysis of clinical samples, it was found that gain-of-function mutations in NRF2 exist in carcinomas of esophagus, skin, and larynx, while loss-of-function mutations in KEAP1 are observed in carcinomas of lung, gall bladder, ovary, breast, liver, and stomach [51]. Therefore it can be surmised that continuous activation and accumulation of NRF2 due to perturbed regulation and mutation will lead to chemotherapeutic resistance [61]. The double faced function of NRF2 in different contexts indicates that NRF2 can be both antitumorigenic and protumorigenic.

5. Concluding Remarks

By now, we have discussed the molecular mechanisms underlying the detoxification of intracellular ROS and how H_2O_2 participates in the activation of signal transduction and contributes to cell proliferation and growth. We have also provided structural insights demonstrating how KEAP1 regulates the NRF2 stability and coordinates the adaptive defensive responses against oxidative stress. Finally, we have provided an evidence that an increased NRF2 activity in normal cells is protective and beneficial against oxidative stress, but cancer cells harness the ability of NRF2 to survive under stress conditions. Due to this contradictory role of NRF2 in cancer, it is important to determine whether NRF2 genotype could be beneficial or detrimental in the development of other chronic diseases, considering a broader implication of oxidative stress in the pathogenesis of numerous human diseases. The existence of various genetic tools, including NRF2 knock-out mice model, can help to address this issue.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Extracellular Superoxide Dismutase: Growth Promoter or Tumor Suppressor?

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Extracellular superoxide dismutase (SOD3) gene transfer to tissue damage results in increased healing, increased cell proliferation, decreased apoptosis, and decreased inflammatory cell infiltration. At molecular level, *in vivo* SOD3 overexpression reduces superoxide anion (O_2^-) concentration and increases mitogen kinase activation suggesting that SOD3 could have life-supporting characteristics. The hypothesis is further strengthened by the observations showing significantly increased mortality in conditional knockout mice. However, in cancer SOD3 has been shown to either increase or decrease cell proliferation and survival depending on the model system used, indicating that SOD3-derived growth mechanisms are not completely understood. In this paper, the author reviews the main discoveries in SOD3-dependent growth regulation and signal transduction.

1. Introduction

Extracellular superoxide dismutase (EC-SOD, SOD3) [1, 2], similar to cytosolic CuZn-SOD (SOD1) [3] and mitochondrial MnSOD (SOD2) [4, 5], catalyzes the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) (in this review reactive oxygen species refer to O_2^- and H_2O_2), which to date is the only reported physiological function of the enzyme. Thus, the cellular effects of SOD enzyme activity are caused by changes in the local concentrations of O_2^- and H_2O_2, which are second messengers in signal transduction that have an impact on growth capacity and the transformation of primary cells. Although the enzymes have a significant therapeutic potential their delivery to injury site is challenging due to limitations in gene transfer efficiency. Hence researchers have developed SOD mimics that function similarly with SOD enzymes regulating redox balance with consequent impact on growth, differentiation, and death [6–10]. The importance of local regulation of reactive oxygen species (ROS) by SOD3 has been highlighted by our previous studies of local and systemic delivery of *sod3* via adenovirus to sites of cardiovascular injury: both gene transfer methods increase plasma SOD activity, but only the local gene delivery demonstrates a therapeutic response [11]. The data is supported by observations reporting that Arg-213-Gly mutation at C-terminal end of SOD3 reduces the affinity of the enzyme to heparan sulphate proteoglycans of endothelial cells thus increasing plasma SOD3 concentration by 10-fold [12, 13]. The mice carrying Arg-213-Gly mutation have tissue level changes, such as increased neutrophil mediated inflammation, cellular degeneration and premature aging, abnormal gait, and reduced lifetime that may be result of increased neutrophil ROS production [14]. Based on the abovementioned data decreased SOD3 content at cell membranes impairs life-supporting cellular functions. Notably, H_2O_2 can have toxic effects on cellular functions at high concentrations, thus suggesting a need to regulate ROS production in the tissue environment. Indeed, a number of reports have demonstrated tight regulation of SOD3 expression at the transcriptional, posttranscriptional, and posttranslational levels [12, 15–23]. This regulation is influenced by various factors, most importantly by the level of O_2^- substrate and the reaction end product H_2O_2 [23–25].

2. Therapeutic Effects of SOD3 Overexpression

One of the first milestones in SOD3 research was the discovery of the tissue-protective nature of the enzyme in cardiovascular models. The earliest observations reported reduced cardiovascular damage by recombinant SOD3 administration [26–30]; these observations were confirmed by a series of gene transfer studies [11, 24, 31–39] and later reviewed in [40–44]. Characteristically, treatment of cardiovascular tissues with SOD3 reduces the extent of the damage, increases the healing process, improves cardiac function, reduces the remodeling of vasculature, attenuates apoptosis, inhibits inflammatory and smooth muscle cell migration, and increases cell proliferation and endothelial cell layer recovery. The role of SOD3 in neoangiogenesis is less clear. We have reported increased endothelialization and reduced macrophage and smooth muscle cell migration with consequent long-term inhibition of neointima formation in rabbit denudation and in rabbit in-stent models [11, 38], suggesting a role for the enzyme in vascular cell proliferation and inflammatory cell migration. We have further demonstrated, using rat hind limb injury model, SOD3-dependent increases in tissue injury recovery that were mediated by activation of mitogen signal transduction with consequent increased satellite cell proliferation in muscles [24]; by activation of antiapoptotic signaling that involved increased extracellular signal regulated kinase 1/2 (ERK1/2), protein kinase B (AKT), and forkhead box O3a (FOXO3a) activation [39]; and by reduction of macrophage-specific inflammation, which was correlated with reduced expression of the inflammatory cytokines tumor necrosis factor α (TNFα), interleukin 1α (IL1α), interleukin 6 (IL6), macrophage inflammatory protein 2 (MIP2), and monocyte chemotactic protein 1 (MCP-1) and the adhesion molecules vascular adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), P-selectin, and E-selectin [36]. Although we did not observe increased neoangiogenesis by overexpressing SOD3, another recent study performed in SOD3 knockout mice suggested defective vessel formation in the absence of the enzyme. The authors demonstrated that SOD3 does not directly promote vascular endothelial growth factor receptor (VEGFR) activation but it is able to enhance the ability of VEGF ligand to phosphorylate VEGF-R [45]. Thus, *in vivo* data suggest that SOD3 expression activates growth-promoting, antiapoptotic, and anti-inflammatory signal transduction pathways in cardiovascular models (Figure 1).

The function of SOD3 in lung models has been investigated using SOD3 null and transgenic mice. The earliest observations suggested that SOD3 null mice had a significantly shortened life span and experienced death associated with lung edema under conditions of hyperoxia [46]. These observations were confirmed in conditional knockout mice that showed reduced survival associated with disorders resembling adult respiratory distress syndrome, such as thickening of alveolar septa, increased inflammation, hemorrhage, and loss of patent alveoli [47]. Hence, the lung model data support results obtained from cardiovascular damage models, suggesting survival-supporting and growth-promoting roles for SOD3 in the tissue environment.

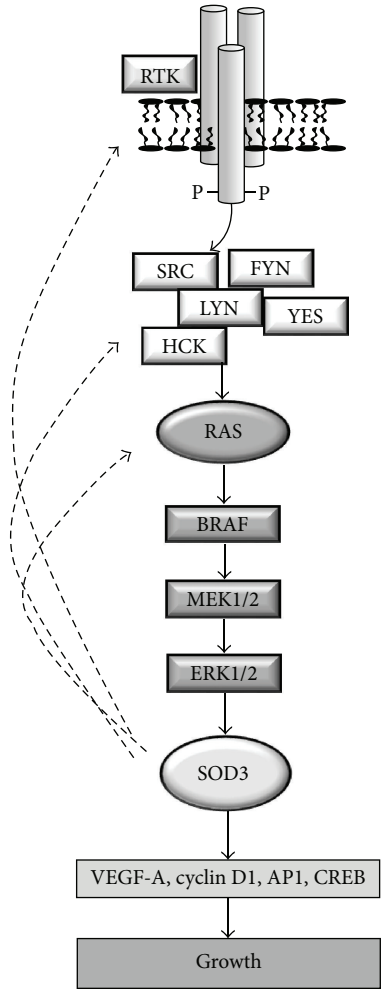

Figure 1: Suggested positive feedback loop in SOD3 signal transduction. Phosphorylation of RTKs activates the cell membrane associated SRC proto-oncogene family members that contribute to RAS GTP loading and stimulation of mitogenic signal transduction to BRAF, MEK1/2, and ERK1/2 kinases. *In vitro* transient transfection of *RAS*, *BRAF*, *MEK1/2*, and *ERK1/2* increases both *SOD3* mRNA and protein expression hence suggesting mitogen pathway induced SOD3 synthesis. SOD3 production results in increased synthesis of growth promoters, such as VEGF and cyclin D1, and increased activation of activator protein 1 (AP1) and cAMP response element-binding protein (CREB). Importantly, SOD3 activates cell surface receptor tyrosine kinases (RTKs), increases phosphorylation of SRC family members, and regulates the GTP loading to small GTPases, such as RAS.

The most dramatic prosurvival effect of SOD3 has been observed in total body irradiation (TBI) studies. In one study, intravenous administration of 0.5×10^6 mesenchymal stem cells (MSCs) previously transduced with SOD3-expressing adenovirus multiplicity of infection (MOI) 2000 resulted in a 90% survival rate 35 days after 9 Gy TBI without hematopoietic stem cell (HSC) transfusion, whereas 90% of control animals died [48]. These data were confirmed in a study of mice receiving 5.81 Gy TBI, which showed a similar survival rate [49]. In this study, in the absence of HSC transplantation,

the transfusion of MSCs (1×10^6) transduced with SOD3-expressing adenovirus (MOI 50) resulted in a 90% 30-day survival rate compared to a 20% survival rate in control animals. Blood value analysis 10 days after TBI demonstrated that there were eightfold higher white blood cell counts (1.1×10^8 in controls versus 8.9×10^8 in the SOD3 group), 40-fold higher platelets values (2.4×10^9 in controls versus 97×10^9 in the SOD3 group), and significantly increased hemoglobin levels (105 g/L in controls versus 128 g/L in the SOD3 group) in SOD3-treated animals compared to controls [49]. Although the authors concluded that the increased survival was caused by significantly decreased apoptosis in SOD3-treated animals, another possible survival mechanism could be increased cycling of primitive HSCs with consequent hematopoietic cell differentiation. The data provided by Gan and coworkers suggested that the gene expression of members (i.e., *p53*, *p21*, and *p16*) of the *p53*-mediated growth arrest pathway was reduced in MSC-SOD3-transplanted animals [49], supporting the hypothesis that increased SOD3-driven mitogen stimulus in the bone marrow together with reduced apoptosis might explain the increased survival after TBI observed in SOD3-treated animals. Previous studies have suggested a common bone marrow niche and homing site for HSCs and MSCs [50, 51], thus indicating that SOD3-treated MSCs could have a paracrine effect on quiescent HSCs, inducing them to proliferate and to differentiate by directly affecting primitive progenitor cell cycling or via erythropoietin signaling [52]. Hence, the *in vivo* data observed from various animal models suggest that SOD3 maintains normal tissue homeostasis by promoting cell survival and proliferation.

3. Hydrogen Peroxide Action in Signal Transduction

Hydrogen peroxide regulates a number of cellular functions, such as cell proliferation, differentiation, migration, and survival. The first evidence that H_2O_2 could function as a second messenger came from studies demonstrating increased H_2O_2 production in association with increased platelet-derived growth factor (PDGF), epithelial growth factor (EGF), and vascular endothelial growth factor (VEGF) receptor tyrosine kinase phosphorylation with simultaneously reduced protein tyrosine phosphatase (PTP) activity [53–55]. In general, ROS are able to affect cell signaling by two mechanisms: (1) by inactivating PTPs, thereby increasing tyrosine kinase phosphorylation, and (2) by directly oxidizing tyrosine kinase receptors, causing their phosphorylation [56, 57].

H_2O_2 and $O_2^{\bullet-}$ are known to be involved in the initiation of tumorigenesis and in malignant transformation [58]. In addition to increasing cell proliferation, survival, and migration, H_2O_2 activates SRC family proto-oncogenes, which regulate vascular development and vascular permeability [59], the latter of which is an early step in tumor stroma development [60]. The role of H_2O_2-derived signaling in the later phase of tumor development allows cancer cell survival in hypoxic environments by maintaining activation of the AKT pathway, with a consequent increased expression of hypoxia inducible factor 1α [61].

4. SOD3 Expression in Tumorigenesis

Previous data have suggested that there is a correlation between increased *sod3* mRNA production and increased growth of benign tumors [62], indicating a role for SOD3 in early tumorigenesis. *In vitro* studies have supported this conclusion by demonstrating that moderate overexpression of SOD3 stimulates mouse primary embryonic fibroblast (MEF) cell proliferation, mimicking the RAS oncogene response in primary cells [63] and further corroborating the close relationship of SOD3 expression and cellular growth. Consistent with these results, *sod3* mRNA synthesis is upregulated at low RAS activation levels; however, *sod3* mRNA expression, which negatively correlates with *mir21* expression, is strongly downregulated when the RAS activation level increases to ≥10-fold relative to parental cells [23]. In contrast to the case in benign growth, SOD3 expression is progressively downregulated in a number of cancers and cancer cell lines [62, 64–67], correlating with the RAS activation level [23], which suggests that *sod3* could be a prognostic differentiation marker. Silencing of the *SOD3* gene can be divided into reversible immediate events and stable late events. Immediate events following RAS activation occur via SOD3 self-regulation through small RAS GTPase regulatory genes, *mir21* upregulation, and p38 MAPK phosphorylation [23, 68–72], whereas late regulatory events consist of DNA methylation and histone acetylation [15, 16, 72–74]. The correlation of decreased *SOD3* expression with increased malignancy has led to the hypothesis that SOD3 could function as a tumor suppressor that must be silenced to allow the progression of carcinogenesis [66]. Although the hypothesis is feasible based on conventional tumor suppressor gene silencing mechanisms, the mechanisms of how reduced *SOD3* expression could increase transformed cell proliferation have not been fully elucidated.

5. SOD3 as a Growth Promoter in Tumorigenesis

As mentioned above, SOD3 has been shown to promote normal primary cell proliferation in various model systems [11, 24, 33, 34, 38, 63]. The close connection of SOD3 to growth-associated signal transduction was demonstrated in a recent microarray functional KEGG and GO pathway analysis suggesting that the highest number of SOD3-affected genes was in the MAPK signaling (254 genes, $p < 0.02$) and endothelial cell proliferation pathways (33 genes, $p < 0.018$). Other significantly affected pathways included various cancer-associated signal transduction and cell proliferation pathways [75]. We have previously shown that RAS-BRAF-MEK1/2-ERK1/2, a major signal transduction pathway in cancer, activates *SOD3* mRNA expression and enzyme activity *in vitro* and *in vivo*, which then increases GTP loading to RAS [24]. These data suggest the existence of a positive feedback loop that maintains the mitogen pathway in a phosphorylated

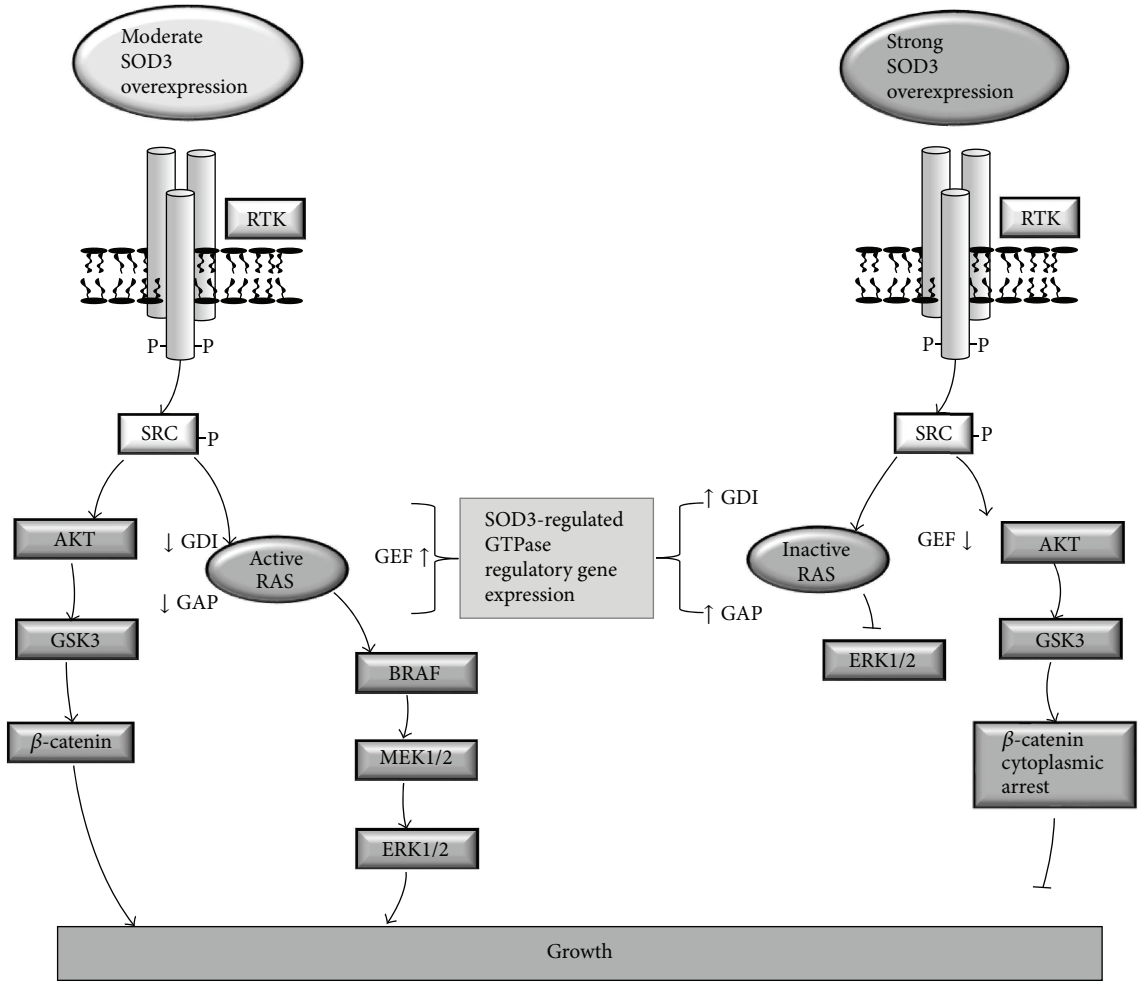

Figure 2: Suggested model for dose-dependent effect of SOD3 on RAS activation and β-catenin cellular localization. Moderately increased SOD3 expression at cell membranes promotes cell membrane bound RAS GTP loading by activating GEF expression and by inhibiting GAP and GDI synthesis causing increased RAS-ERK1/2 signaling. Robustly increased SOD3 expression inhibits RAS GTP loading by inhibiting GEF expression and by activating GAP and GDI synthesis causing decreased RAS-ERK1/2 signaling. Moderately increased SOD3 expression promotes AKT and GSK3 phosphorylation and β-catenin nuclear entry, whereas robustly increased SOD3 expression arrests β-catenin to cytoplasm by increasing the expression of *WWTR1*, *SNAI2*, and *AXIN2*. Note that both moderate and robust SOD3 expressions increase the phosphorylation of RTKs, SRCs, AKT, and GSK3. SOD3 dose-dependent signal transduction regulation occurs at the level of small GTPases and β-catenin cytoplasm-nuclear localization.

state, inducing growth-supporting and antiapoptotic signal transduction pathways in injured tissues [24, 39] (Figure 1). We have further shown that thyroid stimulating hormone (TSH), cAMP-PKA, and PLC-Ca^{2+} increase *sod3* production in thyroid cells, demonstrating that SOD3 in the thyroid contributes to cell proliferation and differentiation [62]. The role of SOD3 in growth promotion was further strengthened by data indicating that expression of SOD3 induces the activation of AP-1, c-Jun, and CRE promoter regions; increased expression of FOXO3a and FOXQ1 transcription factors; and increased expression of the cell cycle proteins cyclin D1, cell division cycle 25A (CDC25A), and proliferating cell nuclear antigen (PCNA) [24, 39, 63]. Importantly, H_2O_2 treatment of cells has been shown to stimulate *SOD3* mRNA synthesis and mimic SOD3 function in cells that were treated with N-acetylcysteine (NAC) and diphenyleneiodonium (DPI), suggesting substrate specific regulation of the enzyme [23, 24, 75].

Signal transduction studies have suggested that cell membrane bound SOD3 increases the phosphorylation of the cell membrane tyrosine kinase (RTK) receptors, epidermal growth factor receptor (EGFR), erb-b2 receptor tyrosine kinase 2 (ERBB2), receptor-like tyrosine kinase (RYK), anaplastic lymphoma kinase (ALK), Fms-like tyrosine kinase 3 (FLT3), Ephrin A10 (EPHA10), and VEGF-R [45, 67, 75]; cell membrane-associated signaling molecules, such as the SRC proto-oncogene family members HCK, FYN, SRC, YES, and LYN; and cytoplasmic signaling molecules, including AKT, glycogen synthase kinase 3 (GSK3), and β-catenin [75]. Hence, SOD3 overexpression activates two main growth-related signal transduction pathways, RAS-ERK1/2 and β-catenin cascades (Figure 2).

Interestingly, recent studies have indicated that reducing the expression of SOD3 to physiological levels can increase the growth of transformed malignant cells *in vitro* and *in vivo*. Based on *in vivo* data, VEGF-C-driven SOD3 expression increases the tumorigenesis and metastasis of xenografted mammary cells [67]. Additionally, knockdown of VEGF-C in mammary cancer cell lines significantly reduces the expression of SOD3, tumor formation, and metastasis of the cells, whereas restoration of SOD3 expression in VEGF-C knockdown cells to the levels of control cells with carcinogenic characteristics partly recovers the aggressiveness of the cells, increasing both primary tumor growth and metastasis [67]. The growth-promoting effects of SOD3 are further supported by studies in cancer cell lines harboring decreased endogenous *SOD3* expression; transfection of *SOD3* into these cell lines results in *in vitro* and *in vivo* growth selection of cells, favoring those with modestly increased *SOD3* levels. *In vitro* transfection of high *SOD3* concentrations into cancer cells followed by mixed population long-term culture results in apoptosis of cells with high supraphysiological concentrations of *SOD3* plasmid, whereas cells that contained moderately increased *SOD3* expression compared to control cancer cells took over the culture due to their increased proliferation capacity. *In vivo* studies with xenografted *luciferase*-marked cells support this observation, showing an initial decrease in tumorigenesis and in luciferase signal in tumors derived from SOD3-transfected cancer cells containing a strong increase in *SOD3* mRNA expression. The initial decreased growth phase was followed by faster tumor development and *in vivo* selection of cells, which contained moderately increased *SOD3* mRNA expression levels compared to control cell-derived tumors [63]. Thus, SOD3, by affecting local ROS concentrations, might have progrowth characteristics in early tumorigenesis as a mediator of the RAS oncogene and, in certain cellular environments, may work in coordination with other growth factors that stimulate cancer cell proliferation.

6. SOD3 as a Growth Suppressor in Cancer

Various studies have demonstrated cancer growth suppression caused by supraphysiological *SOD3* overexpression *in vitro* and *in vivo*. In general, these studies have been performed using cells transfected with *SOD3* at high concentrations or using cells transduced with adenovirus expressing *SOD3* [63, 75–79], which induces strong mRNA production, thus reaching supraphysiological SOD3 and H_2O_2 levels for three to four days [80]. High expression of *SOD3* results in DNA damage and activation of the DNA damage response (DDR), including phosphorylation of histone H2AX, phosphorylation of checkpoint kinase1/2 (CHK1/2), phosphorylation of p53, increased production of p21, and consequent growth arrest and apoptosis [63]. Additionally, supraphysiological SOD3 expression in anaplastic cancer cells activates AKT-GSK3-β-catenin signaling but prevents β-catenin nuclear transfer by increasing the gene expression of WW domain-containing transcription regulator protein 1 (*WWTR1*), snail homolog 2 (*SNAI2*), and axis inhibition protein 2 (*AXIN2*) [75], which are responsible for β-catenin cytoplasmic arrest, binding, and degradation, respectively [81–83].

At the tissue level, supraphysiological SOD3 overexpression correlates with reduced oxidative stress marker 4-hydroxynonenal staining in xenografted tumors and decreased intracellular dihydroethidium staining in cancer cells transduced with adenovirus expressing *SOD3* [77, 78]. Functionally, supraphysiological overexpression of SOD3 inhibits the nuclear localization of NF-κB, reduces VEGF-A expression, decreases cell proliferation, inhibits tumor growth, decreases metastasis (suggesting a reduction of *in vivo* cancer cell migration), and increases apoptosis [76–78]. Furthermore, SOD3 has been shown to affect hypoxia inducible factor HIF-1α signaling, which enables vascular growth, thus regulating tumor progression. supraphysiological *SOD3* overexpression by adenovirus (MOI 50–100) decreases HIF-1α levels by inducing degradation, whereas virus doses of MOI 25 or less have minor or no impact on HIF-1α levels [84].

7. SOD3 Affects Growth in a Dose-Dependent Manner

SOD3 overexpression studies have demonstrated a dual role for the enzyme in growth regulation depending on the expression level of the enzyme: rescued or moderately increased SOD3 expression promotes cell proliferation, whereas supraphysiological overexpression of the enzyme causes growth arrest and apoptosis [63, 67, 75, 77, 78]. Notably, moderately increased SOD3 levels stimulate cell proliferation, mimicking the function of the RAS oncogene in primary cultures and causing mitogenic burst followed by growth arrest-related senescence, immortalization of primary cells, and even transformation of the cells together with additional changes in cellular signaling [63, 85–87]. Interestingly, our data have suggested the existence of SOD3 dose-dependent regulation of downstream signal transduction at the level of RAS small GTPases [24, 75]. A moderate twofold increase in SOD3 activity in tissues markedly increases RAS GTP loading and downstream growth signaling [24, 75], whereas robust supraphysiological SOD3 overexpression decreases RAS, RAC, RHO, and CDC42 activation by regulating the gene expression of regulators of these small GTPases. Mechanistically, moderate SOD3 expression increases the mRNA expression of guanine nucleotide exchange factors (GEFs) responsible for GTP loading to GTPases and decreases the expression of GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors, which are responsible for maintaining GDP-RAS association and inhibiting localization of GTPases to cell membranes, where they are activated [88–90]. Robust supraphysiological expression of SOD3, which has been shown to reduce primary tumor growth metastasis and cancer cell proliferation, reduces the expression of GEFs and increases the expression of GAPs and GDIs, resulting in inhibition of the activation of downstream ERK1/2 kinases [75] (Figure 2). Hence, modification of the gene expression of regulators of these small GTPases is a cornerstone in SOD3-derived control of the RAS-ERK1/2 mitogen pathway activation and

cellular growth. It is important to note that, in line with the effect of SOD3 on cellular functions, the effect of H_2O_2 is concentration dependent. Low physiological (<0.7 μM) concentration induces growth, whereas concentrations above 50 μM induce DNA damage and senescence [91]. Therefore, SOD3-driven signal transduction resembles H_2O_2-activated signaling.

8. Conclusions

The role of SOD3 in tumorigenesis is only partly solved. Recent studies have suggested that SOD3 has dose-dependent effects on primary tumor growth and metastasis activity that, however, may depend on the ability of different kinds of tumor cells to detoxify ROS differently. Thus, several avenues of research must still be pursued. Most importantly, the *in vitro* results that showed that moderate SOD3 mRNA overexpression induces primary cell immortalization and transformation should be repeated using *in vivo* model systems, and the mechanism of this effect should be further elucidated. Second, increased *SOD3* mRNA expression correlates with increased benign growth and decreased *SOD3* mRNA expression correlates with increased malignant progression, thus creating a dilemma regarding the signal transduction differences between primary and transformed cells. The presented hypothesis suggesting increased aggressiveness of cancer cells caused by decreased SOD3 mRNA expression requires a mechanistic explanation. Furthermore, as strong expression of *SOD3* mRNA induces apoptosis and death of cancer cells, it would be of great interest to determine if *SOD3* gene regulation in certain cellular conditions allows supraphysiological expression of the enzyme, causing cellular death, thus suggesting tumor suppressor characteristics for SOD3. Although the function of SOD3 as a regulator of cellular growth has been well established by a number of studies, the enzyme itself might not be a suitable cancer drug or druggable target molecule. Rather, SOD3-related signal transduction studies might indicate mediators of tumor progression, which could then be useful targets for preclinical and clinical studies.

Competing Interests

The author has no conflict of interests.

Acknowledgments

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Review Article

ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases

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The aging process worsens the human body functions at multiple levels, thus causing its gradual decrease to resist stress, damage, and disease. Besides changes in gene expression and metabolic control, the aging rate has been associated with the production of high levels of Reactive Oxygen Species (ROS) and/or Reactive Nitrosative Species (RNS). Specific increases of ROS level have been demonstrated as potentially critical for induction and maintenance of cell senescence process. Causal connection between ROS, aging, age-related pathologies, and cell senescence is studied intensely. Senescent cells have been proposed as a target for interventions to delay the aging and its related diseases or to improve the diseases treatment. Therapeutic interventions towards senescent cells might allow restoring the health and curing the diseases that share basal processes, rather than curing each disease in separate and symptomatic way. Here, we review observations on ROS ability of inducing cell senescence through novel mechanisms that underpin aging processes. Particular emphasis is addressed to the novel mechanisms of ROS involvement in epigenetic regulation of cell senescence and aging, with the aim to individuate specific pathways, which might promote healthy lifespan and improve aging.

1. Introduction

The reduced rate of birth and mortality is the motive of the older population growth in western industrialized countries, where advanced age remains the fundamental risk factor for most chronic diseases and functional deficits. As an example, it is estimated that the individuals of age 65 and above in the USA will reach 20% by 2030, while they constituted 12.4% in 2004 [1]. Human aging is developed from such an accumulation of physical, environmental, and social factors that the definition of the molecular mechanisms that trigger the aging means a difficult task. Some theories associate various factors with aging rate, as changes of metabolic control [2] and gene expression patterns [3] and production of high levels of Reactive Oxygen Species (ROS) [4]. Low ROS level has been, instead, associated with lengthening of organismal lifespan [5]. Current studies aim at deepening how cell senescence process, so far experimented *in vitro*, may be extended to *in vivo* studies. Increasing evidence for causal role of cell senescence has been demonstrated in age-related dysfunctions and pathologies [6]. Senescent cells proliferate in aging, as a stress response primed by a number of "counting mechanisms," like telomeres shortening, DNA damage accumulation, abnormal oncogenes activities, metabolic alterations, and excessive ROS generation [7]. These mechanisms cause cell proliferating arrest and generate features, as constitutive production of high ROS levels, critical for the senescent phenotype maintenance. Despite increasing modestly, as a number, the senescent cells are implicated in age-related diseases promotion, through the restriction of the regenerative pool of the tissue

stem cells [8]. Some observations indicate that senescent cells do not necessarily induce mechanisms that promote aging and can be efficiently removed from the human body [9]. The general consensus on cellular damage accumulation, as aging initial event, suggests that cell senescence process is a major question regarding biological and clinical aging aspects [10].

Here, we review evidences on novel molecular mechanisms of the "ROS signaling" during aging and related pathologies, because they suggest a way of promoting healthy lifespan and improve human aging.

2. ROS Physioma Homeostasis

The ROS physioma is a family of highly reactive molecules which includes free oxygen radicals, like superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and nonradical oxygen derivatives, like the stable hydrogen peroxide (H_2O_2). The superoxide radicals react to form other ROS, namely, hydrogen peroxides and hydroxyl radicals, and interconvert with reactive nitrogen species (RNS), which generate effects similar to ROS [11]. The inefficient electron transfer in mitochondrial respiratory chain is believed to be a main ROS source, among diverse possible enzymatic and nonenzymatic sources [12]. Increased expression of catalase and peroxiredoxin-1 molecules are considered as OS markers. The family comprises seven transmembrane members, namely, Nox1–5 [13–15] and Duox1-2 [16]. ROS are generated by oxygen metabolism (i.e., cellular respiration) in all the cells that utilize oxygen, as inevitable consequence of aerobic life, and may derive from exogenous metals, recycling of redox compounds, radiation, chemotherapeutic agents, carcinogens (estrogenic molecules), and other dietary and environmental means. Generally, the ROS increasing levels cause nonlinear cellular responses [17]. A fine balance between oxidant-antioxidant mechanisms leads to continuous modulation of ROS production, location, and inactivation, in both physiological and pathological conditions. Endogenous antioxidants, like the enzymes of catalase family, glutathione group, thioredoxin-related group, and superoxide dismutase [18], together with exogenous antioxidant as reduced glutathione [19], carotenoids, and vitamins C and E, constitute the indispensable ROS detoxifying system. Nevertheless, imbalance of redox homeostasis may occur, usually in favor of oxidants, so that ROS shift from physiological to potentially harmful levels, named oxidative and nitrosative stress (OS/NS). Increased expression of catalase and peroxiredoxin 1 molecules are considered as OS markers [20–22].

2.1. ROS Measurement Techniques. ROS are so highly variable and freely diffusible molecules that the detection of ROS and antioxidants, to obtain a picture of the cellular redox status, still represents a challenge. We stress some specific points and sensitive methods that are subjected to continuous improvement. Probes and antibodies have been developed to recognize oxidative damage by ROS/RNS [23–25]. The tools allow revealing antioxidant enzymes [26] and a variety of oxidative products, as lipid peroxidation products, protein carbonyls [27], oxidized DNA products [28], and nitrotyrosine [29]. Combinations of diverse approaches will prove essential for understanding ROS involvement in aging and age-related diseases [30]. An innovative method simultaneously assesses glutathione, hydrogen peroxide, and superoxide levels in a single cell, together with cell viability alterations, thus allowing for defining both oxidant-antioxidant balance and cell death, after the administration of a specific stimulus [31]. A wide range of pathways and molecular mechanisms that involve ROS suggests determining the redox state of thiols in ROS targets, which compose the "cellular oxidative interface" [32, 33]. ROS oxidize specific protein residues of cysteine into sulfenic acid, reversibly. This molecule functions as OS/NS sensor within enzymes and transcriptional regulatory factors and may allow priming the routes of the versatile ROS action [34–36].

2.2. ROS Functions. The increasing comprehension of mechanisms, underlying the oxidant milieu of the cell, shows ROS as signaling molecules, besides metabolic byproducts. They act in a myriad of pathways and networks, mediated by hormones, which ranges from protein phosphorylation to transport systems, for example. ROS do not influence single steps of multistep processes; rather, they influence all the steps at the same time, by reacting with several compounds and taking part in several redox reactions. Depending on ROS concentration, molecular species, and subcellular localization, cell components and signaling pathways are affected positively or negatively. ROS levels are believed to be a "redox biology" that regulates physiological functions, including signal transduction, gene expression, and proliferation. "Redox biology," rather than OS, has been proposed to underlie both physiological and pathological events [37]. Data in the literature on slow and constant ROS increases have to be integrated with data on fast and stepwise ROS increases, typical of signaling events, which deliver messages among cellular compartments. Questions related to ROS dynamics and specificity, as the effects of their waves of concentration on networks with other signaling pathways, are investigated in single cells and across different cells. Proteins are the major target of ROS/RNS signaling and undergo reversible or irreversible modifications of their functions, which result in cell death, growth arrest, and transformation. The modulation of the reversible oxidation of redox-sensitive proteins plays basic roles in sensing and transducing the oxygen signal. Receptor-dependent or nondependent tyrosine kinases, AMP-activated protein kinases, adaptor protein p66SHC, and transcription factors as FOXO (forkhead homeobox type O), Nrf2 (nuclear factor E2-related factor 2), p53 (tumor suppressor 53), NF-κB (nuclear factor kappa B), AP-1 (activator protein-1), HIF-1a (hypoxia inducible factor-1a), PPARγ (peroxisome proliferator-activated receptor gamma), and β-catenin/Wnt signaling are listed in Table 1 [38–81]. ROS mediate *in vitro* response towards intra- and extracellular conditions, such as growth factors, cytokines, nutrients deprivation, and hypoxia, which regulate cell proliferation, differentiation, and apoptosis, besides being important cancer hallmarks [82]. Intrinsic and extrinsic factors control ROS regulation on cellular self-renewal, quiescence, senescence, and apoptosis, during the *in vivo* tissues homeostasis and repair [83] and in ROS

Table 1: Selected ROS sensitive proteins that are involved in cell signaling transduction mechanism. Indicative examples of possible effects and processes they promote after being directly and/or indirectly modified by ROS (the references are indicated inside the square brackets).

ROS sensitive proteins: *oxidative interface*	(1) Effects of ROS sensitive proteins after being redox modified	(2) Physiopathological processes in which ROS sensitive proteins are involved
Protein kinases		
Receptor/nonreceptor tyrosine kinases (Src, TRK, AKT, c-Abl, MAPK, CaMKII, PKG, ATM, and Ask1)	(i) Interactions between kinases pathways [38, 39] (ii) Signal of ROS production feedback [40]	Control of cell cycle progression [56] Mitosis for anchorage-dependent cells [57] Cellular homeostasis [43, 57]
AMP-activated protein kinases (AMPK)	(i) Regulation of cell ROS/redox balance [41, 42]	Myocyte adaptation to energy requirement [42] Adipocyte differentiation [58] Lipid metabolism ("fatty liver") [59] Hyperglycemic damage [60] Cell fate (autophagy and apoptosis) [61]
Adaptor proteins		
p66Shc	(i) Signaling start in the aging process [43]	Apoptosis [43]. Prolonged life span [43, 62] Cardiovascular diseases and obesity [63] Diabetic endothelial dysfunction [64]
Nuclear receptors		
PPARγ	(i) Redox sensor function [43] (ii) Regulation of genes that modulate ROS increases [44]	Neurodegenerative diseases [65, 66] Lipid dysfunction (fatty liver) [59]
Membrane receptors		
Elements in Notch1 pathway	(i) Notch signaling modulation in association with Wnt/beta-catenin signal [45]	Cell fate control in vascular development [45] Biological clocks in embryonic development [67]
Transcription factor		
p53	Modulation of cell redox balance (prooxidant/antioxidant effects) [46–48]	Cell fate signaling [68] Autophagy and apoptosis [61, 69]
Nrf2	Cell adaptation to ROS resistance [49, 50]	Apoptosis [70] Neurodegenerative diseases [71] Cardiovascular diseases [72]
FOXO3A	Cell coordination in response to OS [51]	Metabolic adaptation to low nutrient intake [73] Cancer development [73] Diabetes [74] Atherosclerotic cardiovascular disease [75]
Components in β-catenin/Wnt pathway	Regulation of Wnt signaling via nucleoredoxin [76]	Early embryonic development [76] Vascular development [45]
HIF-1a	Cell adaption to oxygen tension modifications [52]	Cell proliferation; angiogenesis [77] Cell transformation [78, 79]
Components in JAK–STAT pathway	(i) Cell adaption to OS [53] (ii) Mediation of ROS mitogenic effect [53]	Stress response gene expression [51] Systemic/pulmonary hypertension [80]
NF-κB	Regulation of redox-sensitive gene expression [54, 55]	Rheumatoid arthritis, dyslipidemia, atherosclerosis, and insulin resistance [81]

induction of stem cells proliferation and differentiation. ROS act as a rheostat, which senses and translates environmental cues in stem cells response, thus balancing cellular output (function) with cellular input (nutrients, cytokines). The stem cells may undergo exhaustion depending on ROS levels [84]. Mitochondrial ROS may activate an adaptive response (mitohormesis), which, as defensive mechanism, promotes health to extend the lifespan through diseases prevention and delay [5, 85]. ROS is integral in the development of physiopathologic events like mitochondrial death signaling [86] and autophagy [87], besides inflammation and infection [55, 88], in which they impart immunological changes. High ROS levels are generated by professional cells (lymphocytes, granulocytes, and phagocytes) in defense against microbes [89, 90]. Differently, any event which contributes to chronic OS or NS, through its increased generation or defective detoxification, dysregulates signaling networks, alters lipids and protein and nucleic acids, and activates mechanisms to face the changes. ROS overproduction hampers damaged nuclear and mitochondrial DNA repair, at multiple steps, contributing to cell genomic instability [91]. ROS are recognized as key modulators in processes that accumulate oxidized molecules chronically, as diabetes, cardiovascular diseases, atherosclerosis, hypertension, ischemia, reperfusion injury, neurodegeneration, and rheumatoid arthritis [17]. Also, ROS participate in cancer development through their

effects on cellular proliferation, mutagenesis, and apoptosis inhibition [56]. The cross talk between ROS, p53, and NF-κB plays crucial roles in tumorigenesis. OS is allied with energy metabolism to stimulate the growth of cells transformed by oncogenes or tumor suppressors [92–94]. The deregulated ROS productions in cancer cells and the consequent constitutive OS may cause the cellular invasive phenotype [57].

Although ROS functions remain difficult to investigate, multiple pharmacological investigations are in progress to maintain ROS homeostasis through both OS decrease and antioxidant defense increase [95, 96].

3. ROS in Aging and Age-Related Diseases

Poor knowledge of basic processes in aging interferes with interventions to prevent or delay age-related pathologies, like diabetes, cardiovascular disorders, neurodegenerative disorders, and cancer, which, consequently, impact human independence, general wellbeing, and morbidity [97–99]. Recently, interest has been focused on stem cells, because their decline impairs tissues homeostasis maintenance, leading to the organism weakening and the age-related diseases [84]. Aging mechanisms have been collected into two classes. The first class presents aging as genetically programmed by developmental processes, like the cell senescence, the neuroendocrine alterations, and the immunological alterations. The second class presents aging caused by random damage, that is, accumulation of somatic mutations and OS. The separation between the classes is no longer considered clear, because pathways involved in aging often share features with specific diseases [100]. The genetic heredity contributes no more than 3% to aging, while epigenetic processes and posttranslational processes imprint a significantly different aging rate among diverse populations, as well as among diverse anatomical sites of a single organism. In the onset of aging, telomere erosion, OS, and cell senescence are crucial events that originate from the disorganized homeostasis of cell metabolism. For example, mitochondria-nucleus interplay [101] and alterations of mitochondrial homeostasis drive age-dependent modifications [102, 103]. Ineffective ROS control on mitochondrial supercomplexes causes ROS signaling alteration, thus mediating cell stress responses towards age-dependent damage [104]. A progressive ROS scavengers decrease shifts aged cells towards a prooxidant status [105, 106]. In parallel, all the suggested methods to prolong lifespan, as caloric restriction and increased activity of SIRT1, share the OS reduction effect [107]. It is known that chronic muscular exercise protects older persons from damage caused by OS and reinforces their defenses against it. On the other hand, acute exercise increases ROS production and damage from ROS [108]. High levels of mitochondrial ROS contribute to aging of genetically modified animals, in a mechanistic way. Superoxide dismutase-deficient animals, SOD1- [109] and SOD3-deficient animals [110], and p66SHC-deficient animals show mitochondrial dysfunctions that generate oxidative damage and related phenotypes, resembling premature aging features. Similarly, mice that overexpress mitochondrial catalase counteract oxidative damage and live longer. The incidence of age-related diseases and pathologies in animal models, after they have been submitted to disparate patterns, suggests that OS influences old age aspects significantly [111]. The observations have been extended to humans, even if rate and distribution of mitochondrial mutations may deviate from animals. The conclusions regarding OS effect on aging in animals from mitochondrial genetic manipulations are still conflicting. SOD+/− mice have reduced ROS detoxifying ability and high ROS level, while they exhibit a quite normal lifespan. OS effect on worms' lifespan depends on where ROS are produced: high mitochondrial or cytoplasmic levels are associated with increased and decreased lifespan, respectively [109, 112]. It remains to define whether models' longevity is entirely associated with response to OS, because their lifespan is not affected by modulation of the antioxidant defense. The complex genetic manipulation of the models might weaken their support to the "OS theory of aging." Interventions to ROS lowering, by both scavenging free radicals and enhancing antioxidant defenses, are widely proposed as an antiaging strategy. However, positive association between supplementation with pharmacological or natural compounds and health beneficial effects has not been evidenced. Some antioxidants may be eventually useless or even harmful [113, 114]. Moreover, a number of ROS-independent mitochondrial dysfunctions appear so involved in aging that doubts arise that OS is the most concrete contributor to fuel aging [115]. Based on the consideration that mitochondrial DNA (mtDNA) is a precise marker to detect total mitochondrial OS, methods have been developed to measure mtDNA replication defects and the oxidative damage level, simultaneously. The errors in mtDNA replication and repair, which accumulate through clonal expansion in advanced age, result in a major source of mtDNA mutations, rather than the errors acquired through ROS-dependent vicious cycles [116]. Summarizing, ROS are involved in elderly lesions that concern (i) DNA insufficiency, which is partly responsible for premature aging and apoptosis [117]; (ii) RNA involvement in the onset of chronic-degenerative diseases [118]; (iii) nuclear lamins that participate in cell proliferation and longevity [119]. The variations of speed and quality in the aging of each organism may reflect the peculiar alterations that have been accumulated in DNA, proteins, and lipids [120], following the organism exposition to chronic stressors. Low ROS levels improve the defense mechanisms by inducing adaptive responses, which contributes to stress resistance and longevity, while high ROS levels induce insufficient adaptive responses, which may contribute to aging onset and progression [121].

In conclusion, accumulated mutations, decreased mitochondrial energy metabolism, and increased OS may significantly contribute to the human aging and the related diseases.

4. ROS-Dependent Epigenetic Modifications

Intra- and extracellular environments change hereditary characters at the epigenetic level, without altering genes sequence [122]. The interplay between modified histones, DNA methylation, regulator noncoding RNAs, and other reversible processes constitutes the epigenetic machinery that regulates genes transcription and expression [123]. The epigenetic modulation provides the essential and flexible interface

between organism and environment, which is essential for all the cell functions. The extent to which epigenome has shaped, and might shape, human populations over generations is investigated by an International Human Epigenome Consortium (http://www.ihec-epigenomes.org/). Both long- and short-acting stimuli lead to epigenetic effects that result in 13 being long-term (heritable) or short-term (nonheritable), respectively. These features suggest epigenetic modifications as more attractive target for therapeutic interventions in humans than genetic modification, throughout the entire life [124]. ROS operate modifications on histone and DNA, by acting in interconnected epigenetic phases, during mitochondrial and nuclear DNA regulation [125, 126]. A clinical example of ROS-dependent epigenetic modifications is demonstrated in "nonalcoholic fatty liver" disease. The pathology represents the most common cause of chronic liver disease in western countries and affects one-third of the population. Altered redox mechanisms mediate the link between increased accumulation of triglycerides in hepatocytes and epigenetic modifications that are recognized as crucial factors in the pathophysiology of this disease [127]. About the basic mechanisms of ROS action, Afanas'ev proposes that ROS might cause epigenetic activation and repression, by acting like nucleophilic compounds, which accelerate and decelerate hydrolysis and esterification reactions. The hypothesis suggests a ROS role different from free radicals, because the last molecules cause an irreversible damage of the compounds with which they react [128].

4.1. ROS-Induced DNA Methylation. Usually, condensed chromatin structure (heterochromatin) is associated with genes repression by hypomethylation processes, while open chromatin (eu-chromatin) is associated with genes activation by acetylation processes [129]. The epigenetic marking modulates the genes expression by altering the electrostatic nature and the protein binding affinity of chromatin. DNA methylation causes gene silencing through inhibiting the transcriptional activators access to the target binding sites, or through activating the methyl-binding protein domains. The last function interacts with histone deacetylases and promotes chromatin condensation into transcriptionally repressive conformations. Hypo- and hypermethylation stages occur consecutively, indicating how DNA methylation and the correlate mechanisms of DNA binding are complex. ROS-dependent modifications are related to DNA methylation and demethylation, directly or indirectly. The NF-κB binding to DNA, which is methylation dependent, results in being altered in SOD (Cu/Zn)-deficient mice. The observation associates ROS-dependent modifications with altered methylation processes, although indirectly, and suggests that modifications linked to altered redox mechanisms may fit into cell signaling pathways [130]. Also, the oxidation of deoxy-guanine of CpG nucleotides to 8-hydroxy-2′-deoxyguanosine (8-OHdG) is believed to be a surrogate marker of oxidative damage, in various human diseases [131]. The 8-OHdG adducts interfere with DNA restriction nucleases and DNA methyl transferases (DNMT), thus altering transcription factors binding to DNA and causing general DNA hypomethylation. *In vitro* [132] and *in vivo* [133] studies demonstrate that ROS induce general genome hypomethylation and specific DNA promoters hypomethylation, via the DNMT upregulation and the DNMT complexes generation. Moreover, recent studies show that a ROS-mediated pathway causes repression of the protein kinase C epsilon gene, through its promotor methylation. The events are important in heart hypoxia, *in utero*, which leads to heightened heart vulnerability to ischemic injury, later in people's life [134].

4.2. ROS and DNA Methylation in Aging and Age-Related Diseases. Starting from the observation that both defective genome and DNA repair processes promote phenotypes of premature aging, the "aging epigenetics" has been developed as emerging discipline, which concerns genes and processes impacting aging (Figure 1) [135]. ROS effects on epigenetic mechanisms have been discussed as cause and consequence of aging and age-related DNA modifications [128]. Recent studies demonstrate that global DNA hypomethylation is deeply included in aging gene expression [136], and, at the same time, cancer is the age-related disease that shows the most significant effects of ROS-dependent DNA methylation [137]. Tumor progression is induced by general hypomethylation of the DNA and hypermethylation of tumor suppressor genes that lead to aberrant genes expression [138–140]. Abnormal and selective DNA methylation may constitute a potential biomarker and a tool to assess therapeutic treatments at the same time. The data on OS-mediated alterations in DNA methylation, which have been so far obtained, motivate chemoprevention trials, to reduce OS in cancer diseases [141–143]. In human aging, the telomerase reverse transcriptase (hTERT) controls the mitochondrial function and the cellular metabolism, besides the telomeres structure. The enzyme is regulated by DNA methylation. Various observations demonstrate that hTERT may confer major sensitivity towards OS [144] and reduce ROS increase in aging and age-related diseases [145]. Examples of both ROS levels and DNA methylation, which seems to change with age, suggest that they are potentially linked [146, 147]. ROS-induced methylation at SOD2 gene promoter causes the decreased expression of the gene, which may be associated with the disruption of the cardiorespiratory homeostasis, a typical problem of the old humans. Treatments with DNA methylation inhibitors, in preclinical studies, can prevent the hypoxic sensitivity that leads to the respiratory dysfunction [148]. Also, both ROS-induced 8-OHdG and 5-methyl cytosine generate abnormal GC regions in the DNA, which undergo further methylation and oxidation, thus hampering DNA repair enzymes. These regions have been demonstrated to hit gene expression and DNA susceptibility to damage in Alzheimer's pathology [149].

In complex, ROS are involved in DNA methylation processes in different conditions, occurring in the human aging. The epigenetic machinery operates as OS sensor, which contributes to the OS control and, at the same time, orchestrates the progressive homeostasis impairment, which shapes the cardiovascular, respiratory, and nervous systems of old human beings [146]. The ROS signaling in the DNA methylation during the aging process deserves to be more deeply studied.

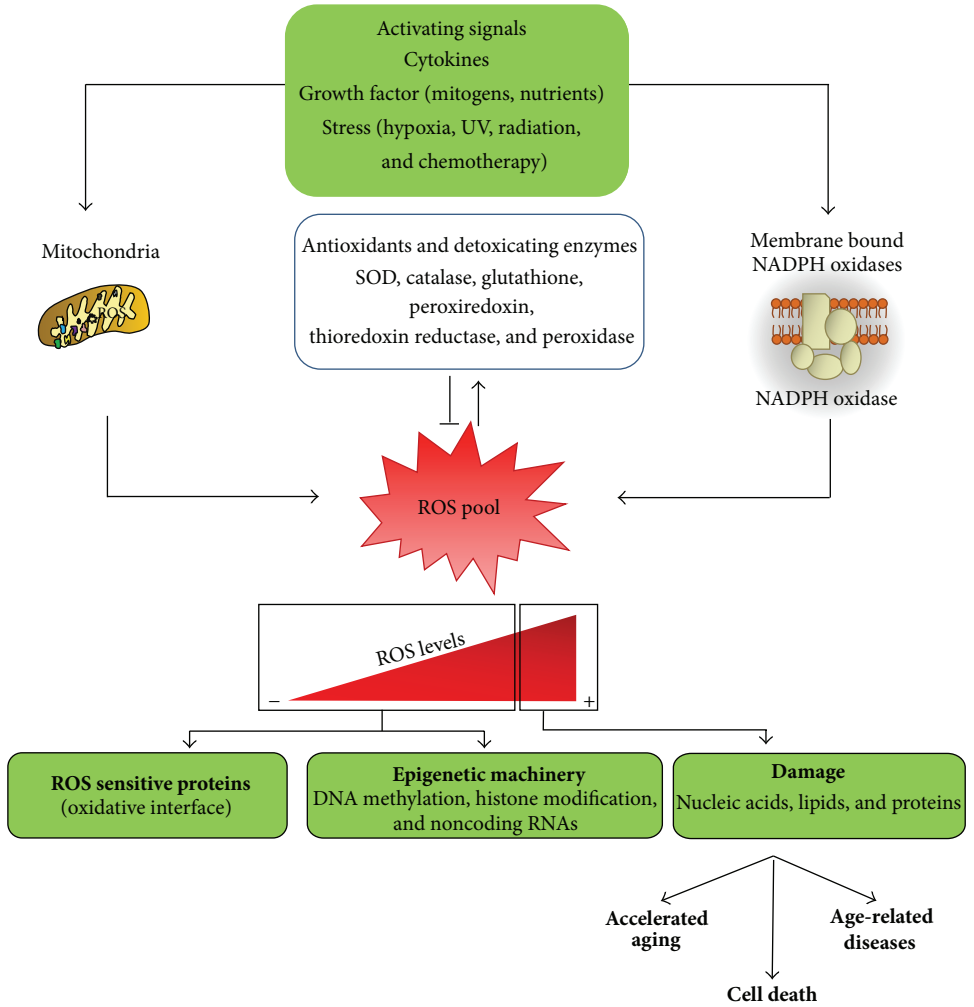

Figure 1: Schematic representation of ROS signaling in physiological and pathological conditions. Low and medium ROS levels produced by mitochondria and NADPH oxidase activate cell ROS sensitive proteins and epigenetic machinery. High ROS level causes nucleic acids, lipid, and proteins damage possibly involved in accelerated aging, cell death, and age-related diseases.

5. ROS in Cell Senescence

The cell senescence has indicated the irreversible G1 growth arrest of normal primary cells, which occurs after the cells have accumulated time-dependent damage, during extensive culture passages ("replicative senescence"). The cells resist apoptosis and face malignant progression through cytostasis, thus causally contributing to cell senescence induction and maintenance. The senescent cells are able to diversify constantly, like cancer cells, but missing proliferation as a driver [7, 9]. Large and flat shape, rich cytoplasmic and vacuolar granularity, high levels of lysosomal β-galactosidase activity (SA-βgal), p16, p21, macroH2A, IL-6, phosphorylated p38MAPK, and "double-strand breaks" are the most common senescent cells features in *in situ* assays [9]. The exact mechanisms underlying the cell senescence onset and stabilization are still obscure. OS, mitochondrial deterioration, DNA damage, oncogenes expression, and loss of tumor suppressor genes, like PTEN, RB1, NF1, and INPP4, can induce cell senescence [9]. "Replicative senescence," which is provoked by endogenous stimuli, is distinct from "stress-induced premature senescence," which is provoked by exogenous stimuli. The two processes share molecular and functional features, although they are dependent, or not, on telomeres status, respectively. Intrinsic and extrinsic events can induce either the cell senescence or the apoptosis process, depending on the level of the impairment of the cell homeostasis [150] and the p53 activity [47]. The molecules secreted by senescent cells (secretoma) cooperate deeply to maintain the tissues homeostasis, through autocrine and paracrine activities [151], by acting at multiple levels: epigenome [152], gene expression, protein processing, and metabolic control [153]. Moreover, specific mitochondrial pathways contribute to priming the senescence process, through the alteration of the mitochondrial redox state [6, 151]. The senescence secretoma acts in physiological and pathological events, as tissue remodeling during embryogenesis, tissue repair in wound healing, and induction of aging, as well as age-related diseases of different organisms. The secretoma develops beneficial effects on carcinogenic DNA lesions of precancerous cells,

by both preventing their uncontrolled cell proliferation and reacting with specific anticancer compounds [154]. However, the secretoma may provide indispensable cytokines for the cancer cells growth, thus promoting tumorigenesis in definite conditions, which are partly related to the cellular metabolic state [155]. Cause-effect relationships between cellular ROS production and cell senescence have been investigated through diverse pathways that comprise the following.

(i) Mitochondrial DNA (mtDNA) Damage. ROS contribute to cellular senescence onset and progression by damaging mtDNA directly or in synergy with modifications of the telomerase reverse transcriptase (TERT) enzyme and the p53 and Ras pathways activity [9]. Also, ROS production by serial signaling through GADD45-MAPK14 (p38MAPK)-GRB2-TGFBR2-TGFb is both necessary and sufficient for the stability of growth arrest, during the establishment of the senescent phenotype [156].

(ii) Signaling Pathways via Ras, p53, p21, and p16. The pathways generate ROS, which act as signaling molecules, without causing oxidative DNA damage. ROS result as a tightly regulated signaling process for the induction of the cell senescence [157].

(iii) Autophagy. High ROS levels mediate p53 activation that induces autophagy inhibition. This event generates mitochondrial dysfunction, which in turn generates cell senescence. The autophagy inhibition causes the senescent cells to aggregate oxidized proteins and protein carbonyls with products of lipid peroxidation and protein glycation into the lipofuscin [158].

(iv) miR-210 and miR-494. The induction of these microRNAs by ROS generates mitochondrial dysfunction and autophagy inhibition [159].

The (iii) and (iv) pathways generate vicious loop cycles in ROS production. Autophagy inhibition causes lipofuscin accumulation, which activates further autophagy impairment and ROS production, consequently. All the factors (i), (ii), (iii), and (iv) may add to DNA damage and dysfunctions of both mitochondria and cell metabolism homeostasis [159]. *In vitro* and preclinical experiments show that ROS decreasing interventions influence cell senescence progression, via the slowdown of telomere shortening and the extension of the cell lifespan. Replicative telomere exhaustion, DNA damage, and OS prime the cell senescence by sharing the activation of the "DNA Damage Response." ATM or ATR kinases of these signaling pathways cause p53 stabilization and transcriptional activation of the p53 target, p21 [9]. p53 triggers cell cycle arrest by upregulating p21, which inhibits the cell cycle regulator cyclin-dependent kinases Cdk4 and Cdk2 [159]. Whereas high OS levels induce the prosenescence function of p53, the mild OS levels that are induced by the physical exercise in humans have a positive effect on cell and mitochondrial homeostasis. p53 exerts a dual effect on cell senescence because of its ability to both decrease and increase the cellular OS level [160]. In parallel to "DNA Damage Response," the mitochondrial p38-MAPK replenishes the short-lived DNA damage foci, via a ROS feedback loop, and induces the senescent secretoma [161].

The occurrence of the ROS role in cell senescence onset and maintenance might be relevant for therapeutic interventions, which aim to modulate ROS levels in cancer cells, as well as in aging processes [156]. Human kidney dysfunctions exemplify progressive stages of ROS-induced cell senescence. ROS act like a sensor in regulating the oxygen-dependent gene expression of the kidney and play a leading role in the inflammatory processes, to which the organ is especially sensitive [162]. In conclusion, the ROS signaling has highlighted key factors for the cell senescence induction and maintenance, which are the object of intensive investigations.

5.1. Cell Senescence in Aging and Age-Related Diseases (ROS Effect). The "replicative cell senescence" is considered an aging hallmark on the basis of two motives: (1) the senescent cells accumulate in organismal tissues, by rate and proportion, which parallel the age advancement; (2) the senescent cells accelerate the age-related decrease of tissue regeneration, through the depletion of stem and progenitors cells [8, 97]. While the sequence of proliferative arrest (senescence), recruitment of immune phagocytic cells (clearance), and promotion of tissue renewal (regeneration) results in being beneficial upon a damaged tissue, for instance, the sequence is inefficiently completed in aging tissues, causing senescent cells to undergo chronic accumulation [163]. Also, a delicate balance exists between cell senescence positive effects on tumor suppression and negative effects on aging related processes [164]. The transcription factor and tumor suppressor p53 are involved in DNA repair and cellular stress response, as well as cellular cycle control. In addition, p53 modulates both the cell senescence and the aging process, through the coordination of specific cellular pathways [165, 166]. It is not clear whether p53 mechanisms in cell senescence and aging are common [160]. An increased senescence secretoma causes detrimental effects over the years and contributes to the typical disruption of aged tissues [8, 167, 168]. Senescent cells endowed with the semiselective marker of senescence p16 drive age-related pathologies, which are delayed or prevented by the selective elimination of the senescent cells [169]. A partial list of suggested markers of cell senescence in human tissues, both aged and affected by age-related pathologies, is reported in Table 2 [170–197]. Lungs show a typical example of cell senescence associated with the progressive, age-related organ dysfunction. The OS generated by the potent cigarette oxidants is a key element in the pathogenesis of the pulmonary emphysema, induced by the chronic smoking. The fibroblasts that provide essential support and matrix for lung integrity show reduced proliferation rate and increased SA-βgal activity in patients affected by pulmonary emphysema. These senescent fibroblasts contribute to the lung disease by affecting the tissue homeostasis. Also, senescent features of the endothelial cells in chronic smokers associate with premature vessels atherosclerosis. In patients with severe coronary artery disease, OS accelerates the senescence of endothelial cells, which is related to risk factors for cardiovascular disease [198]. A further example

Table 2: Clinical examples of senescence-associated biomarkers detected in organs and tissues of patients affected by age-related diseases.

Organ/tissue	Senescence-associated biomarkers	Clinical references
Cardiovascular diseases		
Aged vascular tissues	Telomeres length, SA-βGal, p16, and p21	[170, 171]
Atherosclerosis		
Systolic heart failure		
Malignant tumors		
Lung cancer	Telomeres length, SA-βgal	[172, 173]
Breast cancer	SA-βgal, p21, p16, DEP1, NTAL, EBP50, STX4, VAMP3, ARMX3, B2MG, LANCL1, VPS26A, and PLD3	[174, 175]
Neuroblastoma	SA-βgal	[176]
Astrocytoma	SA-βgal	[177]
Mesothelioma	SA-βgal, p21	[178]
Melanoma	SA-βgal, p16, and p21	[179]
Prostate cancer	SA-βgal, Glb1, and HP1g	[154, 180]
Liver cancer	Telomeres length, SA-βgal	[181]
Colorectal cancer	Short telomeres	[182]
Fibrosis		
Idiopathic pulmonary fibrosis	Telomeres length, IGFBP5, and SA-βgal	[183, 184]
Cystic fibrosis	Telomere length, p16	[185]
Liver fibrosis	Telomere length, IGFBP-5, SA-β-gal, and p21	[183, 186]
Renal fibrosis	p16	[187, 188]
Neurological disorders		
Alzheimer's disease	SA-β-gal	[189, 190]
Other diseases		
Chronic obstructive pulmonary disease	Telomere length, p16, p21, and SA-βgal	[191, 192]
Pulmonary hypertension	p16, p21	[192, 193]
Emphysema	Telomere length, IGFBP-3, IGFBP-rP1, p16INK4a, and p21	[194, 195]
Benign prostatic hyperplasia	SA-βgal	[196, 197]

of aging dysfunction related to cell senescence is shown by the scaffolding protein Caveolin 1 (Cav1), which controls molecular signaling in caveolar membranes. Cav1 promotes cellular senescence in age-related pathologies, by mediating p53 activation with EGF modulation, focal adhesion, and small Rho GTPase-dependent signaling. The upregulation of the Cav1 promoter by high ROS levels contributes to explaining how OS promotes cell senescence effects in aging and age-related diseases [198]. In addition, the interplay between different conditions of mitochondrial homeostasis and ROS-dependent signaling pathways contributes to aging process, through the cell senescence induction and stabilization [199]. Yet ROS-independent signaling pathways link dysfunctions in mitochondria and aging, through the cell senescence process [6, 151]. As a new approach, preclinical and clinical studies demonstrate the therapeutic effects of the aging inhibitor rapamycin, whose signaling pathway is involved in cellular senescence [160, 200].

In conclusion, cell senescence reduces the age-related tumor development and contributes to human aging, suggesting that aging might be switched for tumorigenesis [201, 202]. ROS may modulate tumor suppression process, which is induced by the senescence, thus participating in anticancer mechanisms, although ROS may act as tumor promoters in definite conditions [48]. With the cell senescence and aging controlled by cells and cellular environment, the possibility is suggested that the two processes may be subjected to interventional therapies [203, 204].

5.2. Epigenetic Mechanism in Cell Senescence (ROS Involvement). The epigenetic control of acute and chronic cellular senescence allows for the two processes that are involved in various conditions that lead to the cells longevity preventing cell death and tumorigenesis [205]. The abrogation of tumor suppressor pathways, as p53 and p16/Rb, bypasses the cell senescence, thus leading to the tumorigenic phenotypes acquiring [206]. The mechanisms that balance the transcriptional state of the chromatin are not fully understood. Some regulative changes involve the histone proteins that coordinate the DNA accessibility, through transcription factors, besides the DNA replication and repair. The Polycomb Repressor Complex 2 (PRC2) initiates and preserves specific histone methylations, thus acting as an epigenetic mark that mediates targeted genes [207]. The repression of the histone activity by the Polycomb Group (PcG) proteins causes gene silencing, but it can be countered by specific demethylases,

which erases the methyl mark [208]. The upregulation of many PRC target genes leads to global epigenetic changes [209–211]. Specific transcription factors [212], as well as long noncoding RNAs [213], are involved in the recruitment performed by PRC. PRC2 takes a crucial part in silencing the locus of p16, the marker that is upregulated during cell senescence [212]. The reversal of chromatin epigenetic pattern via deacetylation, demethylation, and dephosphorylation is significantly involved in underscoring both flexible and dynamic nature of histone modifications [214]. The histone demethylases JMJD3 produce diverse outputs of biological function, depending on the action of their transcriptional complexes. Different expression of these demethylases, which have tumor suppressor activities during the "stress-induced senescence" [215, 216], is reflected into cellular phenotype changes and variations associated with cellular senescence [217]. The JMJD3 gene is located near the p53 tumor suppressor gene, that is, a genomic area that is frequently lost in various malignancies. The SIRT1 histone deacetylase (SIRT1) is a known regulator of age-related diseases that regulates the senescence secretoma components, by silencing their promoter regions epigenetically. SIRT1 plays a pivotal role in stress modulation also through p53 deacetylation, acting against aging and age-related diseases. As indicated above, the high ROS levels activate p53, which, in turn, activates p53-mediated apoptosis and cell senescence. Moreover, SIRT1 regulates the ROS-dependent FOXO factors, which are responsible for cell growth, proliferation, and longevity. The characteristic ROS increase during aging may be responsible for the decreased SIRT1 activity, which facilitates the senescent-like phenotype. SIRT1 causes oxidant effects, as well as antioxidant effects, by acting on epigenetic modifications, which include acetylation and deacetylation (see references in [128, 146]). Experiments on cell senescence induction show different molecular mechanisms in acute versus chronic senescent cells. A better knowledge of the order in which epigenetics mechanisms change during the cell senescence progression, from initial towards full senescence, is believed to be vital for finding therapies against age-related disorders [9].

5.2.1. Noncoding RNA. Latest genomics tools and sequencing approaches have helped unravel large chromosomes stretches, which were previously deemed not transcribed [218, 219]. These sequence regions contain noncoding RNA (ncRNA), which is known as long lncRNAs, and short ncRNAs. Among short ncRNAs, the microRNAs (miRNAs) have emerged as being able to control the gene expression, either by blocking targeted mRNA translation or by mRNA degrading [220, 221]. Recently, ncRNA role is gaining more importance in age-associated dysfunctions as cardiovascular diseases [222, 223]. The senescence-associated lncRNAs are differentially expressed in proliferating and senescent fibroblasts, as assessed by RNA sequencing [224–226]. Toxicological studies associate increased ROS production with increased expression of a set of 115 lncRNAs, which significantly affect p53 signaling pathway [227]. A mitochondrial-transcribed lncRNA is induced in aorta and endothelial cells aging, during the "replicative vascular senescence," which is partly responsible for age-associated cardiovascular diseases, but not in the "stress-induced premature senescence" by ROS [228].

5.2.2. microRNA (miRNA, miR). Normal cellular development and homeostasis are under the control of miRNAs, throughout the entire life [229], since miRNAs regulate the gene expression in biological processes as proliferation, development, differentiation, and apoptosis. Yet several miRNAs families control cell senescence at multiple levels, by regulating the autophagy process and the gene expression involved in ATP and ROS production. Some miRNAs may induce ROS production that generates a self-sustaining ROS vicious cycle [159]. miRNAs constitute a connection between aging, cell senescence, and cancer. The miRNAs dysregulation causes the activation of pathways they normally repress. The event may activate aberrant pathways and also aging mechanism in young individuals [222]. Although current studies are monitoring miRNA tissues and systemic alterations, instead of miRNA changes through lifespan and metabolic modifications, several profiles of miRNA expression demonstrate changes during the aging. As an example, miR-29, which targets the genes of type IV collagen and maintains the structure of the extracellular matrix, increases in elderly mice, thus causing collagen decreasing, a tissues basement membranes weakening [230]. Only few miRNAs have been directly linked to age-related changes in cellular and organ functions, whereas many miRNAs have been directly connected with disease states. It is unclear if the modifications of miRNA profiles are mostly involved in pathological changes onset or if they mark the senescence end, which leads to the organ aging and dysfunction. Altered expression in miRNA activity has been observed in elderly people, as in the case of miR-34a, which belongs to a family with conserved functions in controlling aging and age-related diseases [203, 231, 232]. miR-34a targets ROS scavenger enzymes inducing OS [159]. The miR-34a upregulation or overexpression has been associated with cell proliferation inhibition, subsequent cell senescence induction, and premature death, in both endothelial progenitor and mature cells. miR-34a causes memory function impairment when it is upregulated in aged mice and in models for Alzheimer's disease (AD), while miR-34a targeting restores the memory function [233]. Also, the miR-34 mutation of the loss-of-function delays the age-related decline markedly, thus resulting in extended lifespan and increased resistance to the heat and the OS. The human miR-34a is downregulated in Parkinson's disease brain, while it is upregulated in AD brains [234] and in plasma of Huntington's disease patients [235].

Several miRNA families are modulated by ROS in the development of mitochondria-mediated cell senescence, which are, indirectly or directly, implicated in human pathologies. Little is known about the roles of ROS-modulated miRNAs in cell function. The molecular mechanisms that control neuronal response to OS have been deeply studied in different strains of senescence accelerated mice, based on the consideration that OS plays a critical role in AD etiology and pathogenesis. OS upregulates a group of miRNAs (miR-329, miR-193b, miR-20a, miR-296, and miR-130b), which is associated with affecting 83 target genes. Among the

genes, mitogen-activated protein kinase signaling pathway has been suggested to play a role in pathogenesis of neurodegenerative diseases [233]. OS effects on vascular homeostasis, including angiogenesis in physiological processes and age-related diseases, are largely studied in human umbilical vein endothelial cells (HUVECs), considering that miRNAs modulate endothelial cells response to OS. ROS induce the expression of miR-200 family members (miR-200c, miR-141, miR-200a, miR-200b, and miR-429), which determines apoptosis and cell senescence both in HUVEC cells and in a model of hind limb ischemia, which shows OS-mediated mechanism [236]. The miR-200 family plays a causative role in the vascular diabetic inflammatory phenotype in a diabetic model and in the human vasculopathy disease, suggesting that miR-200 inhibition might represent a therapeutic target to prevent OS negative effects on cell function and survival [146]. Also, miR-200 family has been extensively studied in epithelial-to-mesenchymal transition of cancer cells [236]. Lately, miR-760 and miR-186 upregulation has been associated with replicative senescence in human lung fibroblast cells. These miRNAs cooperate to induce senescence through the ROS-p53-p21Cip1/WAF1 pathway, which depends on the ROS generated by the downregulation of the protein kinase 2 (CK2α). A better understanding of the mechanisms of CK2 regulation might provide new therapeutic options to restore the function of lungs in aged people. An example of the increasing evidence that miRNAs are critically involved in the posttranscriptional regulation of cell functions, including the ROS signaling modulation, is underlined in Figure 2.

6. Conclusion and Future Perspectives

The multifactorial and inexorable phenomenon of aging worsens the human functions at multiple levels, causing a gradual reduced ability to resist stress, damage, and illness. Healthy aging appears to be an ideal healthcare priority that entails a better understanding of aging, with the aim of slowing down the process and preventing or even treating its related pathologies [200]. Indeed, genetic insights combined with findings from animal and cellular models have advanced our understanding of pathways that lead to age-related features, highlighting possible interventional targets [2–5]. The cellular senescence process is considered an aging hallmark, because it drives the cells through longevity, by hampering tumorigenesis and cell death, and is involved in many age-related diseases [97, 205, 206]. The cell senescence is a feature that characterizes somatic cells, except for most tumor cells and certain stem cells [6–10]. The senescent cells produce a specific secretoma that cause beneficial effects, through its autocrine and paracrine mechanisms. When the senescent cell program is inefficiently developed, as it occurs during the aging, the secretoma causes detrimental effects [151–153, 167, 168, 199]. In the recent years, evidence has been accumulating that ROS, which include H_2O_2, superoxide, anion, and hydroxyl radicals, generated from both intrinsic and extrinsic events, inhibit cell growth and induce cell death and senescence in a context-dependent manner [157, 236]. Through the understanding of the ROS role as signaling molecules in a myriad of signaling pathways, ROS levels are no longer considered as mere metabolic byproducts but are believed to be a "redox biology" that regulates physiological functions, including signal transduction, gene expression, and proliferation [37]. Firstly, it has been evidenced that the DNA damage caused by ROS acting as mutating agents contributes to the induction and maintenance of the cell senescence process [9, 156]. More recently, particular attention has been focused on the ROS involvement as signaling molecules in cell senescence induction, without causing DNA damage. Signaling pathways via Ras, p53, p21, and p16 have been defined to generate ROS, which may act as tightly regulated process contributing to the cell senescence induction [20, 157, 158]. Cause-effect relationships between cell ROS production and cell senescence have been investigated through diverse pathways that include the field of mitochondrial DNA and autophagy inhibition and the effects of the microRNAs miR-210 and miR-494 in various mitochondrial processes [159]. These pathways highlight ROS contribution to prime cell senescence at diverse levels, among which epigenetic level is attracting more and more attention in studies aimed at the senescence control [227, 233, 236]. Indeed, the epigenetic modulation provides the essential and flexible interface between the organisms and the environment, which results in being essential for all the cell functions [122, 123, 129], throughout the lifespan [135–137]. A major breakthrough in the last decades has been the understanding that epigenetics contribute to human diseases development.

In parallel, the "OS theory of aging" remains the most documented mechanistic hypothesis of aging, although it does not necessarily imply ROS imbalance as the earliest trigger or the main cause of aging [98–103]. Therapeutic ROS modulation is suggested as relevant in aging and related events [95, 96, 114]. Also, the senescent cells have been identified as a novel potential therapeutic target in the aging and age-related diseases [169, 171]. Further research is needed to define when and where cell senescence results in being favorable or unfavorable to organismal health. Both pro- and antisenescent therapies can be equally helpful, when they are opportunely modulated and balanced. Prosenescent therapies contribute to minimize damage in the cancer disease and in the active tissue repair by limiting proliferation and fibrosis, respectively, while antisenescent therapies may help to eliminate accumulated senescent cells and to recover tissue function. The current research points to a double objective: to define the changes about the redox-sensitive cell pathways and to define the OS role in linking environmental factors with epigenetic modifications.

Particular emphasis is addressed to novel mechanism of ROS and epigenetics in cell senescence and aging [160, 165, 166]. The histone demethylases network is often synergizing with the action of histone deacetylases, histone methyl transferases, and various nuclear transcriptional complexes, thus ensuring that the chromatinic environment is correct for the cell [128, 146]. Preclinical and clinical examples of ROS-dependent epigenetic modifications [125–127, 130–134, 138] extend their effects to aging [135, 136] and age-related diseases [137, 142–144, 146–149], particularly towards cancer disease [139–141, 145]. Among the noncoding RNAs, miRNAs families provide a broad silencing activity of mRNA

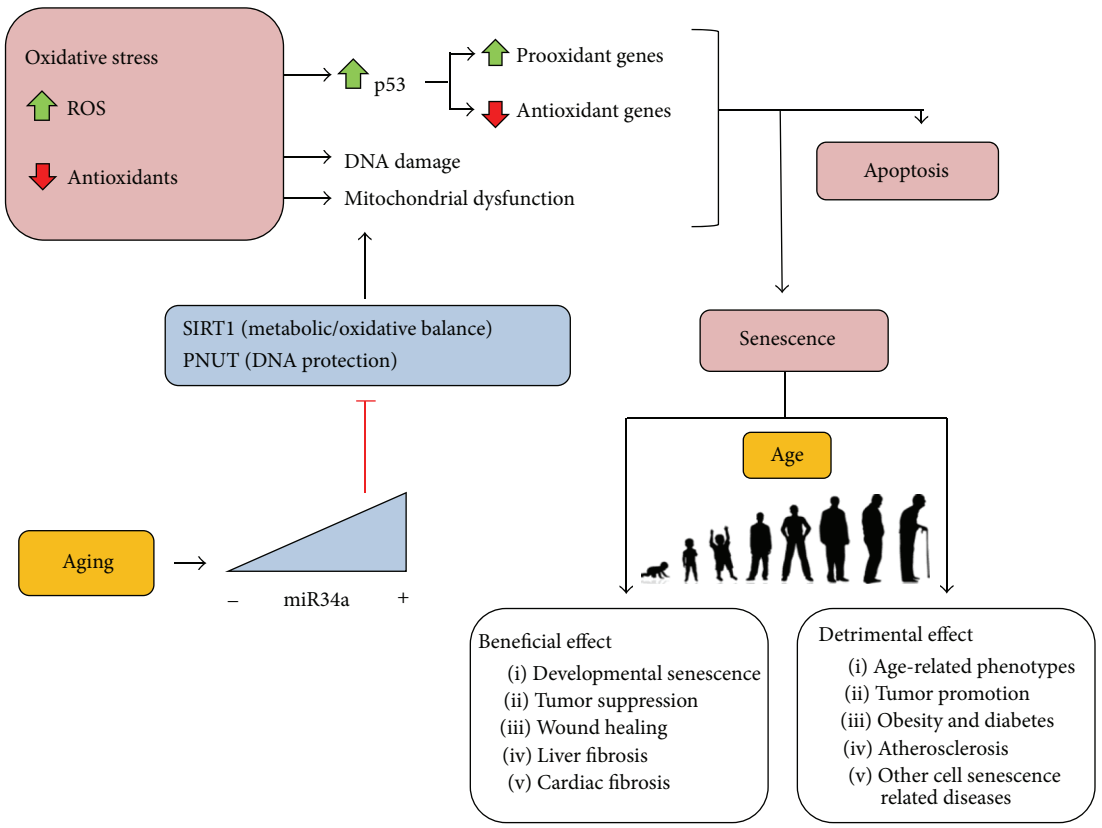

Figure 2: ROS-mediated senescence. Besides causing DNA damage and mitochondria dysfunction, OS activates p53 that, in turn, induces prooxidant genes and imbalances antioxidant genes induction. The set of alterations caused by ROS lead to induction of cell senescence, which, in turn, can develop both positive and negative effects; miR34a expression increases with aging in many tissues downregulating SIRT1 protein activity (a longevity promoting factor) and PNUT protein (a DNA protecting factor which prevents telomere attrition and is involved in tissues repairs).

targets, in a sequence dependent fashion that modulates the stress response [159]. Accumulating evidences show that stressors, including ROS, potentially alter the function of miRNA-processing in aging organisms, which renders the cells even more prone to stress, linking aging and cancer. Several miRNAs families induce ROS level increase in aging or target factors involved in the ROS signaling. In addition, ROS increase highly correlates with a specific miRNA dysregulation, which mediates the cross talk between p53, NF-κB p65, and ROS. All these events have been associated with cell senescence [203, 231, 232]. At the same time, certainly several miRNAs families are modulated by ROS in the development of mitochondria-mediated cell senescence, which are, indirectly or directly, implicated in human pathologies [159, 233, 236]. Because epigenome is so tightly regulated and complex, understanding individual modifications and their network of interaction offers the potential to design drugs that are very effective therapies against a number of diseases [124, 203–205, 219–222]. More reliable OS biomarkers, as well as OS related epigenetic mechanisms, have emerged over the last years as potentially useful tools to design therapeutic approaches aimed at modulating *in vivo* enhanced OS.

Abbreviations

AP-1:	Activator protein-1
DDR:	DNA Damage Response
FOXO3a:	Forkead homeobox type O
HIF-1a:	Hypoxia inducible factor-1a
hTERT:	Human telomerase reverse transcriptase
miRNA, miR:	MicroRNA
JAK/STAT:	Janus kinase/signal transducers and activators of transcription
Nox:	NADPH oxidases
NF-κB:	Nuclear factor kappa B
NS:	Nitrosative stress
Nrf2-ARE:	NF-E2-related factor 2 binding to the antioxidant responsive elements
p53:	Tumor suppressor p53
OS:	Oxidative stress
PPARγ:	Peroxisome proliferator-activated receptor gamma
RNS:	Reactive Nitrosative Species
ROS:	Reactive Oxygen Species
SA-βgal:	Senescence-associated β-galactosidase
SOD:	Superoxide dismutase.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Cross Talk Mechanism among EMT, ROS, and Histone Acetylation in Phorbol Ester-Treated Human Breast Cancer MCF-7 Cells

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Epithelial-mesenchymal transition (EMT) plays a pivotal role in the progression of cancer, and some transcription factors including Slug and Snail are known to be involved in EMT processes. It has been well established that the excess production of reactive oxygen species (ROS) and epigenetics such as DNA methylation and histone modifications participate in carcinogenesis; however, the cross talk mechanism among EMT, ROS, and epigenetics remains unclear. In the present study, we demonstrated that the treatment of human breast cancer MCF-7 cells with phorbol ester (TPA), a protein kinase C activator, significantly induced cell proliferation and migration, and these were accompanied by the significant induction of Slug expression. Moreover, the TPA-elicited induction of Slug expression was regulated by histone H3 acetylation and NADPH oxidase (NOX) 2-derived ROS signaling, indicating that ROS and histone acetylation are involved in TPA-elicited EMT processes. We herein determined the cross talk mechanism among EMT, ROS, and histone acetylation, and our results provide an insight into the progression of cancer metastasis.

1. Introduction

Accumulated evidence shows that the excess generation of reactive oxygen species (ROS) elicits oxidative stress in cells and tissues and leads to various diseases, such as cancer, atherosclerosis, and type 2 diabetes [1–3]. A recent study demonstrated that epithelial-mesenchymal transition (EMT) plays a pivotal role in cancer metastasis [4], including breast cancer, which is the most common malignancy in Japanese women. The expression of Slug and Snail, which are key transcription factors in EMT processes, was previously found to be increased in cancer tissues and has been closely associated with EMT phenomena [5–7]. EMT is characterized by the loss of epithelial-like properties including the tight-junction proteins, E-cadherin and N-cadherin [8–10], and the acquisition of mesenchymal properties such as the extracellular matrix protein fibronectin-1 [11, 12]. These processes increase aggressiveness and enhance the metastatic spread of breast cancer [13]; therefore, identifying key molecules in EMT and elucidating the mechanisms underlying it may ultimately result in the suppression of breast cancer malignancy.

Epigenetics, such as DNA methylation and histone modifications, are typically referred to as mitotically heritable changes in gene expression that do not involve any changes in DNA sequences [14]. DNA methyltransferases (DNMTs) 1, 3A, and 3B are known to play critical roles in DNA methylation processes by using S-adenosyl methionine as a methyl donor [15]. Previous studies demonstrated that global DNA hypomethylation and regional hypermethylation are related to the initiation and progression of tumorigenesis [16–18]. Hypermethylation of the *p53* promoter region, which decreases its expression, has been suggested to lead to tumor progression [19–21]. On the other hand, histone modifications including acetylation and methylation at arginine or lysine residues are also associated with gene expression and silencing [22–24]. Among histone modifications, the histone acetylation status is regulated by histone deacetylase (HDAC) and/or histone acetyltransferase (HAT) [25–27]. Recent studies showed that the expression of E-cadherin was regulated by its DNA hypermethylation in hepatocellular carcinoma (HCC) tissues [28]; however, the role of histone

modifications in EMT processes, especially in the regulation of the expression of transcriptional factors, remains unclear.

In the present study, we examined the induction of Slug expression in phorbol ester- (TPA-) treated human breast cancer MCF-7 cells. The results obtained indicated that the TPA-elicited induction of Slug expression is associated with histone H3 acetylation within its promoter region, and these processes are due to the excess production of NADPH oxidase- (NOX-) derived ROS. Taken together, these results contribute to a deeper understanding of the significant role of ROS in EMT processes and epigenetic gene regulation and may lead to the development of novel epigenetic therapies for breast cancer.

2. Materials and Methods

2.1. Materials. TPA and HRP-conjugated goat anti-rabbit (A6154) and anti-mouse (A4416) IgG were purchased from Sigma-Aldrich Co. (St. Louis, MO). A PKC inhibitor (GF109203X) and actinomycin D (ActD) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cyclopentylidene-(4-(4′-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2) was purchased from Calbiochem (San Diego, CA). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFHDA) and dihydroethidium (DHE) were purchased from Molecular Probes (Eugene, OR). Diphenyleneiodonium (DPI) and garcinol (Gar) were purchased from Enzo Life Sciences Inc. (Farmingdale, NY). Trichostatin A (TSA) was purchased from Cayman Chemical (Ann Arbor, MI). An anti-phospho-PKC (pan) (βII Ser660) rabbit polyclonal antibody (#9371) and normal rabbit IgG (#2729) were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin mouse monoclonal antibody (MAB1501) and anti-acetyl-histone H3 (#06-599) and H4 (#06-598) rabbit polyclonal antibodies were purchased from Millipore Co. (Billerica, MA).

2.2. Cell Culture. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator. Cells were grown to confluence on a 96-well plate (seeded at 1×10^4 cells/well or 2×10^4 cells/well), 4-well plate (seeded at 5×10^4 cells/well), 6 cm culture dish (seeded at 3×10^5 cells/dish), or 10 cm culture dish (seeded at 1×10^6 cells/dish) and treated with the reagents described below.

2.3. Wound-Healing Assay. After cells became confluent, a wound field was prepared using a pipette tip. Culture medium was then replaced with fresh medium containing 20 μM DPI and incubated for 1 h. The wound field 24 or 72 h after the TPA treatment (1 nM) was examined under a microscope.

2.4. Cell Proliferation Assay. MCF-7 cells were seeded on 96-well plate at 1×10^4 cells/well and grown for 12 h. After that, the cells were treated with or without 1 nM TPA for 12 h following incubation for 2 h with Cell Counting Kit-8 (CCK-8) assay reagent (Dojindo, Japan). The colorimetric intensity at 450 nm of each well was measured using the iMark™ microplate reader (BioRad Lab, Hercules, CA).

Table 1: Primer sequences used in RT-PCR.

Gene	Sequences
Slug	S: 5′-AGCCAAACTACAGCGAACTG-3′ AS: 5′-GGTCTGAAAGCTTGGACTGT-3′
Snail	S: 5′-CCAATCGGAAGCCTAACTAC-3′ AS: 5′-CTCCAAGGAAGAGACTGAAG-3′
TGF-β	S: 5′-ATCGACATGGAGCTGGTGAA-3′ AS: 5′-GTTCAGGTACCGCTTCTCGG-3′
E-cadherin	S: 5′-AGAATGACAACAAGCCCGAAT-3′ AS: 5′-CGGCATTGTAGGTGTTCACA-3′
Fibronectin-1	S: 5′-CCAACCTACGGATGACTCGT-3′ AS: 5′-GCTCATCATCTGGCCATTTT-3′
β-actin	S: 5′-CAAGAGATGGCCACGGCTGCT-3′ AS: 5′-TCCTTCTGCATCCTGTCGGCA-3′

2.5. PCR Analysis. After MCF-7 cells had been treated, they were lysed in 1 mL TRIzol® reagent (Invitrogen, Carlsbad, CA). The cDNA preparation and RT-PCR were performed using the methods described in our previous study [29]. The primer sequences used in the present study are shown in Table 1. These PCR products were loaded onto a 2% (w/v) agarose gel for electrophoresis, and a densitometric analysis of PCR products was performed with Multi Gauge version 3.0 (Fuji Film, Tokyo, Japan).

2.6. Western Blotting. Whole cell protein from MCF-7 cells was prepared as described in our previous study with minor modifications. Briefly, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NAF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, and 1 mM PMSF), followed by sonication using the ultrasonic homogenizer Vivracell VC100 (Sonic & Materials, Danbury, CT). Cytosolic and membrane fractions were isolated as described previously [30]. Whole cell, cytosolic, or membrane protein concentrations were measured by a BCA protein assay. Twenty micrograms of protein was boiled with SDS buffer (62.5 mM Tris-HCl, pH 6.8, containing 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue (BPB)) for 5 min. Core histones were isolated as described in our previous report [29] and boiled with SDS buffer for 5 min. Whole cell, cytosolic, or membrane protein (20 μg) or isolated histones from approximately 5×10^4 cells were separated by SDS-PAGE on a 12 or 15% (w/v) polyacrylamide gel, followed by their transferal electrophoretically onto PVDF membranes. The membranes were then incubated with anti-phospho-PKC (pan) (βII Ser660) rabbit polyclonal antibody (#9371, 1 : 1,000), anti-acetyl-histone H3 rabbit polyclonal antibody (#06-599, 1 : 1,000), anti-acetyl-histone H4 rabbit polyclonal antibody (#06-598, 1 : 1,000), or anti-actin mouse monoclonal antibody (MAB1501, 1 : 3,000). The blots were incubated with HRP-conjugated goat anti-rabbit (A6154) or anti-mouse

(A4416) IgG (1 : 5,000). Bands were detected using ImmunoStar®LD and imaged using LAS-3000 UV mini (Fuji Film).

2.7. ChIP Analysis. ChIP assays were performed as described in our previous report with minor modifications [29]. The sheared genomic DNA was immunoprecipitated with normal rabbit IgG (#2729), anti-acetyl-histone-H3 (#06-599) or anti-acetyl-histone-H4 rabbit polyclonal antibody (#06-598) for overnight followed by incubation with Dynabeads protein G (Invitrogen) for 2 h. The abundance of Slug or Snail promoter regions in ChIP precipitates was quantified using a PCR analysis. The primer sequences used in the ChIP assay were as follows: Slug: sense 5′-GAG GTT CCT CTC TTG AAA ATA CT-3′, antisense 5′-GCA AGA AAG ATC CAA TCA CA-3′; Snail: sense 5′-CGC TCC GTA AAC ACT GGA TAA-3′, antisense 5′-GAA GCG AGG AAA GGG ACA C-3′. After amplification, these PCR products were loaded onto a 1.2% (w/v) agarose gel for electrophoresis and visualized using FLA5100, and a densitometric analysis of PCR products was performed with Multi Gauge version 3.0.

2.8. Measurement of HDAC Activities. HDAC activities were measured in TPA-treated MCF-7 cells using the HDAC Cell-Based Activity Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. Briefly, after MCF-7 cells had been treated with 1 nM TPA for 1 h, they were incubated at 37°C for 2 h in the presence of the HDAC substrate. Lysis/developer solution was added to the cells, followed by incubation at 37°C for 15 min. The fluorescent intensity (excitation 365 nm, emission 410–460 nm) of each well was measured using the GloMax®-Multi Detection System (Promega, Madison, WI).

2.9. Determination of Intracellular ROS Accumulation. After MCF-7 cells had been treated, the cells were incubated with PBS containing 5% paraformaldehyde and 10 μM carboxy-H_2DCFHDA or 10 μM DHE for 20 min at 37°C in a humidified 5% CO_2 incubator. The cells were visualized under an HS All in One fluorescence microscope BZ-9000 (Keyence, Osaka, Japan).

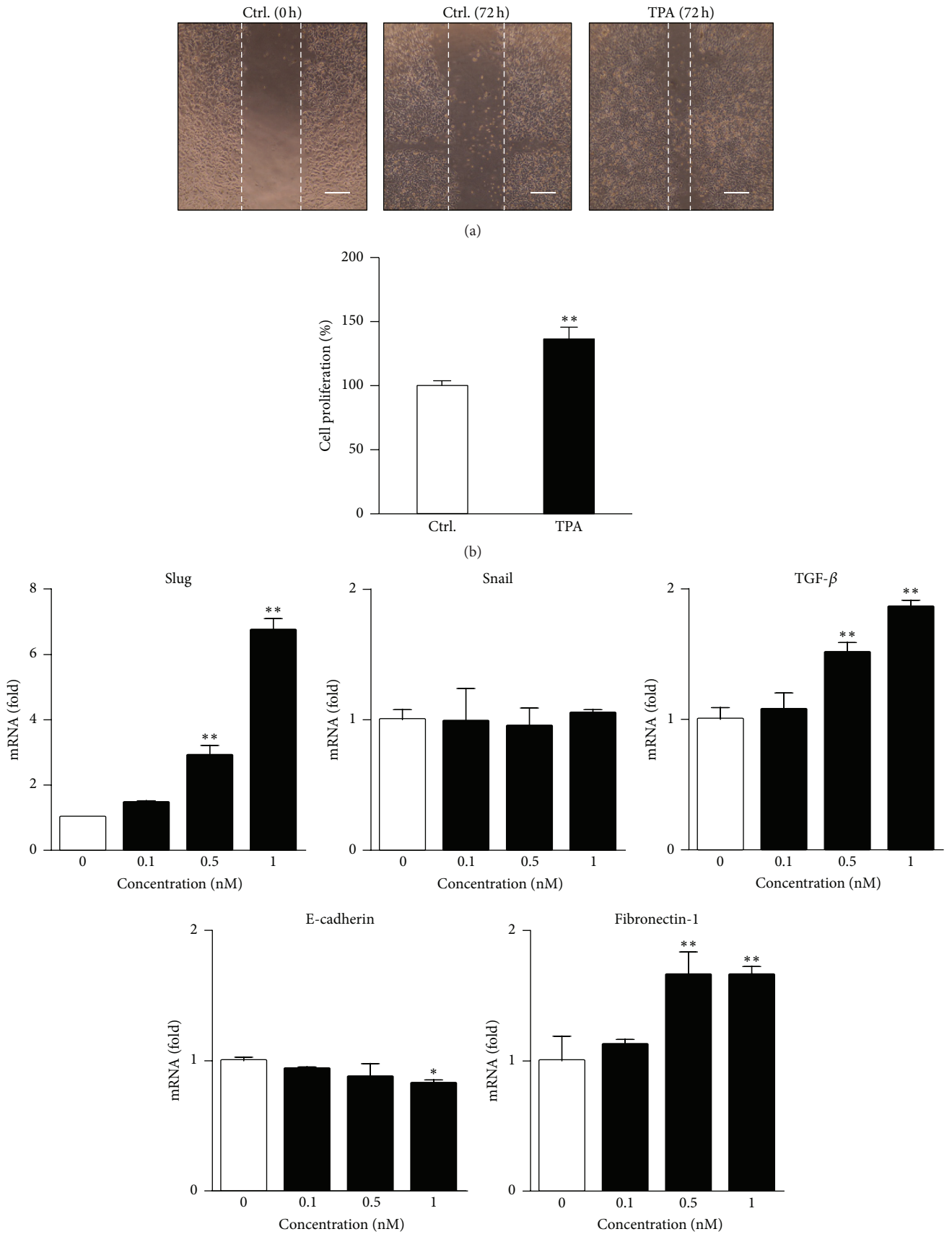
2.10. Statistical Analysis. Data are expressed as the means ± SD of three independent experiments. Statistical evaluations of the data obtained were performed using an ANOVA followed by Bonferroni *post hoc* tests or Student's t-test for Figures 3(b) and 3(d). A p value less than 0.05 was considered significant.

3. Results

3.1. Treatment with TPA Induces EMT Processes in Human Breast Cancer MCF-7 Cells. PKC plays a pivotal role in many physiological processes including cancer metastasis [31, 32], which is associated with cell proliferation and migration, the induction of EMT-related genes, and reductions in tight-junction molecules. Therefore, we investigated MCF-7 cell migration after the TPA treatment using the wound-healing assay. MCF-7 cell migration was not observed under serum-free conditions; however, the treatment with TPA significantly induced its migration (Figure 1(a)). Moreover, it was determined that treatment with TPA significantly induced MCF-7 cells proliferation (Figure 1(b)), suggesting that TPA functions as a potent cell migration and proliferation inducer in this model. Slug and Snail, major EMT-related transcription factors, are known to induce fibronectin-1 and decrease E-cadherin expression during the progression of tumor metastasis. As shown in Figures 1(c) and 1(d), the treatment with TPA significantly induced the expression of Slug, whereas it did not induce that of Snail in MCF-7 cells. Moreover, we demonstrated the induction of transforming growth factor-β (TGF-β) and fibronectin-1 and reductions in E-cadherin in TPA-treated MCF-7 cells, suggesting that TPA induces EMT processes through Slug-related signaling in MCF-7 cells.

3.2. Involvement of PKC in TPA-Induced EMT Processes in MCF-7 Cells. In order to determine the involvement of PKC in TPA-induced EMT processes, we demonstrated the membrane translocation of phospho-PKC in TPA-treated MCF-7 cells. As shown in Figure 2(a), the treatment with TPA induced the membrane translocation of phospho-PKC more rapidly than in control cells, indicating that TPA activated PKC signaling, which is consistent with previous findings. We then investigated the inhibitory effects of GF109203X, an inhibitor of PKC, on TPA-elicited gene alterations. The pretreatment with GF109203X suppressed the induction of TPA-elicited Slug and fibronectin-1 and reductions in E-cadherin (Figure 2(b)), suggesting that PKC signaling is involved in TPA-elicited EMT processes. Moreover, the TPA-elicited induction of Slug expression was completely suppressed by the pretreatment with actinomycin D (ActD), an inhibitor of transcription, indicating that its induction was regulated at the transcription level.

3.3. TPA-Elicited Induction of Slug Expression Was Regulated by Histone H3 Acetylation within Its Promoter Region. We previously reported that TPA induced superoxide dismutase 3 (SOD3) expression through epigenetics such as histone acetylation in human leukemic THP-1 cells [29]; therefore, TPA-elicited Slug induction may also be regulated by epigenetics. As expected, the TPA treatment induced histones H3 and H4 acetylation in a time-dependent manner (Figure 3(a)), and this induction was stronger than that elicited by TSA. Moreover, the treatment with TSA significantly induced the expression of Slug (Figure 3(b)), and our ChIP assay determined the significant enrichment of acetylated histone H3 within the proximal promoter region of *Slug* (Figure 3(c)). However, the treatment with TSA, but not TPA, significantly induced Snail expression. These results suggest that the treatment with TPA selectively induced histone acetylation within the *Slug* promoter region in MCF-7 cells. It has been well established that HDAC and HAT play critical roles in histone acetylation [25–27]; however, the treatment with TPA did not decrease HDAC activities in our model (Figure 3(d)). Furthermore, HAT inhibitors such as Gar and CPTH2 did not suppress the TPA-elicited induction of Slug expression



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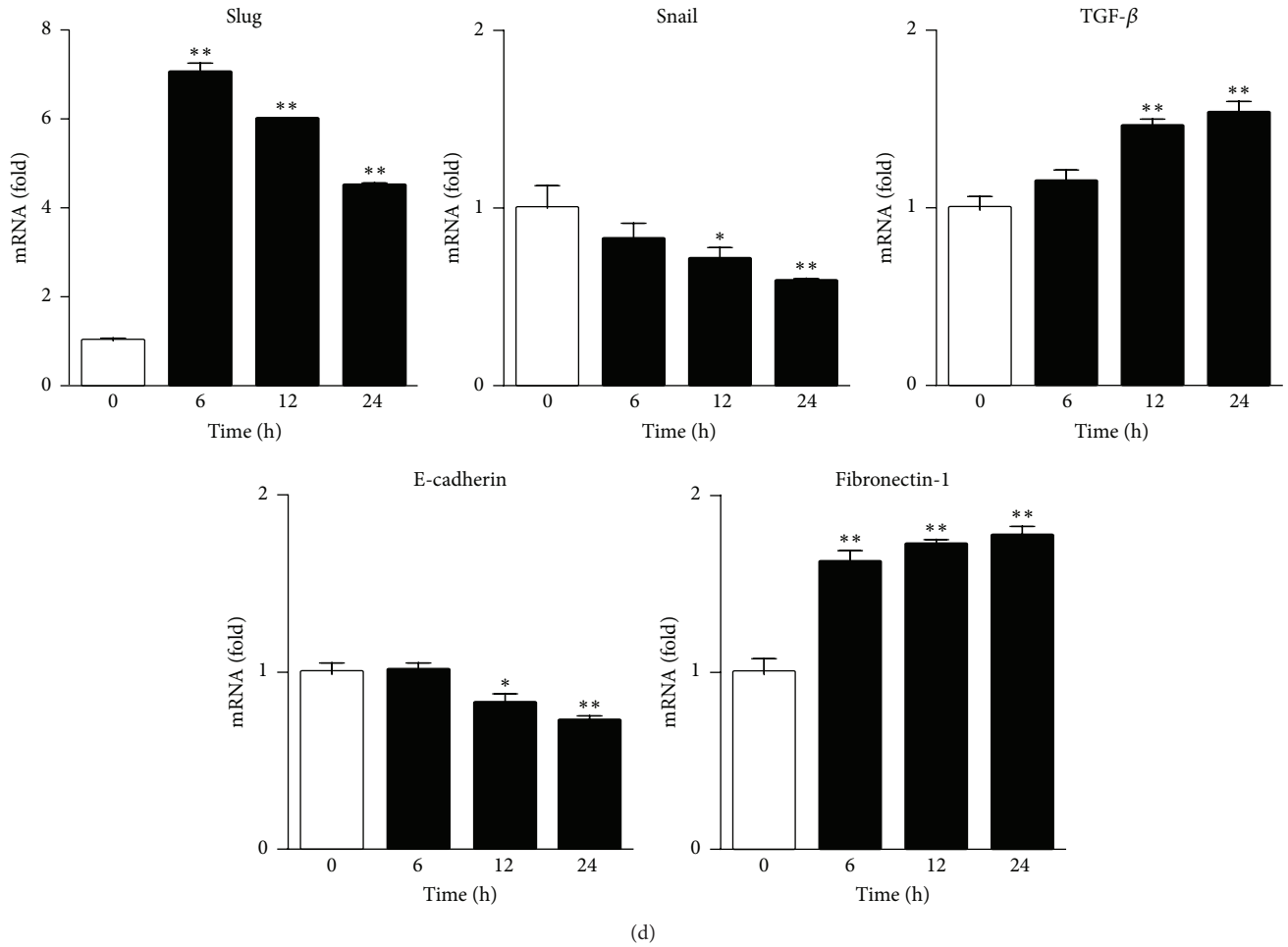


Figure 1: TPA treatment induces EMT processes in human breast cancer MCF-7 cells. (a) MCF-7 cell migration into the wound field was monitored by the method described in Section 2. The scale bars show 200 μm. (b) MCF-7 cells were treated with 1 nM TPA for 12 h. After that, the cell proliferation was evaluated by CCK-8 assay ($^{**}p < 0.01$ versus vehicle). MCF-7 cells were treated with the indicated concentrations of TPA for 24 h (c) or 1 nM TPA for the indicated times (d). RT-PCR was then carried out. RT-PCR data were normalized using β-actin levels ($^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle (c) or 0 h (d)).

(Figure 3(e)), indicating that TPA induced its expression in a HDAC- and HAT-independent manner.

3.4. Involvement of Intracellular ROS in TPA-Elicited EMT Processes in MCF-7 Cells. NOX2, the expression of which is the strongest in monocytes/macrophages, plays an essential role in innate host defenses and is now known as a signaling molecule [33, 34]. We previously reported that NOX2-derived ROS after a TPA treatment functioned as key signal molecules in human leukemic U937 cells [30] and THP-1 cells [35]. In the present study, intracellular ROS generation was enhanced by the treatment with TPA but was suppressed by the pretreatment with DPI, an inhibitor of NOX2 (Figure 4(a)). Moreover, in the presence of DPI, the TPA-elicited induction of Slug was significantly blocked (Figure 4(b)), and this was accompanied by the inhibition of histone H3 acetylation (Figure 4(c)), indicating that NOX2-derived ROS participate in the TPA-elicited induction of Slug in MCF-7 cells. We then investigated the inhibitory effects of DPI on TPA-elicited MCF-7 cell migration. As shown in Figure 4(d), the pretreatment with DPI markedly suppressed TPA-elicited MCF-7 cell migration, suggesting that NOX2-derived ROS are involved in cell migration as well as the induction of Slug expression.

4. Discussion

EMT, which is associated with the loss of epithelial-like properties and the acquisition of mesenchymal properties, is considered to be involved in tumor metastasis and tissue fibrosis [36, 37]. We herein demonstrated that a treatment with TPA, an activator of PKC, induced breast cancer EMT processes through the significant induction of Slug, but not Snail expression. PKCs α, ε, η, ζ, and δ are known to be significantly involved in cell proliferation, migration, and invasion, particularly in breast cancer [31, 32, 38–42]. Our results showed that PKC signaling plays an important role in the TPA-elicited induction of Slug expression (Figure 2) and

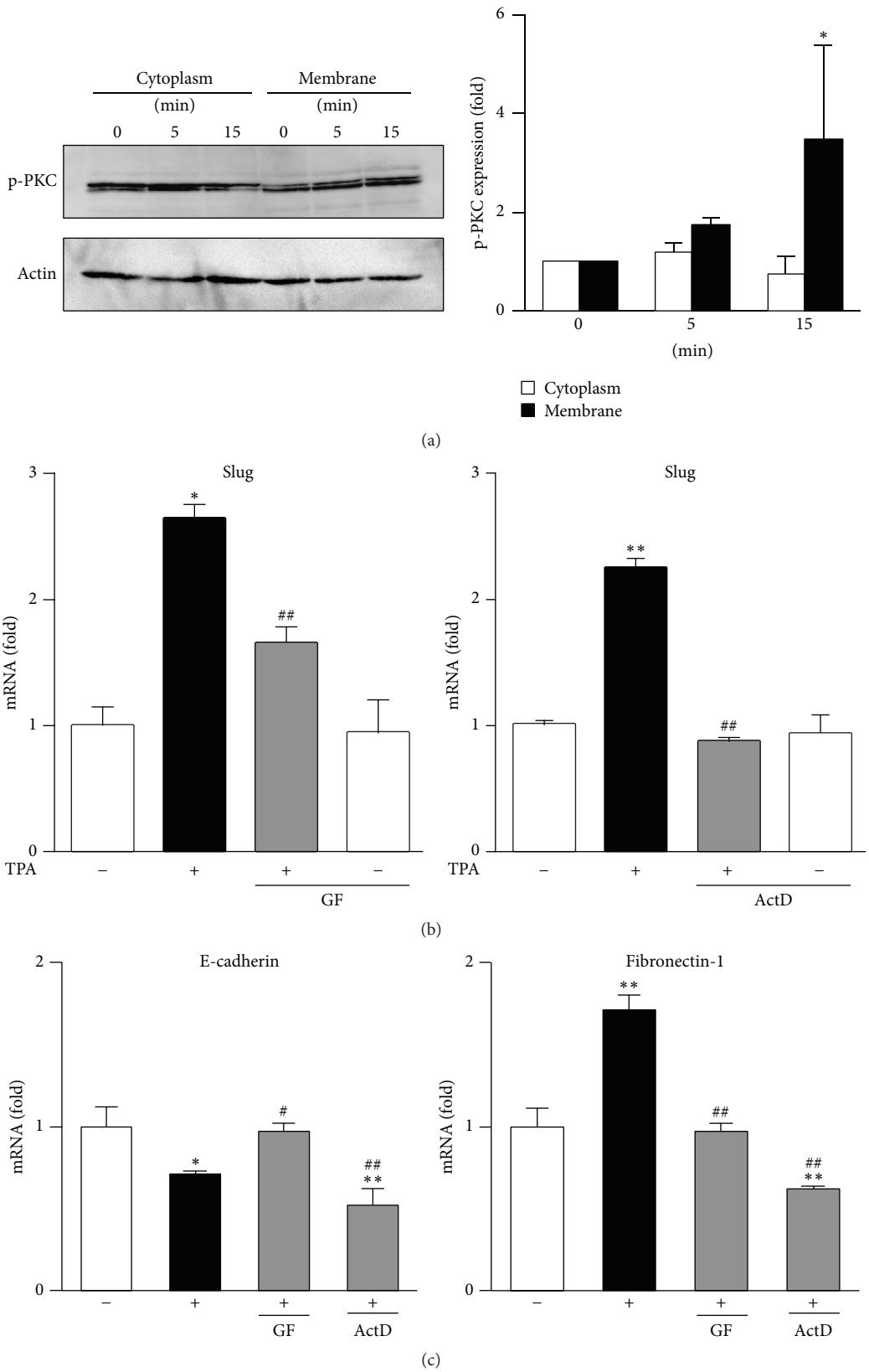
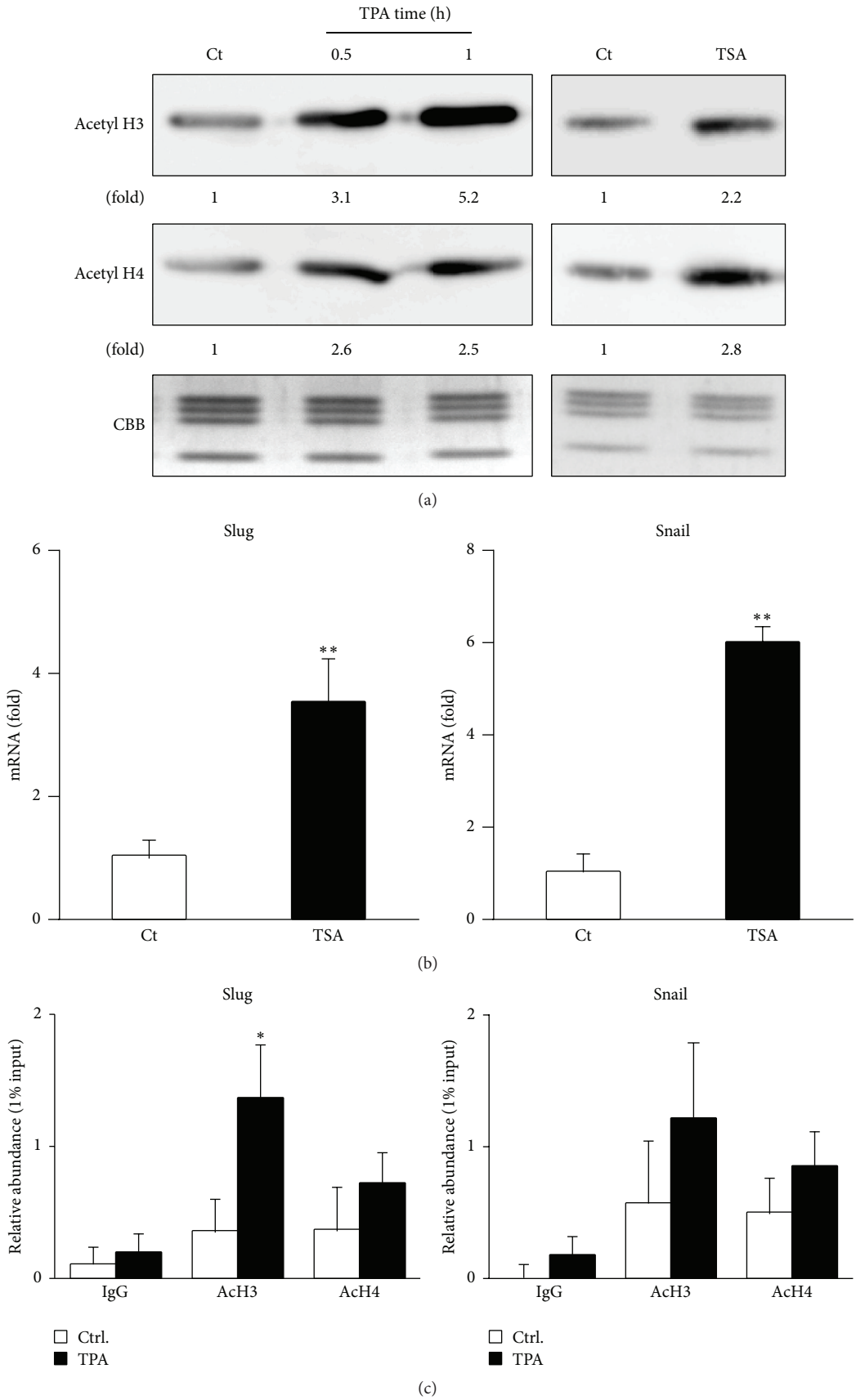


Figure 2: Involvement of PKC in TPA-induced EMT processes in MCF-7 cells. (a) MCF-7 cells were treated with 1 nM TPA for the indicated times. The membrane translocation of phosphorylated PKC was then determined by Western blotting. Values are the mean of fold changes from vehicle-treated cells ($n = 3$, $^{*}p < 0.05$). The cells were pretreated with 5 μM GF109203X (GF) or 10 μg/mL actinomycin D (ActD) for 1 h and were then treated with 1 nM TPA for 6 h (b) or 24 h (c). RT-PCR was carried out. RT-PCR data were normalized using β-actin levels ($^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus TPA-treated cells).



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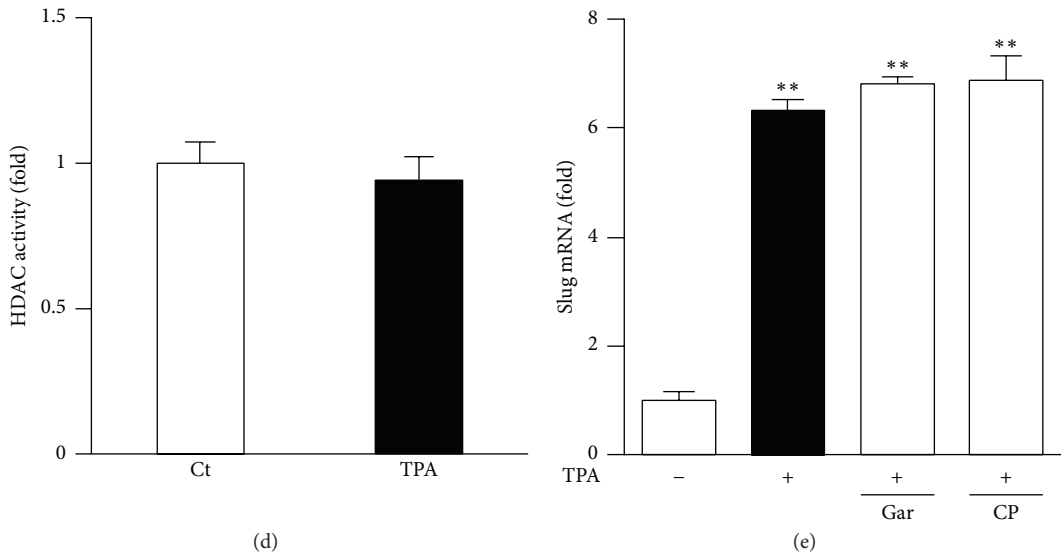

Figure 3: TPA-elicited Slug induction is regulated by histone H3 acetylation within its promoter region. (a) MCF-7 cells were treated with 1 nM TPA or 1 μM of TSA for the indicated times (for TPA) or 1 h (for TSA). Acetylated histones H3 and H4 were then determined by Western blotting. Values are the mean of fold changes from vehicle-treated cells ($n = 3$). (b) Cells were treated with 1 μM TSA for 6 h, followed by RT-PCR. RT-PCR data were normalized using β-actin levels ($^{**}p < 0.01$ versus vehicle). (c) Cells were treated with 1 nM TPA for 1 h. A ChIP assay was then performed. Relative binding to the promoter region is expressed as the percentage amount over input (%) ($^{*}p < 0.05$ versus vehicle). (d) After cells had been treated with 1 nM TPA for 1 h, HDAC activities were measured. (e) Cells were pretreated with 30 μM garcinol (Gar) or 50 μM CPTH2 (CP) for 30 min and then treated with 1 nM TPA for 6 h, followed by RT-PCR. RT-PCR data were normalized using β-actin levels ($^{**}p < 0.01$ versus vehicle).

is consistent with previous findings suggesting the critical role of PKC signaling in tumor initiation and progression. On the other hand, we also found the significant induction of TGF-β (Figure 1(c)). Elevated plasma TGF-β1 levels in breast and prostate cancer patients are considered to correlate with poor outcomes [43–45]. TGF-β-elicited EMT processes are essential for normal embryonic development but are considered to contribute to tumor cell invasion and metastasis. Therefore, we speculated that elevated TGF-β levels might be involved in TPA-elicited EMT processes in MCF-7 cells.

The excess production of ROS is known to contribute to the progression of atherosclerosis, asthma, and cancer [1–3]. Moreover, ROS play a critical role in the induction of Snail expression in HCC tissues and have been closely associated with reductions in E-cadherin [28]. Therefore, a clearer understanding of the role of ROS and regulation of redox homeostasis may lead to the development of novel cancer therapies. In the present study, we determined the involvement of NOX-derived ROS in the TPA-elicited induction of Slug expression, which was closely associated with histone H3 acetylation within its promoter region (Figure 3). These results provide direct evidence for excessively produced ROS regulating the expression of various genes through chromatin remodeling. On the other hand, our results showed that TPA did not induce Snail expression (Figure 1). Nevertheless, the treatment with TSA, an inhibitor of HDAC, significantly induced Slug and Snail expression (Figure 3). These results suggest that TPA selectively induces the expression of Slug through histone H3 acetylation. We previously reported that a treatment with TPA activated HAT including p300 and GCN5, which contribute to the TPA-elicited expression of SOD3 in human leukemic THP-1 cells [29], suggesting that TPA-elicited Slug expression might be associated with the activation of HAT. However, we were unable to determine the involvement of HAT in its induction (Figure 3(e)). Taken together, these results showed that the treatment with TPA did not decrease HDAC activities (Figure 3(d)). A recent study reported that reductions in the levels of Sirtuin 1 (SIRT1), a highly conserved NAD-dependent deacetylase, in breast cancer and kidney tubular epithelial cells promoted tumor metastasis and kidney fibrosis, respectively [46, 47]. Furthermore, SIRT1 deacetylates and suppresses Smad4, a key molecule in TGF-β signaling, which lowers the expression of target genes [47]. Therefore, it raises the possibility that inhibition of SIRT families might regulate TPA-elicited histone H3 acetylation and Slug induction in MCF-7 cells; however, some additional experiments are needed in order to elucidate the exact mechanisms governing TPA-elicited EMT processes.

In the present study, we identified a critical role for ROS in the histone H3 acetylation within the Slug promoter in MCF-7 cells. Taken together with previous findings, our results provide the informative evidence for NOX-derived ROS inducing epigenetic modifications. It remains unknown how ROS selectively regulate histone H3 acetylation within the Slug promoter region; however, a clearer understanding of the role of ROS may lead to the development of novel epigenetic therapies for breast cancer.

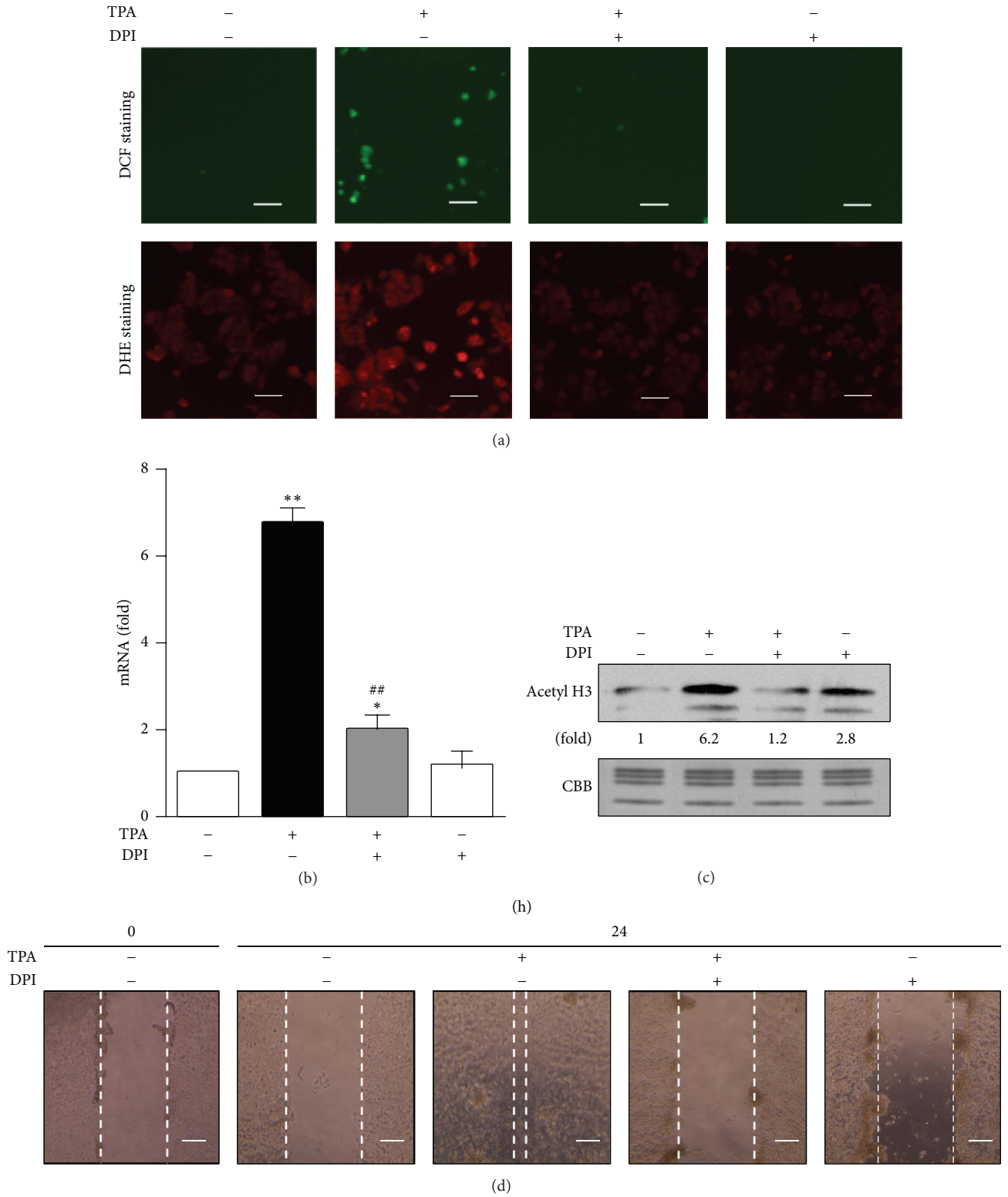

Figure 4: Involvement of intracellular ROS in TPA-elicited EMT processes in MCF-7 cells. MCF-7 cells were pretreated with 20 μM DPI for 1 h and were then treated with 1 nM TPA for 30 min (a), 1 h (b), 6 h (c), or 24 h (d). Intracellular ROS accumulation (a), Slug mRNA expression (b), acetylated histone H3 levels (c), and cell migration (d) were determined. Scale bars show 200 μm. Values (b) are the means of fold changes from vehicle-treated cells ($n = 3$). RT-PCR data were normalized using β-actin levels ($^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle, $^{\#\#}p < 0.01$ versus TPA-treated cells).

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Zinc Chelation Mediates the Lysosomal Disruption without Intracellular ROS Generation

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We report the molecular mechanism for zinc depletion caused by TPEN (N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine) in neuroblastoma cells. The activation of p38 MAP kinase and subsequently caspase 3 is not due to or followed by redox imbalance or ROS generation, though these are commonly observed in literature. We found that TPEN is not responsible for ROS generation and the mechanism involves essentially lysosomal disruption caused by intracellular zinc depletion. We also observed a modest activation of Bax and no changes in the Bcl-2 proteins. As a result, we suggest that TPEN causes intracellular zinc depletion which can influence the breakdown of lysosomes and cell death without ROS generation.

1. Introduction

N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is a membrane-permeable hexadentate compound which chelates metal ions such as Cu^{2+}, Zn^{2+}, and Fe^{2+} [1, 2], but this intracellular chelator has shown a high affinity for zinc [3–5]. This special property permits the use of TPEN in a variety of settings, often as a tool to probe the functions of zinc in cell cultures.

Studies using TPEN have demonstrated that intracellular zinc depletion causes oxidative stress and DNA damage [6], as well as apoptosis in some cells in culture [5, 7, 8]. TPEN could also inhibit the neurotoxic effects of zinc *in vivo* [9, 10]. Previous studies have shown that TPEN induced ROS formation by intracellular zinc depletion and, consequently, DNA damage [4, 6] and apoptosis [7–11]. The most known mechanism involves the activation of caspase 11 [12], caspase 3/7 [4], p53 [13], cleaved PARP, and apoptotic bodies [4]. It has been proposed that zinc deficiency can cause increased oxidative stress and, consequently, cell damage or death [14].

The effects of zinc depletion in cells have been widely discussed in literature, and TPEN is one of the most functional chelators used in those studies. The activation of p38 by an excess of zinc has demonstrated that this mitogen-activated protein kinase (MAP kinase) is responsible for the zinc-mediated activation of the mRNA expression of the Th1 cytokines interferon-gamma and interleukin-2 in human T-cells [15, 16]. Zinc was proven to be involved in apoptosis via the activation of a p38 MAP kinase pathway triggered by reactive oxygen species (ROS) and redox regulation [17–19]. The ubiquitination induced by excess zinc also required p38 activation in neuronal cells [20], and this kinase is activated by treatment with a Zn ionophore complex in HL-60 cells [19]. Conversely, the commitment of intracellular zinc deficiency to the regulation of cell death is an intriguing matter to study. When chelation therapy limits zinc access in cultured cells, DNA synthesis ceases, the cell cycle is arrested [21], a redox imbalance can be established [22, 23], and the involvement of both p53 and caspase 11 has been proposed [13, 24]. However, the molecular mechanism of cell death caused by zinc deficiency is not fully understood. Therefore, the use of intracellular specific chelators, such as TPEN, is also very helpful to understand zinc biology.

For that reason, in the present study, we have investigated the detailed mechanisms of the cell death pathway caused by the specific zinc chelator TPEN.

2. Materials and Methods

2.1. Cell Culture. Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection

(ATCC) and grown in Dulbecco's Modified Eagle F12 Medium (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and antibiotics as described [25]. The cells were routinely trypsinized and seeded at a density of 4×10^4 cells/cm^2. Every month, the cells were cultivated in the absence of antibiotics for control purposes and subjected to a routine assay using a MycoAlert Mycoplasma Detection Kit (Lonza Rockland) to ensure that they had not become contaminated with mycoplasma. All SH-SY5Y cells in this study were used at a low passage number (<20).

2.2. Cell Viability Assessment. To determine the levels of TPEN that would promote cell death, concentration-dependent cytotoxicity studies were performed. Typically, the viability of neuroblastoma cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays, as previously reported [26]. SH-SY5Y cells were inoculated in 96-well plates at a density of 8×10^4 cell/cm^2 and incubated for 24 hours under the conditions described above. Aliquots of freshly prepared solutions of TPEN (2.5 mM) were added to the culture medium to attain final concentrations in the 5.0–100.0 μM range, and the plates were then incubated for an additional 12, 24, and 48 hours. The plates were also preincubated with 10 μM SB202190 (p38 MAPK inhibitor) and/or 300 μM antipain dihydrochloride (A.D.: cathepsin inhibitor) for 1 hour and subsequently treated with 25 μM TPEN for 12, 24, and 48 hours. Trypan Blue dye exclusion test was performed to confirm the MTT assay results. SH-SY5Y cells were inoculated in 24-well plate at a density of 4×10^4 cell/cm^2 and incubated for 24 hours under the conditions described above. Aliquots of freshly prepared solutions of TPEN (2.5 mM) were added to the culture medium to attain final concentrations in the 5.0–100.0 μM range, and the plates were then incubated for an additional 48 h. Following incubation, the cells were trypsinized and combined, washed with phosphate buffered saline (PBS; 137.0 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer at pH 7.4), stained with Trypan Blue, and counted under an optical microscope using a Neubauer chamber.

2.3. Determination of Cellular Zinc by Flame Atomic Absorption Spectroscopy (FAAS). The determination of intracellular zinc concentrations was made as previously reported [25]. We employed a flame atomic absorption spectrometer, Model AAS Vario 6 (Analytik Jena AG, Jena, Germany), equipped with a hollow zinc cathode lamp and a deuterium lamp for background correction. SH-SY5Y cells were plated in a 25 cm^2 culture flask at a density of 8×10^4 cells/cm^2 and incubated in the presence or absence of TPEN (5 or 25 μM) for 6, 24, and 48 hours. After incubation, the cells were trypsinized and combined, washed twice with PBS containing 1.0 mM EDTA to remove residual Zn(II), washed three additional times with PBS, dried for 1 week in a desiccator, and then analyzed by FAAS.

2.4. Cell Death Assay. The percentage of cells undergoing apoptosis and necrosis was determined by Annexin V staining using the ApopNexin™ FITC Apoptosis Detection Kit (Millipore) in a flow cytometer (Cytometer FC 500 MPL, Beckman Coulter). SH-SY5Y cells were seeded in 6-well plates and treated for 12, 24, and 48 hours with 5 or 25 μM TPEN and 100 nM staurosporine (positive control). The apoptosis assay was performed according to what is described in Matias et al. [25]. Apoptosis assays were performed at least 7 times in independent replicate experiments.

2.5. Measurement of Intracellular Reactive Oxygen Species. SH-SY5Y cells that had been plated and incubated in the presence or absence of TPEN (5 or 25 μM) as described above were treated with a trypsin/EDTA (1 mM) solution, washed three times with PBS, and resuspended in a 50.0 μM solution of the oxidation-sensitive, nonfluorescent probe 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) [27] with PI [27, 28] or 5 μM dihydroethidium (DHE). 300 μM H_2O_2 and 50 μM DMNQ were used for positive control to DCFH-DA and DHE, respectively. The cells were washed three times with PBS after incubation at 37°C for 45 min [29] to probe DCFH-DA and at 37°C for 30 min to probe DHE, and the levels of intracellular fluorescence were determined immediately by flow cytometry at 530 nm using a Cytometer FC 500 MPL (Beckman Coulter) [30, 31]. Assays were conducted at least in quintuplicate, and >20,000 viable cells from each sample were analyzed per assay.

2.6. Western Blot Analyses. SH-SY5Y cells were plated and incubated in the presence or absence of TPEN (5 or 25 μM) as described above. Then, at 6, 12, and 24 hours after treatment, the cells were harvested, resuspended and lysed in 150 μL of RIPA buffer (150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in 50 mM Tris at pH 7.5) containing a protease inhibitor cocktail for mammalian cells (Sigma-Aldrich), and centrifuged (14000 g, 20 min, 4°C). The supernatants and pellets were transferred to new microcentrifuge tubes and stored at 80°C until required for analysis. The protein concentrations were determined following Lowry's method using bovine serum albumin (BSA) as the standard [32]. Then, 100 μg of extracts was subjected to SDS-PAGE and blotted onto nitrocellulose membranes (GE Healthcare Life Sciences) with the equal loading of the proteins confirmed by the internal mass control blotting of β-actin or α-tubulin. The membranes were blocked for 1 hour in a blocking solution containing 5% nonfat dried milk (Sigma-Aldrich) and 0.0025% sodium azide solubilized in TBS-T (150 mM NaCl, 50 mM Tris at pH 7.5 and 0.05% Tween-20) and then washed twice with TBS-T. The primary antibodies employed were the rabbit anti-caspase 3 (Sigma-Aldrich), mouse anti-β-actin (clone 279 AC-74; Sigma-Aldrich), mouse anti-p38 (A-12: sc-7972; Santa Cruz Biotechnology), rabbit anti-JNK/SAPK1 (Millipore), rabbit anti-Bax (NT, Millipore), mouse anti-Bcl-2 (clone 100, Millipore), mouse anti p-p38 (D-8: sc-7973, Santa Cruz), mouse anti-JNK/SAPK1 (pT183/pY185; clone 41/JNK/SAPK 14 pT183/pY185; BD Biosciences), and mouse anti-α-tubulin (DM1A: sc-32293). The protein complexes that were formed following treatment with the specific secondary antibodies (anti-mouse or anti-rabbit IgG-peroxidase conjugate) were detected using the chemiluminescent substrate (Thermo

Fisher Scientific). Western blottings were conducted at least in triplicate and represent independent replicate experiments.

2.7. Acridine Orange Assay. SH-SY5Y cells were plated and incubated in the presence or absence of TPEN (5 or 25 μM) as described above. At 4, 12, and 24 hours after treatment, the cells were incubated with Acridine Orange (2.5 μg/mL) in DMEN/F12 medium for 15 min at 37°C. After incubation, the cells were trypsinized and washed three times with PBS, and the red (FL3, 670 nm) fluorescence was recorded on a logarithmic scale by flow cytofluorometry using a Beckman Coulter Quanta SC MPL instrument excited at 488 nm. Untreated but AO-stained cells, which were used as control, had a fluorescence intensity set between 10^2 and 10^3 a.u. on a logarithmic scale. As lysosomes are being degraded there is a decrease in the red fluorescence peak and a simultaneous increase of weaker red fluorescence (subpopulation) under 10^2 a.u. Subpopulations of red fluorescence were evaluated to observe the lysosomal integrity/rupture.

2.8. Statistical Analysis. All experiments were repeated at least three times in independent replicates (except where stated otherwise), and the results are expressed as the mean values ± standard deviations. The analysis of variance (ANOVA) with Bonferroni's correction was used to evaluate the differences between the means, with the level of significance set at $p < 0.05$.

3. Results and Discussion

3.1. TPEN Causes a Decrease in Cell Viability and Intracellular Zinc Levels in Neuroblastoma Cells SH-SY5Y. Several studies have indicated that the metal chelator TPEN induces intracellular zinc depletion in several cell types [5, 33, 34] and here the effects of chelator TPEN on the SH-SY5Y neuroblastoma cell viability were assessed using the MTT assay. Raw data from Viability by MTT method are provided in the Supplementary Material available online at http://dx.doi.org/10.1155/2016/6724585. We selected concentrations of 5 and 25 μM TPEN based on the opposite dose-dependent profiles that they can cause in cells (Figure 1(a)).

It was observed that cells treated with 5 μM TPEN showed an increase in cell viability after 48 hours. On the contrary, cells treated with 25 μM TPEN showed a decrease in cell viability during the 48 hours of treatment. The results of the Trypan Blue assay showed the same profile for cells treated with 5 μM TPEN and 25 μM TPEN (Figure 1(b)). The zinc concentration was precisely quantified by flame atomic absorption spectrometry (FAAS) [1]. The decreasing cell viability observed in cells treated with TPEN is related to the changes in the intracellular zinc levels. Our previous studies showed that TPEN can disrupt the intracellular zinc levels in mammalian cells but it cannot disrupt either copper or iron levels [3]. The results point that intracellular zinc levels in cells treated with 25 μM TPEN decreased compared with the control cells (cells with no chelator, Figure 1(c)), whereas cells treated with 5 μM TPEN showed no significant changes in the zinc levels. This concentration showed no toxic effect in cells, as predicted by many previous studies that used even higher concentrations to chelate zinc [35–41]. Our results showed that neuroblastoma cells treated with the intracellular zinc chelator TPEN had a decrease in cell viability. However, after 48 hours of incubation, it was observed that cells treated at a concentration of 5 μM are able to "break" the barrier and return to viability, indicating that a reservoir of intracellular zinc may exist; conversely, cells treated with high TPEN concentrations (25, 50, and 100 μM) showed a lower cell viability. Therefore, we chose the lowest working concentration to relate changes in intracellular zinc deficiency and the molecular consequences in neuroblastoma cells.

3.2. Both Necrosis and Apoptosis Are Caused by TPEN Inducing Intracellular Zinc Depletion. Because cells treated with 25 μM TPEN showed both decreasing cell viability and intracellular zinc depletion, we correlated these effects with the mechanism of cell death. The results were obtained by flow cytometry analysis with Annexin V labeling FITC (axis x) and propidium iodide (PI) (axis y) and were interpreted as necrotic cells (Annexin V^-/PI^+, left upper quadrant) from early apoptotic cells (Annexin V^+/PI^-, right lower quadrant) and late apoptotic cells (Annexin V^+/PI^+, right upper quadrant). Positive control was done by 100 nM staurosporine for 4 hours (Figure 2(a)). Cells treated with 25 μM TPEN showed 13.5% of the cells in the early stages of apoptosis and 4.5% of the cells in necrosis after 12 hours of incubation (Figure 2(b)). However, after 48 hours of incubation, we observed that the percentage of necrotic cells increased to 43.2%, whereas the cells treated with 5 μM TPEN showed no significant results compared with the control cells (Figure 2(b)). The results of flow cytometry revealed that, depending on the time of exposure, zinc depletion caused cell death by either necrosis or apoptosis, with the most part occurring via necrosis. Necrosis was observed as an accidental and unregulated cellular event. However, evidence suggests that both necrosis and apoptosis may also occur by regulation mechanisms that are normally initiated by TNF-α, Fas, or TRAIL and mediated the formation of the two kinase complexes RIP3 and RIP1 [42–45]. It is also known that other signal-controlled mitochondrial dysfunctions such as generation of ROS, ATP depletion, proteolysis by calpains and cathepsins, and membrane rupture processes are also involved in necrosis [46]. Subsequently, we quantified the levels of ROS and lysosomal integrity to understand the mechanism involved in cell death by necrosis when zinc is depleted by TPEN.

To analyze the influence of reactive oxygen species (ROS) on zinc depletion, cells were incubated with the probe DCFH-DA and PI and analyzed by flow cytometry [47]. Cells were treated with PI to discriminate dead cells from the viable ones. Viable cells were gated and the results indicated that both untreated cells (control) and those treated with TPEN exhibited the same fluorescence profile that remained unchanged throughout the incubation period of 12, 24, or 48 hours, indicating that there is no ROS generation to cause probe oxidation (Figures 3(a) and 3(b)). The results of the indirect quantification of ROS suggest that the activation of the observed cell death related to TPEN is not

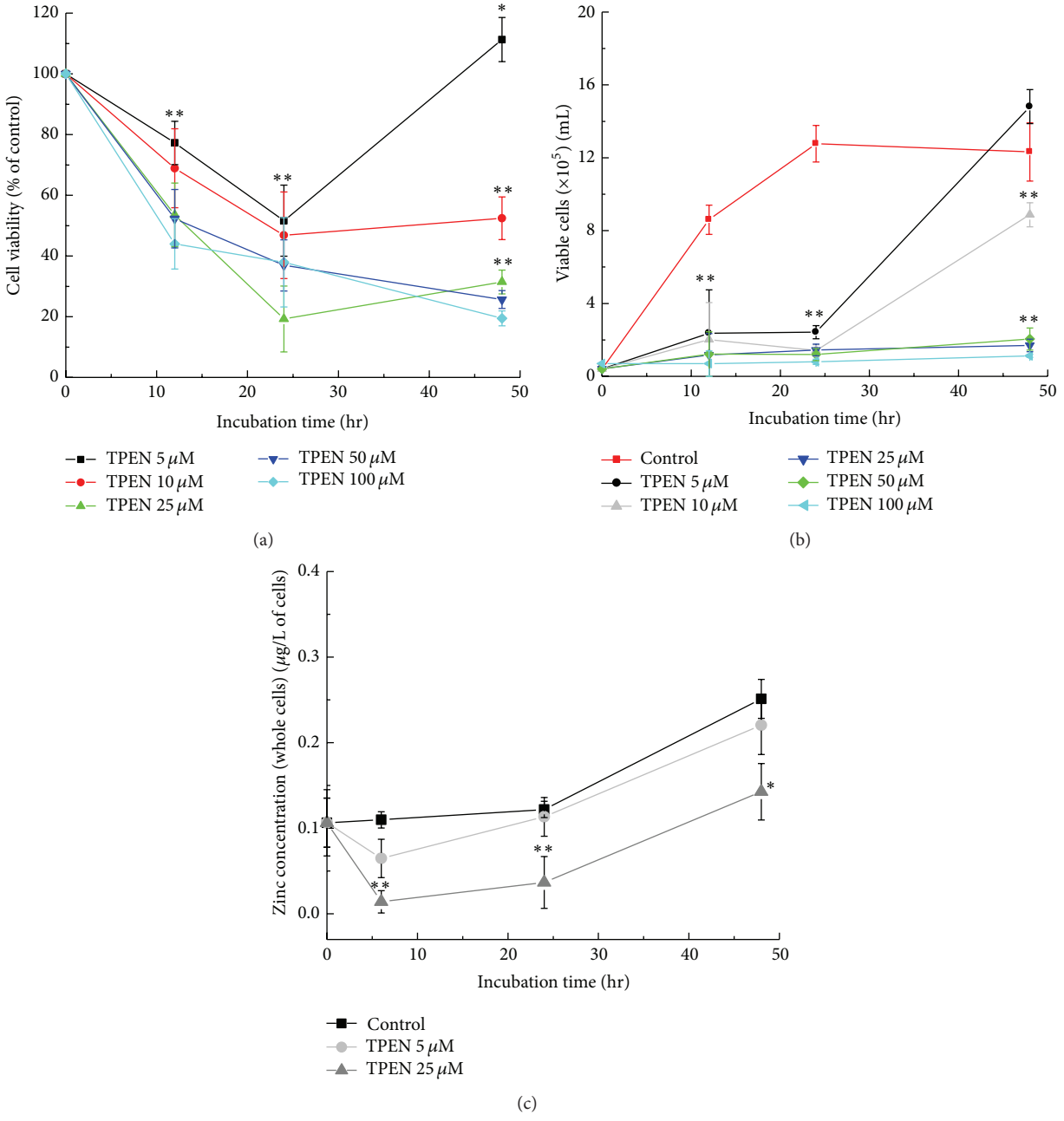


Figure 1: Influence of the TPEN chelator on cell viability and intracellular zinc alterations. (a) Concentration-dependent studies for MTT assay on neuroblastoma. SH-SY5Y cells were treated with varying concentrations of TPEN, while untreated cells were used as control. (b) Concentration-dependent studies for Trypan Blue assay on neuroblastoma. SH-SY5Y cells were treated with varying concentrations of TPEN, while untreated cells were used as control. (c) The concentration of zinc was determined by flame atomic absorption spectroscopy. Data represent the mean values ± standard deviation ($n = 3$), and significant differences between treated and untreated cells were $^{*}p < 0.05$ and $^{**}p < 0.001$, respectively.

involved in redox imbalance, in contrast to many cell death processes.

3.3. Lysosomal Disruption Is Involved in Intracellular Zinc Depletion. Since the decrease of intracellular zinc is not related to ROS but we do see cell death, one hypothesis is that cathepsins, proteins present in lysosome, can lead to cell death by necrosis once extravasated to the cytosol. We analyzed the lysosomal integrity by flow cytometry using the probe Acridine Orange (AO). AO is a metachromatic dye that shows different emission wavelengths when exposed to light at 488 nm. It interacts preferentially with acidic vesicles; for example, when the dye is found in high concentrations in the lysosome, these compartments are labeled dark red. When it occurs in a low concentration in the cytoplasm and nucleus, AO shows a green fluorescence. The lysosomal integrity is

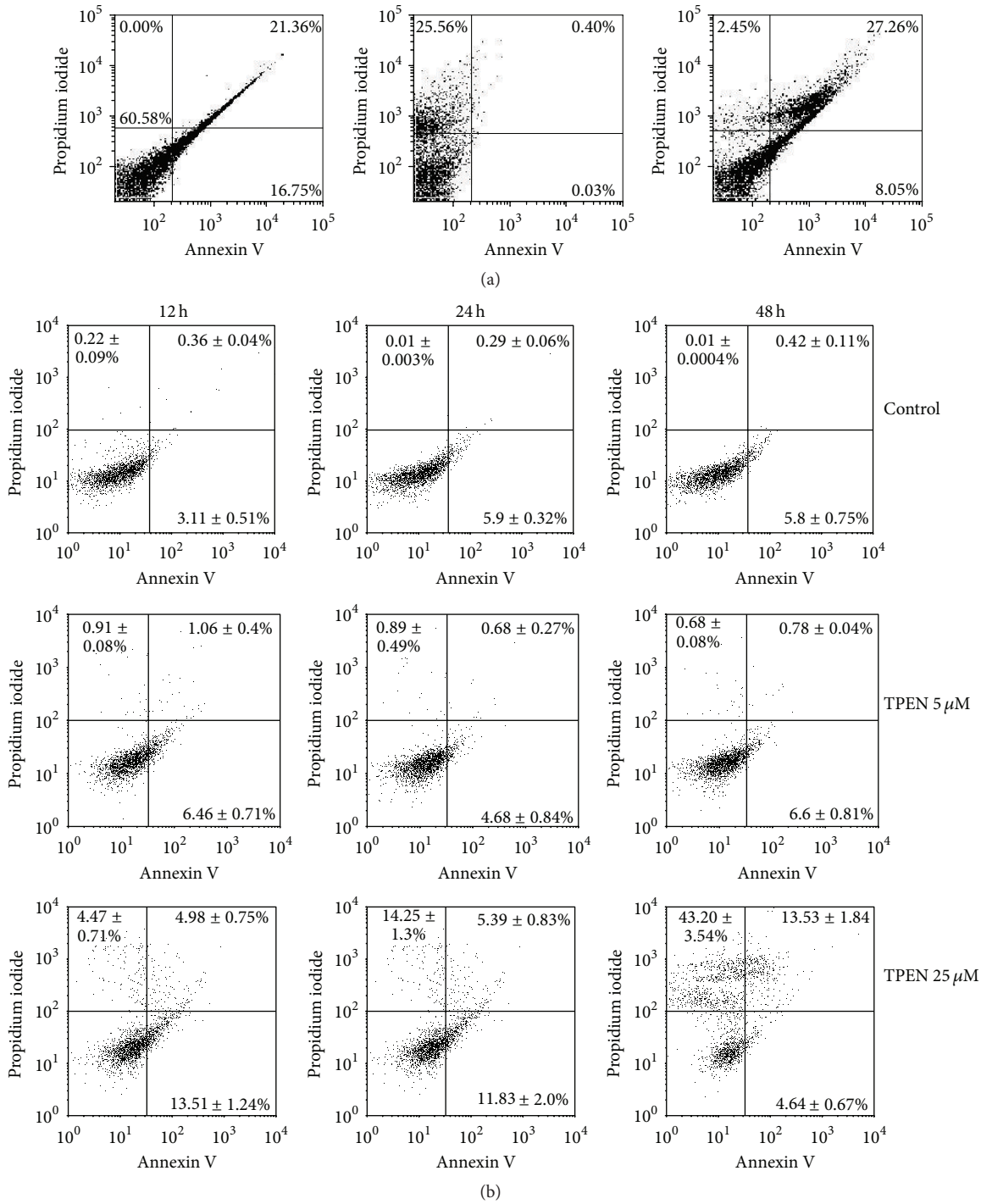


Figure 2: Apoptosis analysis. (a) Dot plot of SH-SY5Y after exposure to 100 nM staurosporine for 4 hours (positive control). Dot plot graphs from left to right show cells treated with (1) 100 nM staurosporine labeled with Annexin V-FITC, (2) 100 nM staurosporine labeled with PI, and (3) 100 nM staurosporine labeled with Annexin V-FITC and PI. (b) Dot plot of SH-SY5Y after exposure to 5 or 25 μM TPEN for 12, 24, and 48 hours and flow cytometry analysis with Annexin V-FITC versus PI. The divisions of the plots distinguish necrotic cells (Annexin V^-/PI^+, left upper quadrant) from early apoptotic cells (Annexin V^+/PI^-, right lower quadrant) and late apoptotic cells (Annexin V^+/PI^+, right upper quadrant). The plots in the figure are representative of five independent experiments. Data represent the mean values ± standard deviations ($n = 3$) and significant differences between untreated cells and cells treated with TPEN 25 μM.

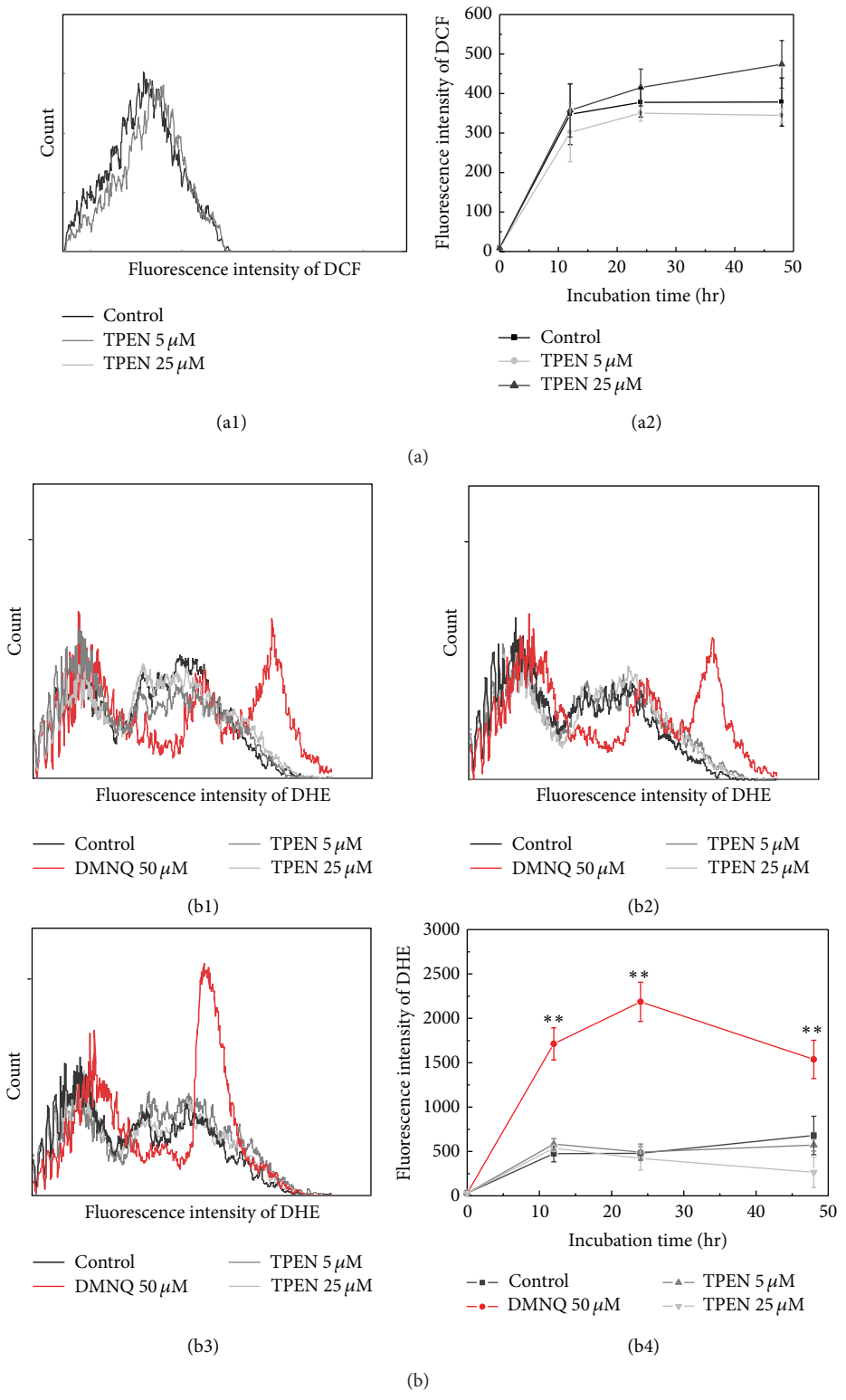


Figure 3: ROS Analysis. (a) Intracellular fluorescence of 2′,7′-dichlorodihydrofluorescein (DCF) in SH-SY5Y cells that were treated or not with 5 or 25 μM TPEN for 12, 24, and 48 hours measured by FACS (a1) flow cytometric data compiled on a single graphic showing no change in DCF fluorescence in different incubation time and concentration of TPEN (a2). Viable cells were labeled with PI to exclude dead cells and only viable cells were analyzed. (b) Dihydroethidium (DHE) in SH-SY5Y cells treated with 5 or 25 μM TPEN for 12 (b1), 24 (b2), and 48 hours (b3). Flow cytometric data compiled on a single graphic showing no change in DHE fluorescence in different treatment time and concentration of TPEN (b4). Data represent the mean values ± standard deviations ($n = 3$). Significant differences between positive control (DMNQ) and treated cell were $^{**}p < 0.001$. There were no significant differences between untreated and treated cells.

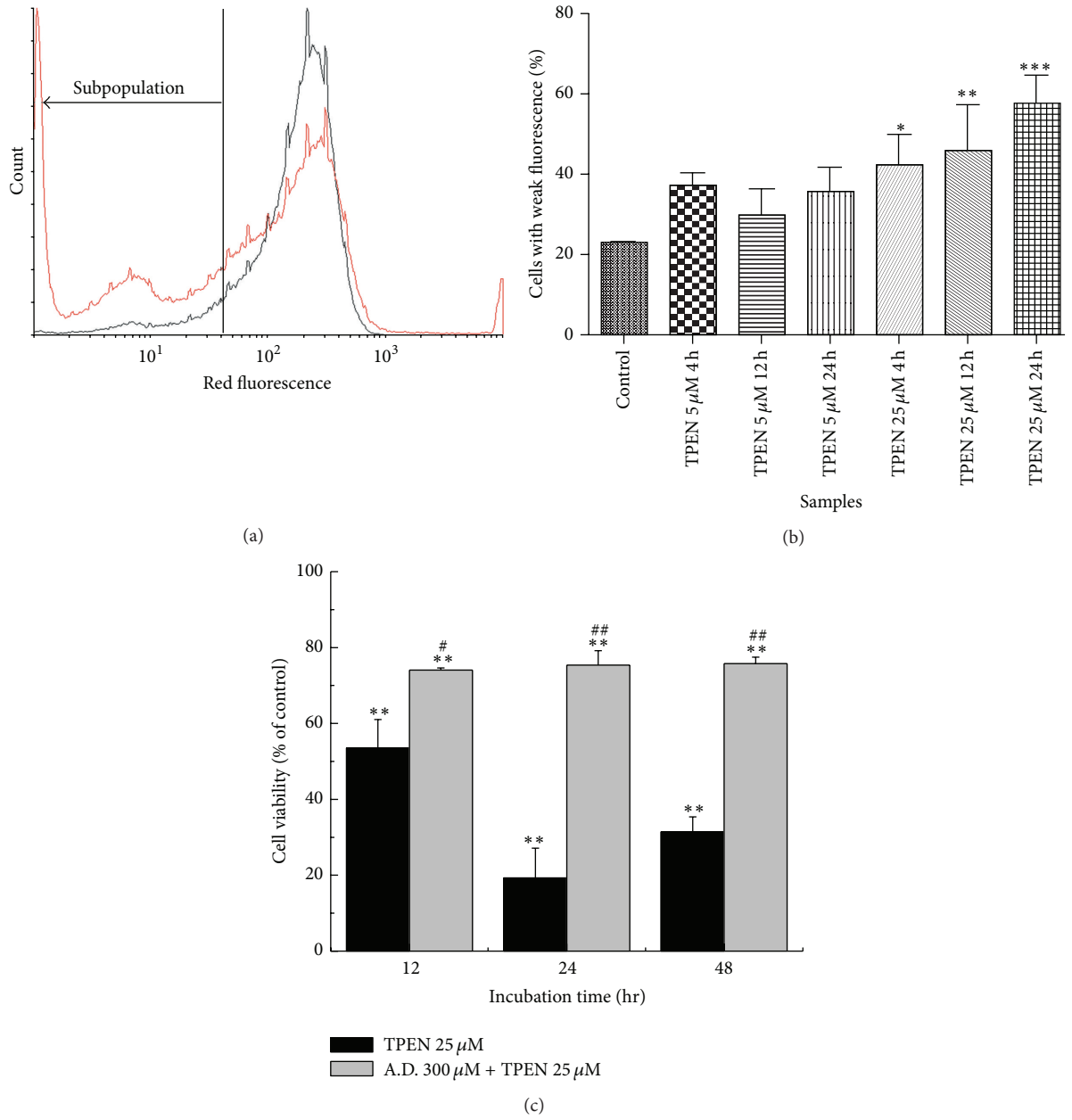


FIGURE 4: (a) Histogram of lysosomal disruption assay with Acridine Orange measured by flow cytometry. Black line shows control population and red line shows increase in the percentage of cells exhibiting a weaker fluorescence (subpopulation). (b) Subpopulation (weaker fluorescence) of 670 nm fluorescence analysis of SH-SY5Y after exposure to 5 or 25 μM TPEN for 4, 12, and 24 hours. (c) SH-SY5Y cells were pretreated with 300 μM antipain dihydrochloride (A.D.: cathepsins inhibitor) for 1 hour and subsequently incubated with 25 μM TPEN for 12, 24, and 48 hours and analyzed by MTT assay. Untreated cells were used as control and treated cells with 25 μM TPEN were used as positive control of cell death. Data represent the mean values ± standard deviation ($n = 3$), and significant differences between treated and untreated cells were $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$; and significant differences between treated and 25 μM TPEN treated cells were $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$.

then monitored by the level of red fluorescence, so when the lysosomal integrity is disrupted, there is an increase in the percentage of cells with weak red fluorescence, also known as the subpopulation [47–51]. An increase in the percentage of cells exhibiting a weaker fluorescence (subpopulation) (Figure 4(a)) at 670 nm in cells treated with 25 μM TPEN during 24 hours was observed, revealing that there was a high level of lysosomal rupture compared with control cells (Figure 4(b)). So it is possible to infer that intracellular zinc depletion is related to lysosomal rupture. AO intercalation into DNA and RNA is too low to disturb the lysosomal integrity assay [48]. There is still undisrupted lysosome, even in cells with moderate treatment. Therefore, the most effective measure of the rupture is in accordance with the change in fluorescence subpopulation, which is modified in the case of lysosomal lysis. The release of cathepsins is one of

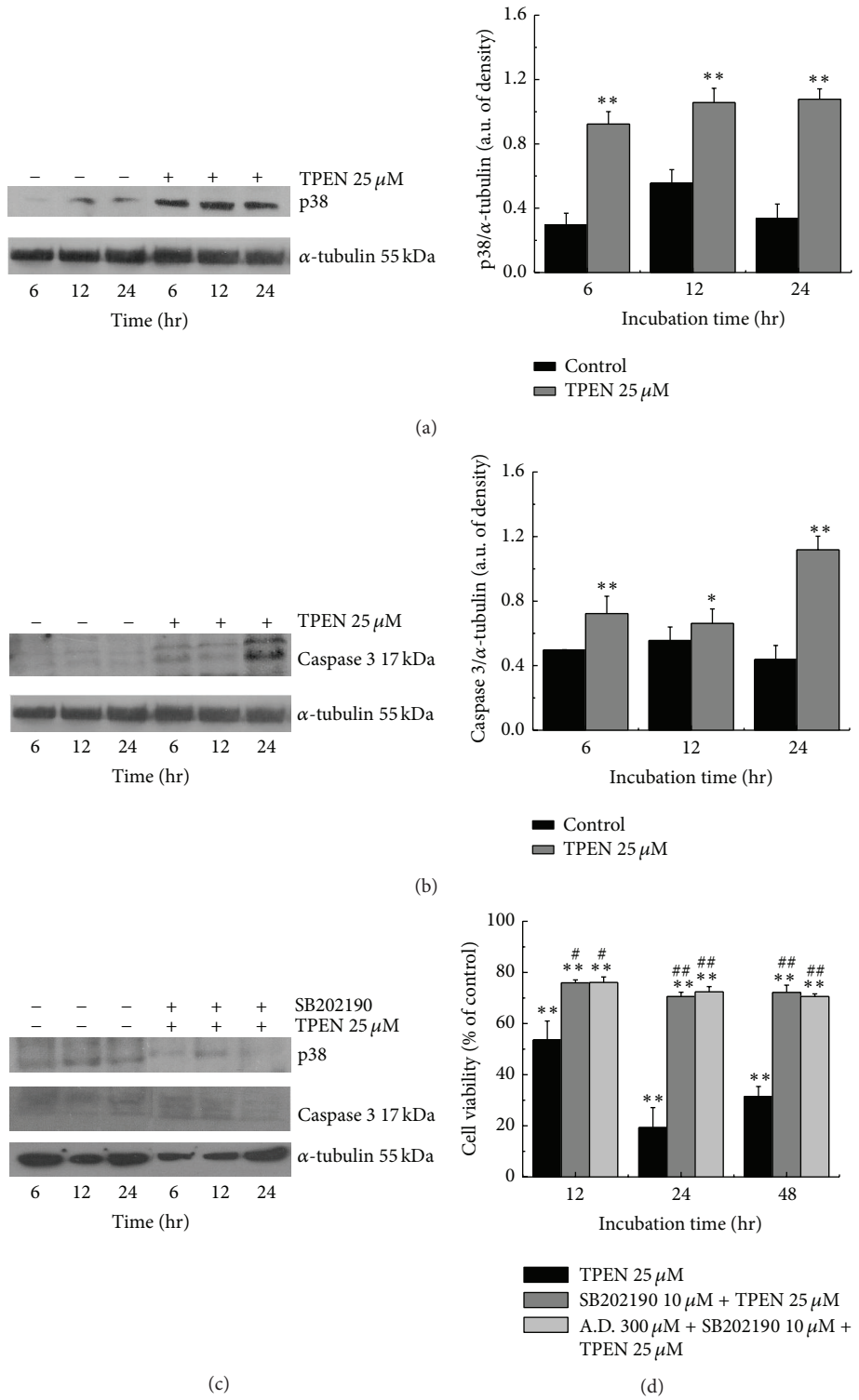
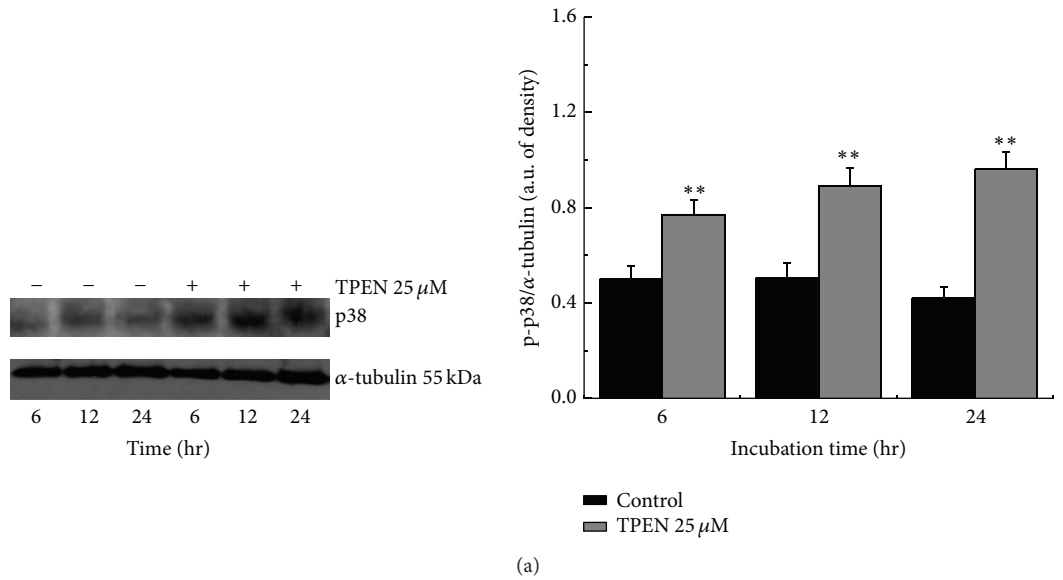
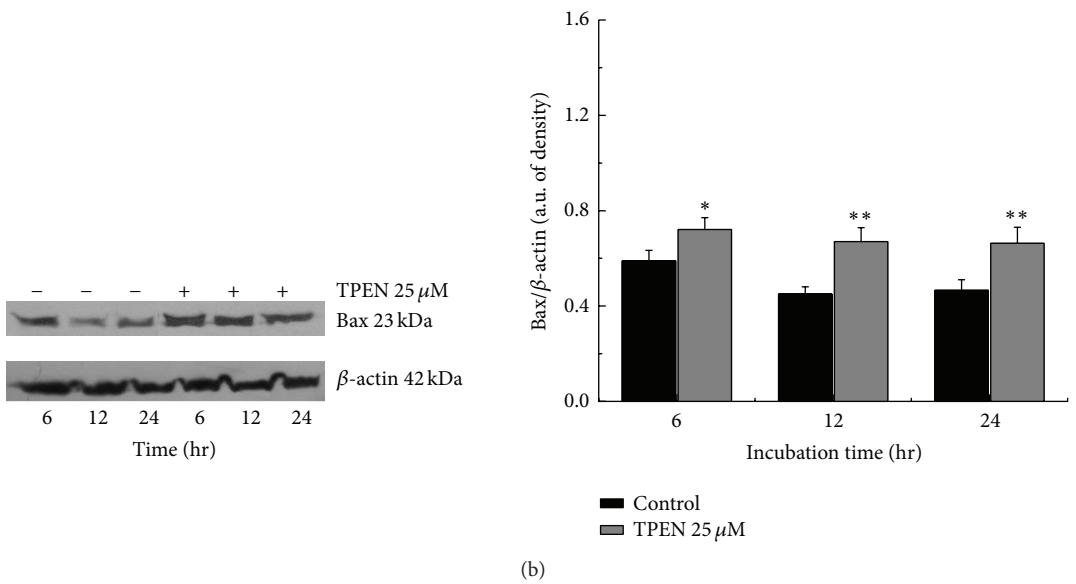


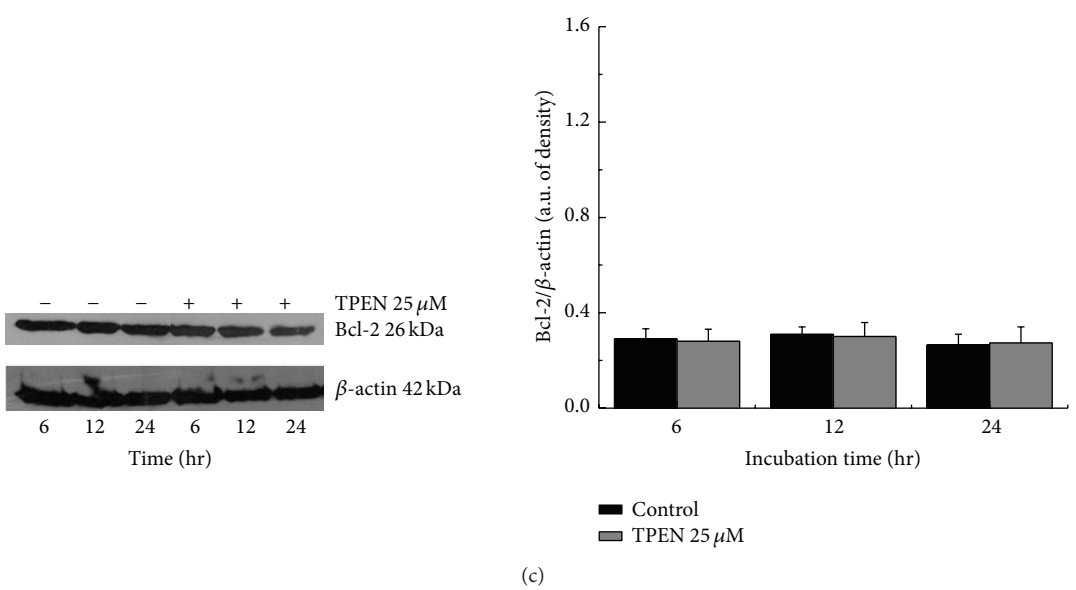
Figure 5: Western blot indicates whether expression levels of apoptotic proteins were upregulated or not during TPEN therapy with zinc depletion in neuroblastoma cells. SH-SY5Y cells were treated with 25 μM TPEN. At each time point, 100 μg of total proteins from the total cell lysates was loaded onto each lane for the detection of p38 (a and c) and caspase 3 (b and c). (c) The p38 MAPK and caspase 3 levels were determined after inhibition of the p38 protein with SB202190. (d) SH-SY5Y cells were pretreated with 300 μM antipain dihydrochloride (A.D.: cathepsins inhibitor) and/or 10 μM SB202190 for 1 hour and subsequently incubated with 25 μM TPEN for 12, 24, and 48 hours. Untreated cells were used as control and cells treated with 25 μM TPEN were used as positive control of cell death in these cases. The Western blot images represent three independent experiments, and significant differences between treated and untreated cells were $^{*}P < 0.05$ and $^{**}P < 0.001$; and significant differences between treated and 25 μM TPEN treated cells were $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$.



(a)



(b)



(c)

Figure 6: Continued.

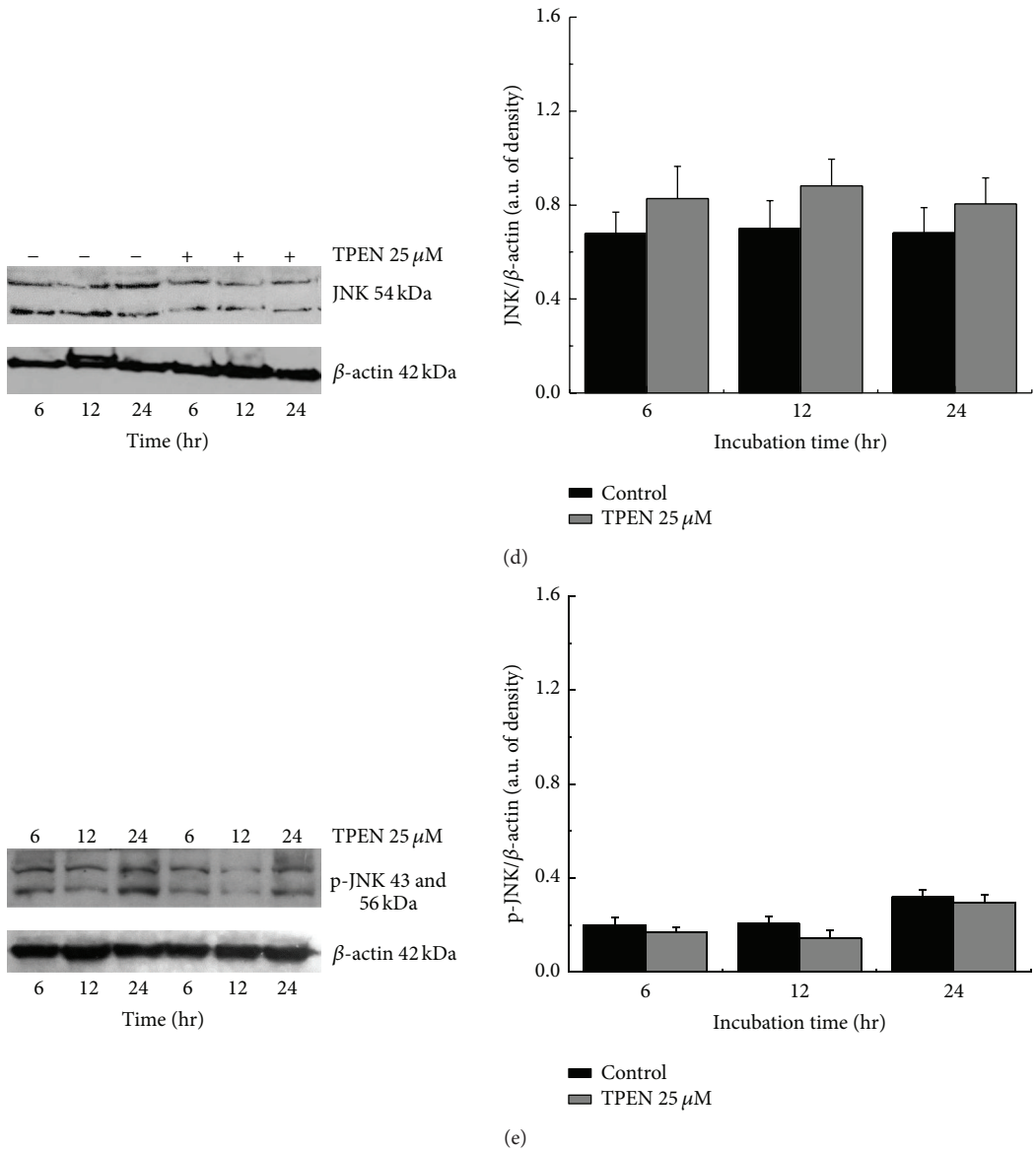


Figure 6: Influence of the TPEN chelator in the expression levels of apoptotic proteins. SH-SY5Y cells were treated with 25 μM TPEN. At each time point, 100 μg of total proteins from the total cell lysates was loaded onto each lane for the detection of p-p38 (a), Bax (b), Bcl-2 (c), JNK (d), and p-JNK (e). β-actin and α-tubulin were used as a loading control. The Western blot images represent three independent experiments, and significant differences between untreated and treated cells were $^{*}p < 0.05$ and $^{**}p < 0.001$, respectively.

the consequences of lysosomal rupture, and this can trigger cell death by apoptosis. To demonstrate that the lysosomal rupture is an event intrinsically correlated to the chelating action of TPEN, it was used to a cathepsin inhibitor (antipain dihydrochloride (A.D.)). The MTT viability assay showed that cells pretreated with 300 μM A.D. presented an increase in cell viability compared with treated cells with 25 μM TPEN (Figure 4(c)). Therefore, it was concluded that TPEN-induced cell death is dependent on the cathepsin release.

3.4. TPEN Activated Bax, Caspase 3, and p38 MAPK. We also determined which pathways are involved in cell death. The JNK and p38 MAP families of protein kinases are involved in stress-induced apoptosis, and some reports have indicated that these kinases are also involved in necrotic cell death [52, 53]. It is also known that proteins of the Bcl-2 family are involved in apoptosis with mitochondrial dysfunction, in cell membrane rupture, and in necrosis, leading us to examine the involvement of Bcl-2 and Bax proteins in cell death. The p38 MAP kinase can also sensitize cells to apoptosis by overexpression of Bax [54, 55]. Therefore, the expressions of p38 MAP kinase and Bcl-2 were also analyzed to understand the mechanism of action of TPEN and its depletion of intracellular zinc.

Conversely, we analyzed the expression levels of another mitogen-activated protein kinase, p38 MAP kinase, and the results showed an increase compared with control experiments at all incubation times in the extracts obtained

from cells (Figures 5(a) and 6(a)). We therefore used a p38 inhibitor to determine whether the activation of the cascade of apoptosis was first regulated by p38 MAP kinase. The compound SB202190 is a pyridinyl imidazole inhibitor that inhibits the activity of p38 MAP kinase through competition with ATP [46] and inhibits the phosphorylation of the protein [56]. When treating cells with an inhibitor of p38 MAP kinase, the highly selective and permeable membrane compound SB202190, it was observed that the compound completely inhibited the expression of both p38 MAP kinase and caspase 3; an increase in cell viability was also observed (Figure 5(d)), indicating the influence of p38 MAP kinase on the subsequent activation of caspase 3 (Figure 5(c)). Additionally, the expression levels of caspase 3 were analyzed, and the results showed that cells with zinc depletion also had an increased expression of this protein, particularly at 24 hours of incubation (Figure 5(b)).

We analyzed the expression levels of Bcl-2 protein by Western blot and observed no changes in Bcl-2 expression in the cell lysates (Figure 6(c)). However, the expression levels of Bax presented an increase following 12 hours of incubation (Figure 6(b)), because it is known that the c-Jun N-terminal kinase (JNK), one of the major mitogen-activated protein kinases (MAPKs), is also involved in cell death via either necrosis [53] or apoptosis [52]. The expression levels of this protein were also analyzed. The results obtained by Western blot showed that the expression levels of total JNK and phosphorylate JNK were not significantly affected when cells suffer zinc depletion with TPEN treatment (Figures 6(d) and 6(e)). It was already reported that JNK stimulates ROS formation, stimulating the protein ferritin to release iron and increase ferric ions in cytoplasm [45]. This increase consequently generates the rupture lysosomal cathepsins and releases the ferric ions to the environment, thus activating the genetically regulated necrosis [43]. However, our results showed that there is neither generation of ROS nor activation of JNK, suggesting that the observed lysosomal rupture is related only to the unbalance of intracellular zinc, which consequently contributes to a predominance of necrotic cell death pathway.

To conclude, our results showed that there is no generation of ROS (Figure 3), but there is a rupture of the lysosomal membrane (Figure 4). The experiments suggest that the rupture of lysosomes is related only to intracellular zinc imbalance. As TPEN induces the both events, activation of p38 MAPK and lysosomal rupture, we suggest that they occur independently of each other. We also observed that the activation of p38 MAP kinase only triggers the activation of the proapoptotic protein Bax. This may inactivate the antiapoptotic protein Bcl-2 and activate Bax protein interconnecting the apoptosis pathway with the lysosomal disturbance [57]. Therefore, because we observed a modest activation of Bax and no changes in the Bcl-2 in cells treated with zinc chelator TPEN, it is suggested that the depletion of intracellular zinc can influence the breakdown of lysosomes as a result of a release of cathepsins.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Redox Imbalance and Viral Infections in Neurodegenerative Diseases

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Reactive oxygen species (ROS) are essential molecules for many physiological functions and act as second messengers in a large variety of tissues. An imbalance in the production and elimination of ROS is associated with human diseases including neurodegenerative disorders. In the last years the notion that neurodegenerative diseases are accompanied by chronic viral infections, which may result in an increase of neurodegenerative diseases progression, emerged. It is known in literature that enhanced viral infection risk, observed during neurodegeneration, is partly due to the increase of ROS accumulation in brain cells. However, the molecular mechanisms of viral infection, occurring during the progression of neurodegeneration, remain unclear. In this review, we discuss the recent knowledge regarding the role of influenza, herpes simplex virus type-1, and retroviruses infection in ROS/RNS-mediated Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS).

1. Introduction

Neurodegenerative diseases are chronic degenerative pathologies of the Central Nervous System (CNS) characterized by progressive loss of specific neurons that lead to a decline in brain functions [1–3]. Despite these pathologies having different clinical features, they possess some common hallmarks, such as the formation and deposition of aberrant protein conformers, synaptic dysfunctions, deficient autophagic processes, oxidative/nitrosative stress, and inflammation [4]. The neurodegenerative diseases present an increase of reactive oxygen species (ROS) production by mitochondria and NADPH oxidase (NOX), which seems to be responsible for tissue injury, inflammation, and neurodegeneration [5, 6].

Substantial evidence indicates that also reactive nitrogen species (RNS) play a key role in most common neurodegenerative diseases although the mechanism of nitric oxide- (NO-) mediated neurodegeneration remains uncertain [7–9]. However, many studies demonstrated that NO is able to modify protein function by nitrosylation and nitrotyrosination, contribute to glutamate excitotoxicity, inhibit mitochondrial respiratory complexes, participate in organelle fragmentation, and mobilize zinc from internal stores in brain cells, contributing to neurodegeneration [10–13]. In response to increased oxidative and nitrosative stress the brain cells (i.e., microglia, astrocytes) activate redox-sensitive transcription factors, including nuclear factor-kβ (NF-kβ) and activator protein-1 (AP-1) [14, 15]. Next to this, it was also observed that the free radical increase, observed during neurodegeneration, may be also due to alteration of endogenous antioxidants. In particular, some antioxidant enzymes, such as superoxide dismutases (SODs), catalase, glutathione peroxidase, and glutathione reductase, have reduced activity in certain brain regions of AD patients [16]. Moreover, a reduction in amount of glutathione (GSH) level has been found in postmortem brain tissue from the *substantia nigra* of PD patients [17, 18]. Similarly, catalase and glutathione reductase activity, as well as GSH levels, were found to be significantly reduced in ALS patients [19]. Many of these antioxidant systems are regulated by nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 transcription factor. In normal conditions, NFE2L2 is associated with Kelch-like ECH associating protein 1 (Keap1) in the cytoplasm. This bond prevents the nuclear translocation of NFE2L2 and promotes its degradation *via* Ubiquitin Proteasome System (UPS). On the contrary, the presence of oxidative stress can

induce the detachment between Keap1 and NFE2L2, due to the modification of the reactive cysteine in Keap1 [20]. These conformational changes determine a release of NFE2L2 and its nuclear translocation, where it binds the ARE consensus sequences and coordinates the transcription of antioxidant and phase II detoxification genes [21]. Alterations of NFE2L2-pathway have been observed in postmortem brain of patients with neurodegenerative disorders [20]. In particular, many studies have showed an increase of NFE2L2 nuclear translocation in dopaminergic neurons of PD patients, but this induction is not sufficient to counteract the oxidative stress [22]. On the contrary, a decrease of NFE2L2 expression has been observed in hippocampus neurons in AD cases [22]. Moreover, a reduction of mRNA and protein levels of NFE2L2 was also found in the motor cortex and spinal cord in ALS patients [23]. Thus, the activation of NFE2L2-ARE pathway constitutes a valuable therapeutic tool to combat oxidative stress that occurs during neurodegenerative disease.

Recently, it has been demonstrated that infection agents can reach the CNS crossing the blood-brain barrier, by infected migratory macrophage or by intraneuronal transfer from peripheral nerves [24, 25]. In particular, these infections can affect the immune system resulting in a variety of systemic signs and symptoms [26]. The virus replication into the CNS produces molecular hallmarks of neurodegeneration, such as protein misfolding, deposition of misfolded protein aggregates, alterations of autophagic pathways, oxidative stress, neuronal functional alterations, and apoptotic cell death [26–28]. These effects associated with genetic alteration and other environmental factors contribute to the pathogenesis of neurodegenerative diseases.

In this review, we will highlight the role of oxidative stress and viral infection in the pathogenesis of Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS).

2. Role of Oxidative Stress in Neurodegeneration: General Aspects

Oxidative stress occurs due to an imbalance in the prooxidant and antioxidant levels. ROS and RNS are highly reactive with biomolecules, including proteins, lipids, carbohydrate, DNA, and RNA [29]. ROS that are particularly abundant during an imbalance of redox state are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), whereas among RNS the most abundant are NO and peroxynitrite ($ONOO^-$). During mitochondrial activity $O_2^{\bullet -}$ is produced in the electron transport chain (ETC), which is immediately converted to H_2O_2 by superoxide dismutase 2 (SOD2) located in the mitochondrial matrix or SOD1 located in the cytosol [30]. H_2O_2 is rapidly converted to water by mitochondrial glutathione (mtGSH) with the participation of GSH reductase and peroxiredoxins [31]. Other sources of free radical are the NOXs, enzymes located in the cell membrane. Several NOXs are expressed in the cells of CNS, such as neurons, astrocytes, and microglia [32, 33]. During infections, activation of NOXs is strongly improved and the resulting ROS increase is particularly important as a host defense mechanism [34]. However, excessive NOXs activation has also been implicated in oxidative stress-mediated neurodegeneration [35].

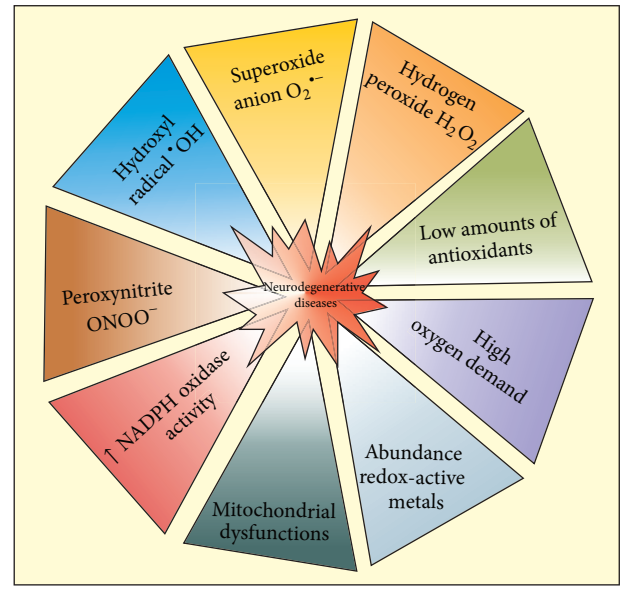

Figure 1: Main characteristics that occur in neurodegenerative diseases.

The brain is particularly prone to oxidative stress-induced damage because of its high oxygen demand, the abundance of redox-active metals (iron and copper), the high levels of oxidizable polyunsaturated fatty acids, and the low amounts of antioxidant enzymes (Figure 1). Another issue is that the neurons are postmitotic cells with relatively restricted replenishment by progenitor cells during the lifespan of an organism [11, 36]. Thus, the brain may be particularly vulnerable to viral infections during neurodegeneration due to different reasons: (i) the blood-barrier is compromised during neurodegeneration; (ii) many viruses can reach the CNS by peripheral nerves; (iii) the mitochondria become dysfunctional during neurodegeneration, preventing neurons from depending on aerobic metabolism and making it very susceptible to oxidative stress [17]. Primarily, in this review, the role of redox imbalance and redox-mediated inflammation in the onset and pathogenesis of neurodegenerative diseases will be discussed.

2.1. Redox Imbalance in AD. AD is a neurodegenerative disorder characterized by progressive decline in cognitive functions leading to memory loss and dementia. It involves degeneration of limbic and cortical brain structures, especially in the temporal lobe. One characteristic of AD is the appearance of senile plaques, which are produced from proteolytic cleavage of the transmembrane amyloid precursor protein (APP) to form β-amyloid peptide (Aβ). Another characteristic of AD is neurofibrillary tangles (NFTs) [37] and aggregates of medium and high molecular weight neurofilaments (NFM and NFH, resp.), as well as the microtubule-stabilizing protein tau, a multifunctional protein involved in

microtubule assembly and stabilization [38, 39]. These hallmarks are altered in ways characteristic of oxidative damage, such as advanced glycation end product- (AGE-) modifications, protein cross-linking, and carbonyl-modifications [40–42]. All these alterations in neurons susceptible to AD play a key role in the irreversible cellular dysfunction that ultimately leads to neuronal death.

Brain autopsy from AD patients has shown oxidative damage markers, such as lipid peroxidation, protein oxidative damage, and glycoxidation in brain tissues [43]. Next to this, a drastic decrease in the intraneuronal content of GSH has been observed in the hippocampus and cortex of AD patients [43, 44]. Thus, the loss of ROS balance produces a chronic oxidative state, which induces a reduction of antioxidants expression and activity, accelerating the neurodegenerative processes. In fact, the alteration of redox homeostasis stimulates the formation of products of advanced glycosylation, an overload of peroxidation of fatty acids, oxidation of cholesterol, insulin resistance, and proteins unfolding [41, 45–49]. Moreover, an increase of Heme Oxygenase-1 (HO-1) and 8-hydroxyguanosine (8-OHG) was found in AD brain as compared with controls [50].

Despite the cause of redox imbalance still being unclear in AD pathogenesis, many studies suggest that the alteration in redox transition metals balance (i.e., iron, copper) is the major cause of neurodegeneration [51–53]. In fact, iron and copper have been found in high concentrations in AD brain. In particular, Zn, Cu, and Fe in senile plaques rims and cores have been found significantly elevated in AD [51]. It has also been demonstrated that the activity of many proteins, such as ferritin and ceruloplasmin, which are important to regulation of metal homeostasis, shows altered expression in AD [54]. Other studies have revealed dramatic drops in the levels of some biometals in the AD brain, which may aid development of senile plaques [55]. In particular, reduced levels of intracellular Cu have been reported in cortical neurons derived from AD transgenic mice and in the most-affected brain region of AD patients [56]. This alteration appears to contribute in part to AD pathogenesis. The dysregulation of biometal homeostasis in AD is a complex pathway, which has contributed to the development of new therapeutic approaches to restore the neuronal functions.

The combination of all these factors could explain how the oxidative stress is linked to the formation of amyloid plaques and NFTs in AD.

2.2. Redox Imbalance in PD. PD is progressive neurodegenerative disease characterized by extrapyramidal movement disorders that manifest as rigidity, resting tremor, and postural instability [57]. PD is also characterized by a progressive loss of dopaminergic neurons in the *substantia nigra*, accompanied by the accumulation of α-synuclein aggregates in Lewy bodies [58]. Lewy bodies are composed not only of α-synuclein, but also of other proteins, such as ubiquitin and neurofilament proteins [59].

Many evidences demonstrate that oxidative stress plays an important role in PD pathogenesis. The *substantiae nigrae* of PD subjects show increased levels of oxidized protein lipid [60], DNA [61], and decreased level of GSH [62]. In particular, oxidized proteins may not be adequately ubiquitinated and recognized by proteasome and thus accumulate within the neurons [63]. Moreover, DNA damage could determine an alteration of many important genes essential for neurons activity and functionality [64]. Increased levels of 4-hydroxynonenal (HNE) were found in the rime of Lewy bodies of PD [65]. HNE, activating caspase-8, caspase-9, and caspase-3 and inducing DNA fragmentation, is able to ultimately provoke apoptosis of dopaminergic cells [66]. HNE inhibits NF-kβ pathway [67], induces PARP cleavage [68], decrease GSH content, and inhibits complexes I and II of the ETC, contributing to the disease progression [69–71].

Mice treated with PD toxins (i.e., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, and rotenone) support the link between oxidative stress and dopaminergic neuronal degeneration. In particular, MPTP causes a depletion of dopamine (DA) levels [72] and reduction of tyrosine hydroxylase (TH) [73]. The monoamine oxidase B (MO B) converts MPTP in 1-methyl-4-phenylpyridinium (MPP+), which blocks mitochondrial complex I and causes ATP depletion and ROS increase. This is thought to be the main cause of MPTP-induced terminal degeneration [74–77]. Consequently, MPTP-treated mice show an induction of glial response and increased levels of inflammatory cytokines and microglial activation, suggesting that the neurodegenerative process is evolving [78].

In the last years, the discovery of genes implicated to familial forms of PD (i.e., α-synuclein, Parkin, and DJ-1) has allowed the identification of new mechanisms, which highlight the importance of oxidative stress in PD pathogenesis. For example, α-synuclein gene mutations are linked with inherited PD and increase the tendency of the protein to aggregate [79]. It is a natively unfolded protein that can associate with vesicular and membranous structures and plays a role in synaptic vesicle recycling storage. Fibrils of α-synuclein in conjunction with DA were found in *substantia nigra*, which lead to an accumulation of cytotoxic soluble protofibrils and an increase of oxidative/nitrosative stress [80, 81].

2.3. Redox Imbalance in ALS. ALS is a relentlessly progressive neurodegenerative disorder, in which increasing muscle weakness leads to respiratory failure and death, which typically develops during the sixth or seventh decade of life [82].

Different studies show an increase of oxidative damage to proteins in ALS postmortem tissues compared to control. In particular, high levels of protein carbonyls have been identified in both spinal cord [83] and motor cortex [84] from ALS cases. Increased 3-nitrotyrosine levels were observed in both sporadic and SOD1 familial ALS patients [85]. Oxidative damage to DNA, measured by levels of 8-OHG, has also been found to be increased in cervical spinal cord from ALS patients [86]. Immunoreactivity to the brain and endothelial forms of nitric oxide synthase (eNOS) was also elevated in ALS motor neurons relative to controls, suggesting that nitration of protein-tyrosine residue is upregulated in motor neurons of the spinal cord of ALS [87].

Transgenic mouse models and cell culture models of ALS based on mutant SOD1 recapitulate the oxidative damage to protein, lipid, and DNA observed in the human disease [88]. Moreover, many studies have suggested that SOD1 mutations could have toxic effects for three different reasons: (i) loss of function leading to increased levels of $O_2^{\bullet-}$, which can react with NO to produce $ONOO^-$ [85]; (ii) a dominant-negative mechanism whereby the mutant SOD1 protein not only is inactive, but also inhibits the function of normal SOD1 expressed by the normal allele [89]; or (iii) increased SOD1 activity leading to increased H_2O_2 levels and $^{\bullet}OH$ [89].

A new pathological feature identified in postmortem tissue of ALS patients consists in neuronal protein deposition of TDP-43 or TAR DNA binding protein with a molecular mass of 43 kDa [90]. In particular, TDP-43 aggregates were found in 97% of ALS cases whether sporadic or familial [91, 92]. TDP-43 is a ubiquitously expressed DNA/RNA-binding protein, which is expressed in cytoplasm and in the nucleus where it regulates RNA splicing and microRNA biogenesis [93–95]. It has been observed that in conditions of oxidative stress TDP-43 is able to translocate in cytoplasm and assemble into stress granules (SGs), which are evident in ALS [96, 97]. SGs are large messenger ribonucleoprotein aggregates that are implicated in the stress-mediated inhibition of mRNA and protein synthesis [98]. An altered control of mRNA translation in stressful conditions may trigger motor neuron degeneration at early stages of the disease. Thus, the presence of TDP-43 in SGs leads to a loss of protein functionality defining an altered control of mRNA translation in stressful conditions triggering neuron degeneration.

3. Redox-Mediated Inflammation in Neurodegenerative Diseases

Recent studies have highlighted the correlation between oxidative damage and *neuroinflammation* in neurodegenerative processes, with the term neuroinflammation meaning the chronic inflammation of the CNS. It is characterized by inflammatory molecules expression, endothelial cell activation, platelet deposition, and tissue edema. Neuroinflammation plays an important role in many common neurodegenerative diseases [99]. Its accompanied by an increase of NO and/or $O_2^{\bullet-}$ with H_2O_2 production [100]. Generally, the inflammation is a protective process that protects the cells from detrimental agents, promoting tissue repair. In uncontrolled conditions the inflammatory process induces inordinate cell damage as it occurs in neurodegenerative disease. In particular, during neuroinflammation, microglia and astrocytes produce many inflammatory genes, including cytokines, chemokines, adhesion molecules, and proinflammatory transcription factors [101]. An increase of some transcription factors involved in inflammation was also found, such as NF-kβ, peroxisome proliferator-activated receptor gamma (PPARγ), and Sp1 in microglia cultures and AD brain [102–104]. Thus, the inflammatory mediators secreted by microglial and astrocytic cells contribute to neuronal dystrophy [105]. In these conditions microglia can produce ROS, NO, and proteolytic enzyme, enhancing the senile plaques and NFTs formation [106]. Furthermore, as a vicious cycle, the senile plaques induce the expression of proinflammatory cytokines and enzymes such as inducible NOS (iNOS) and cyclooxygenase enzyme (COX-2) in microglia cells, suggesting that all these factors can contribute to neurodegeneration [107].

In the case of AD many authors speculate that senile plaques and NFTs constitute the site of activation of a chronic inflammatory response. In fact, an interaction between Aβ peptide and CR3/Mac-1 (CD11b/CD18) on microglia has been observed. This interaction determines the activation of phosphatidylinositol 3-kinase (PI3K), which in turn phosphorylates $p47^{phox}$, inducing the PHOX translocation and activation on microglia membrane increasing the production of $O_2^{\bullet-}$ and causing neuroinflammation [108, 109]. Thus the abnormal activation of microglia disrupts nerve terminals activity causing an alteration and a loss of synapses, which correlates with memory decline, leading to progression of AD [110]. Next to this, some studies have revealed an association between AD and mutations in different genes opening new strategies for comprehension of pathology [111, 112]. For example, genome exome and Sanger sequencing have revealed that heterozygous rare variants in triggering receptor expressed on myeloid cells 2 (TREM2) are associated with a significant increase in the risk of AD [113]. Also genome-wide investigations have revealed many polymorphisms in the human genome of AD patients. In particular, polymorphisms on clusterin (ApoJ, a potent regulator of complement induction) and CR1 (complement receptor) genes are genetically associated with sporadic AD [114, 115]. Moreover, the single nucleotide polymorphisms for cytokines and chemokines genes have been associated with AD risk [116].

In PD the activation of microglia has been amply demonstrated, suggesting an important role of neuroinflammation in the pathophysiology of PD. Activated microglia produce $O_2^{\bullet-}$ and NO, which in turn contribute to oxidative and nitrosative stress in the brain [117]. Notably, activated microglia and T lymphocytes, together with an increase of proinflammatory mediators, have been detected in the brain and cerebrospinal fluids of PD patients [118]. An increase of iNOS has been also revealed in activated microglia of PD subjects [118]. Moreover, the role of DA as being responsible for the ROS-mediated inflammation reaction in neurons was shown [119]. In fact, DA is stable in synaptic vesicles inside the cell; however once DA exists it is easily metabolized by MO. Alternatively, DA can undergo autooxidation determining the ROS production. As a result the microglia became active and produce proinflammatory cytokine, such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) [120], and $O_2^{\bullet-}$ and NO [121], leading the generation of vicious cycle that further increases dopaminergic toxicity in the *substantia nigra*.

As for the other neurodegenerative diseases a characteristic of ALS pathology is the occurrence of a neuroinflammation, which activates microglia, astrocytes, and T-cells. In particular, the autopsy studies have demonstrated a microglia activation and an induction of activator transcription-3 (STAT3) in ALS spinal cord microglia [122]. Moreover,

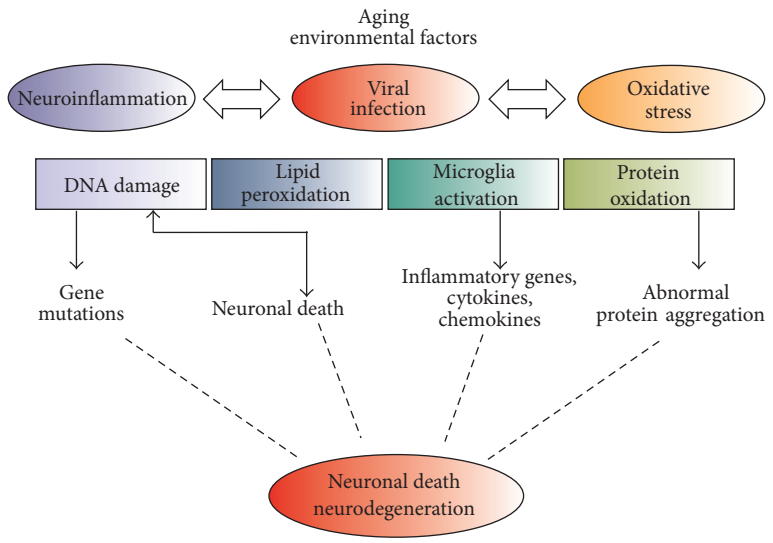

Figure 2: Different genetic and/or environmental factors lead to ROS increase during neurodegeneration. This accumulation triggered the activation of glia cells and the release of proinflammatory markers, stimulating thus a neuroinflammatory response. These events contribute to neuronal damage (DNA damage, lipid peroxidation, and protein oxidation) and axon degeneration, which ultimately caused neuronal death. In addition, virus infection can strengthen the ROS-mediated neurodegenerative signs in neurons and glia cells, producing functional and molecular hallmarks of neurodegeneration.

an upregulation of lipopolysaccharide/Toll Like Receptor 4 (LPS/TLR4) signaling associated genes has been observed in peripheral blood mononuclear cell (PMBCs) from ALS patients, suggesting chronic monocytes and macrophage activations [123]. Studies made on serum and cerebrospinal fluid (CSF) of 20 ALS patients show an increase of MCP1 and IL-8 levels, indicating a stimulation of proinflammatory cytokine cascade after microglia activation [124]. Also increased levels of IL-17, IL-6, and LPS are found in the serum of subjects with ALS [125]. ELISA assays have also demonstrated an increase of IL-15 and IL-12 in serum and CSF of 21 patients with ALS, suggesting that these molecules could be used as potential markers of immune activation in ALS [126]. Moreover, 2D gel electrophoresis analysis highlighted an increased activity of components of complement C3 in serum of ALS patients with respect to controls [127]. All these studies demonstrate the presence of an inflammatory and immune response in subjects with ALS.

4. Viral Infections and Neurodegeneration

As mentioned above, a common feature of neurodegenerative disease is the chronic neuroinflammation and activation of microglia in the brains of patients with PD, AD, and ALS. In the last years, many studies show an association between virus infection and neurodegenerative as another important common feature of these disorders (Figure 2). In the second part of this review we will provide a detailed picture of how some virus infections can guide us to underpin mechanisms in neurodegeneration and amplify the damage mediated by oxidative stress.

Neuronal degeneration can be either directly or indirectly affected by viral infection. Viruses can injure neurons by direct killing, by cell lysis, and by inducing apoptosis. Different pathogens and/or their products may directly induce long-term degenerative effects, such as the deposit of misfolded protein aggregates, increased levels of oxidative stress, deficient autophagic processes, synaptopathies, and neuronal death. Viruses, bacteria, protozoa, and unconventional pathogens such as prion proteins have the ability to invade the CNS as described by De Chiara et al. (2012) [128]. There are different routes of entry of infectious agents into the CNS and they cause acute infections, which in some cases may be fatal or which may progress to become chronic illnesses [129, 130]. When the viruses enter into the nervous system, that is, they are neurotropic, it leads to activation of both innate and adaptive immune responses. Viral antigens preferentially activate the TLRs 3, 7, and 8 driving innate and adaptive immune responses and leading to neuronal damage, which occurs through direct damage, killing, release of free radicals, cellular activation, and inflammation, and induce a number of encephalopathies [58]. In particular, one of the secondary consequences of these encephalopathies can be the Parkinsonism that is both transient and permanent condition.

According to reviewed literature, and as discussed in depth below, a large number of studies demonstrate that the viruses are one of the main causes of degenerative diseases. In particular, as emerging from the review below, a growing interest is devoted to investigating the effects of H1N1 in PD (Section 4.1), of HSV1 in AD (Section 4.2), and of retroviruses in ALS (Section 4.3).

4.1. H1N1 in PD. In the last years, it has emerged that influenza virus has been implicated as a direct and an indirect cause of PD, although it was recently found that influenza

can be considered as PD-like symptoms such as tremor, particularly in the month after an infection, but not with an increased risk of developing idiopathic PD [131].

Influenza virus is a respiratory pathogen contagious to humans, belonging to Orthomyxoviridae family, which are negative sense, single-stranded, segmented RNA viruses. In particular, a viral etiology for PD is based largely on epidemiological studies indicating a possible coincidence of PD with influenza flu pandemics, most notably the 1918-1919 "Spanish" influenza outbreak [132, 133]. In recent studies, Rohn and Catlin have shown the presence of influenza A virus within the *substantia nigra* pars compacta (SNpc) from postmortem PD brain sections [134]. They also identified colocalized influenza A and immune cells with caspase-cleaved Beclin-1 within the SNpc, which clearly indicated the role of neuroinflammation with influenza A virus's involvement in PD pathogenesis. Influenza A virus labelling was identified within neuromelanin granules as well as on tissue macrophages in the SNpc [134]. As mentioned above, the PD hallmark Lewy bodies are also composed mainly of aggregated α-synuclein. The formation of Lewy bodies is due to accumulation of normally produced Ser-129 phosphorylated α-synuclein [135]. It is demonstrated that H5N1 influenza virus progresses from the peripheral nervous system into the CNS and increases the phosphorylation and aggregation of α-synuclein [136]. Reviewed data suggest that influenza virus could have a role in the PD.

4.2. HSV1 in AD. Growing epidemiological and experimental evidence suggests that recurrent herpes simplex virus type-1 (HSV-1) infection is a risk factor for AD. It belongs to the family Herpesviridae, which is a large family of double-stranded DNA viruses. HSV-1 is a virus that primarily infects epithelial cells of oral and nasal mucosa [137]. The concept of a viral role in AD, specifically of HSV-1, was first proposed several decades ago [138, 139]. Several epidemiological studies have reported the presence of the HSV-1 genome in postmortem brain specimens from numerous AD patients, particularly those who carry the type 4 allele of the gene that encodes apolipoprotein E (APOE4), another potential risk factor for AD [140, 141]. Moreover, Wozniak et al. [142] have found the HSV-1 DNA in amyloid plaques of AD brains.

Several studies suggest that HSV-1 could be a possible major cause of amyloid plaques and hence possible aetiological factor in AD. Besides, genes related to HSV-1 reactivation have been detected in the brain of patients with familial AD, associated with β-amyloid deposits [143]. HSV-1 infection has also been shown to promote neurotoxic Aβ accumulation [144–146], tau phosphorylation [147], and cleavage [142] *in vitro*. Several studies have sought anti-HSV-1 IgM as well as IgG in serum from AD patients, showing that the risk of AD is increased in elderly subjects with positive titers of anti-HSV-1 IgM antibodies [148]. Genetic studies too have linked various pathways in AD with those occurring in HSV-1 infection [149].

The presented evidences suggest that HSV1 may have a critical role in AD pathogenesis.

4.3. Retroviruses in ALS. Retroviruses play an important role in the pathogenesis of ALS. In fact, several studies have reported retroviruses to be involved in ALS [150–154]. As found by [155], the reverse transcriptase (RT) enzyme of the retroviruses can convert RNA into complementary DNA. The first demonstration of retroviral involvement in ALS dates back to 1975 when Viola et al. [156] found RT activity in cytoplasmic particulate fraction from two Guamanian ALS but not in brains from two control individuals. At that time, a growing interest was in finding the retroviral.

Other studies showed that the RT is present more frequently in ALS patients' sera compared to that of control and the levels of the activity in ALS patients were comparable to that in HIV-infected patients [157, 158].

ALS-like syndromes are developed in a small percentage of persons infected with the human immunodeficiency virus-1 (HIV-1) or human T-cell leukemia virus-1 (HTLV-1). HIV-infected patients may develop neurological manifestations that resemble classical ALS although it occurs at a younger age and they may show a dramatic improvement following the initiation of antiretroviral therapy. On the other hand, HTLV-1 associated ALS-like syndrome has several features that may distinguish it from classical ALS. However, most patients with probable or definite ALS show no evidence of HIV-1 or HTLV-1 infection [159]. Moreover, studies have shown increased HERV-K expression in both serum and brain tissue in ALS patients [160]. Furthermore, in a recent study it has been shown that HERV-K is activated in a subpopulation of patients with ALS and that its envelope protein may contribute to neurodegeneration [161]. These evidences suggest that retroviruses are involved in the pathophysiology of ALS.

5. Viral Infections and Oxidative Stress in Neurodegenerative Disease

Frequently viral infections cause changes in the redox state in host cells [162–166]. Many viral infections can cause an increased generation of ROS and RNS, which can be caused by both direct effects of virus on cells and inflammatory responses of the chronic viral host. In the presence of surplus ROS, the pathogen-mediated proteins can induce pathologic changes in neural tissue and lead to chronic inflammation of the brain, as seen in classical neurodegenerative diseases.

5.1. HSV1. HSV1-1 when infecting neurons and glia cells induce the production of proinflammatory cytokines produced by microglia and infiltrating macrophages, as well as the production of chemokines and antiviral cytokines [167, 168]. Several studies have shown that during HSV-1 infection into the cell a depletion of GSH, the production of ROS, the induction of mitochondrial DNA damage, and endoplasmatic reticulum stress with consequent alteration of the intracellular redox state towards a prooxidant state occur [166, 169–171].

More data indicate that virus infection induced oxidative damage in the brain. In particular, Schachtele et al. (2010) [172] have shown that HSV-1 induced neural cell oxidative tissue damage and cytotoxicity, which are mediated by microglial cell through a TLR2-dependent mechanism. In other studies increases in ROS levels, lipid peroxidation, and

protein nitrosylation were reported when there is HSV-1 infection [167, 173, 174]. Furthermore, in the recent study Santana et al. (2013) have shown that oxidative stress enhances the accumulation of intracellular Aβ and the inhibition of Aβ secretion induced by HSV-1 infection [175]. Several studies suggest that HSV-1 induced oxidative stress in neuronal cells may trigger β- and γ-secretase activation and, consequently, APP processing and Aβ formation. These findings demonstrate that HSV-1 infection of neuronal cells can generate multiple APP fragments with well-documented neurotoxic potentials [147].

5.2. Influenza Virus. *Influenza* virus uses host cell structures and metabolic pathways for its life-cycle. In particular, intracellular redox state changes, for example, GSH depletion or ROS or RNS increase, have been detected during influenza virus infection [165]. On the other hand, it has been recently demonstrated that NOX4 enzyme, the main source of ROS production during influenza virus infection, regulates specific steps of virus life-cycle [34]. Virus-induced GSH decrease is pivotal for viral replication by allowing the folding and maturation of viral hemagglutinin [176] and activating cellular kinases involved in nucleocytoplasmic traffic of viral proteins [177].

On the basis of these evidences it can be assumed that the infection of influenza virus amplifies the effects of oxidative stress, which contribute to neuronal damage.

5.3. Retroviruses. Garaci et al. [178] demonstrated that *in vitro* HIV infection significantly decreases the GSH content of human macrophages. In addition, recent work has shown that HIV-1 induces ROS production in astrocytes and microglia [179, 180]. Dasuri et al. [180] have shown that oxidative stress is involved in the pathology of HIV-associated neurocognitive disorders. HIV-infected monocytes and T-cell, to enter in the cell, use the glycoprotein gp120. The viral protein gp120 can directly induce apoptosis in neurons and increase oxidative stress through GSH and lipid peroxidation [179].

The increases of ROS plays a role in viral pathogenesis probably because the increase of oxidative stress, generated when viruses infect the aged neuronal cells, may contribute to increasing the production of misfolded proteins and hence to the pathogenesis of neurodegenerative diseases.

Data discussed in this review suggest that viruses can be causative agents or, at least, cofactors of some neurodegenerative diseases. Therefore, much attention should be paid to infectious and, especially, viral agents among the environmental factors contributing to neurodegenerative diseases.

6. Conclusions

Although numerous studies have been made to understand the genetic/molecular mechanisms that underly the different neurodegenerative diseases, the comprehension of how redox imbalance is implicated in viral infection during neuronal damage is still unclear. In particular, understanding whether the redox imbalance is the cause or the effect of an increased propensity of brain cells to infection would be of great importance to develop new therapeutic strategies to target redox/inflammatory markers in brain inflammation and neurodegenerative disorders.

Abbreviations

MPTP:	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+:	1-Methyl-4-phenylpyridinium
HNE:	4-Hydroxynonenal
8-OHG:	8-Hydroxyguanosine
AP-1:	Activator protein-1
STAT3:	Activator transcription-3
AGE:	Advanced glycation end product
AD:	Alzheimer's disease
APP:	Amyloid precursor protein
ALS:	Amyotrophic lateral sclerosis
Aβ:	β-Amyloid peptide
CNS:	Central Nervous System
CSF:	Cerebrospinal fluid
CR1:	Complement receptor
COX-2:	Cyclooxygenase enzyme
DAT:	DA transporter
DOPAC:	Dihydroxyphenylacetic acid
DA:	Dopamine
ETC:	Electron transport chain
eNOS:	Endothelial nitric oxide synthase
GSH:	Glutathione
HO-1:	Heme Oxygenase-1
HSV-1:	Herpes simplex virus type-1
H_2O_2:	Hydrogen peroxide
$^{\bullet}OH$:	Hydroxyl radical
IL-12:	Interleukin-12
IL-15:	Interleukin-15
IL-17:	Interleukin-17
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
IL-8:	Interleukin-8
iNOS:	Inducible nitric oxide synthase
INF-γ:	Interferon-γ
LPS:	Lipopolysaccharide
MIP1α:	Macrophage inflammatory protein 1α
MIP1β:	Macrophage inflammatory protein 1β
NFM, NFH:	Medium and high molecular weight neurofilaments
mtGSH:	Mitochondrial glutathione
MO B:	Monoamine oxidase B
MCP1:	Monocyte chemotactic protein 1
NOX:	NADPH oxidase
NFTs:	Neurofibrillary tangles
NO:	Nitric oxide
NF-kβ:	Nuclear factor-kβ
PD:	Parkinson's disease
PPARγ:	Peroxisome proliferator-activated receptor gamma
$ONOO^-$:	Peroxynitrite
PMBCs:	Peripheral blood mononuclear cells
RNS:	Reactive nitrogen species

ROS: Reactive oxygen species
SNpc: *Substantia nigra* pars compacta
$O_2^{\bullet -}$: Superoxide anion
SOD2: Superoxide dismutase 2
TLR: Toll Like Receptor
TNF-α: Tumor necrosis factor-α
TREM2: Triggering receptor expressed on myeloid cells 2
TH: Tyrosine hydroxylase.

Competing Interests

The authors have no competing interests to declare.

Authors' Contributions

Dolores Limongi and Sara Baldelli equally contributed to this work.

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Research Article

PD98059 Protects Brain against Cells Death Resulting from ROS/ERK Activation in a Cardiac Arrest Rat Model

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The clinical and experimental postcardiac arrest treatment has not reached therapeutic success. The present study investigated the effect of PD98059 (PD) in rats subjected to cardiac arrest (CA)/cardiopulmonary resuscitation (CPR). Experimental rats were divided randomly into 3 groups: sham, CA, and PD. The rats except for sham group were subjected to CA for 5 min followed by CPR operation. Once spontaneous circulation was restored, saline and PD were injected in CA and PD groups, respectively. The survival rates and neurologic deficit scores (NDS) were observed, and the following indices of brain tissue were evaluated: ROS, MDA, SOD, p-ERK1/2/ERK1/2, caspase-3, Bax, Bcl-2, TUNEL positive cells, and double fluorescent staining of p-ERK/TUNEL. Our results indicated that PD treatment significantly reduced apoptotic neurons and improved the survival rates and NDS. Moreover, PD markedly downregulated the ROS, MDA, p-ERK, and caspase-3, Bax and upregulated SOD and Bcl-2 levels. Double staining p-ERK/TUNEL in choroid plexus and cortex showed that cell death is dependent on ERK activation. The findings in present study demonstrated that PD provides neuroprotection via antioxidant activity and antiapoptosis in rats subjected to CA/CPR.

1. Introduction

Cardiac arrest (CA) remains a leading cause of death and long-term disability worldwide, thus representing a major concern to public health and the economy [1]. Despite many years of laboratory and clinical research, treatment of post-cardiac arrest has not reached therapeutic success. Survival rate and neurological injury outcome following CA and cardiopulmonary resuscitation (CPR) remain poor [2]. The patients with successful return of spontaneous circulation (ROSC) have neurological deficits, 80% are comatose or with persistent vegetative state, with only 3% to 7% able to return to their previous level of brain function [3, 4]. The pathogenesis of cerebral ischemia/reperfusion (I/R) injury is very complex and remains incompletely understood; overproduction of reactive oxygen species (ROS) plays vital role in I/R brain damage [5–7]. Overproduction of ROS can cause oxidative damage to biomolecules (lipid, protein, and DNA) in membrane and nucleus, eventually leading to cell death [8]. Furthermore, ROS is able to activate kinases or inhibit phosphatases resulting in stimulation of signaling pathways [9, 10]. ERK belongs to a family of mitogen-activated protein kinases (MAPKs) which is one example for ROS-regulated kinases [11, 12]. ROS can inhibit protein phosphatases resulting in the activation of the ERK1/2 signaling pathway [12]. ROS are also able to stimulate directly growth receptors such as EGFR and PDGFR, which induces the activation of both Ras and ERK1/2 pathways [13]. Evidences showed that ERK1/2 pathway is phosphorylated in the damaged brain after ischemia in animal model [14, 15]. Inhibitors of MEK/ERK 1/2, PD98059 (PD), and U0126 reduce infarct volume and cell death in transient occlusion of the middle cerebral artery in mice [14–16]. By contrast, some research results indicated that the inhibition of ERK1/2 activity aggravates neuronal injury and accelerates apoptosis [17, 18]. These differences in outcome resulting from MEK1/2 inhibition depend on various reasons, including the nature and severity of injury, the drug dosing, the observing time point, and the cell type

expressing activated ERK1/2. So, further research is required to define the roles of ERK pathway involved in cerebral I/R pathophysiological process with various kinds of models.

CA rat models that closely mimic patient cardiac arrest circumstances may be useful in studying the mechanisms of cerebral ischemia injury as well as the efficacy of neuroprotective drugs [19, 20]. Thus, in the present study we used a rat CA/CPR model to assess antioxidant and antiapoptosis effects of PD treatment by detecting the survival rates, NDS, ROS, MDA, and SOD level and the expression of p-ERK1/2 and ERK as well as the apoptosis-associated proteins caspase-3, Bax, and Bcl-2.

2. Experimental Section

2.1. Animal Preparation. Adult Sprague-Dawley male rats (300–400 g) were obtained from the Experimental Animal Center of Guangxi Medical University (China, Nanning). All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. This study protocol had been examined and approved by the animal ethics committee of Guangxi Medical University.

2.2. Experimental Cardiac Arrest Rat Model. The rat cardiac arrest model was established according to a method reported by Chen et al. [21]. All rats, which were fasted for 12 hours but had free access to water before operation, were injected with pentobarbital sodium (45 μg/g, i.p.) and supplemented by additional doses of 10 μg/g at an hour interval. After endotracheal intubation, cardiac rhythm was monitored with a standard lead II ECG. Two 20-gauge catheters, filled with saline containing 5 IU/mL of sodium heparin, were, respectively, inserted into the left femoral artery for haemodynamic monitoring and the left femoral vein for drug delivery. Pressure transducers were connected to a four-channel physiological recorder (BL-420 E Bio-Systems, Chengdu Technology & Market Co. Ltd., China). After 5-minute baseline EEG and physiologic measurements, the rats were induced CA by alternating current AC (12 V) from a stimulator (Chengdu Technology & Market Co. Ltd., China) through a pacing electrode put in esophagus. CA was defined as a loss of aortic pulsation or aortic pulse pressure <10 mmHg. Five minutes after CA, CPR was initiated with effective ventilation (TV 8 mL/kg, respiration rate 40/min, and PEEP 0 cm H_2O) using a volume-controlled small animal ventilator (DH-150, The Medical Instrument Department of Zhejiang University, China), oxygenation (100% O_2), epinephrine (0.02 mg/kg, i.v.), and sternal chest compression (180 compressions/min). Restoration of spontaneous circulation (ROSC) was defined as an unassisted pulse with a mean arterial pressure (MAP) of ≥50 mmHg for ≥1 min. Mechanical ventilation was withdrawn, when spontaneous breathing occurred at ≥40 breaths per minute for ≥1 min within one hour after ROSC and the blood pressure was stable or increased gradually. The rats were injected intravenously with saline (CA group) or ERK inhibitor PD 0.3 mg/kg in DMSO solution (PD group) immediately after ROSC. After resuscitation, rats were placed alone in a cage with dry bedding and housed in an air-conditioned and peaceful room (room temperature 27°C). Rats had free access to water and food. The rats were divided into two experiments after ROSC, one experiment to examine the survival rates and NDS, and the other one for brain harvest to do biochemical analysis.

2.3. Survival Observation and Neurological Evaluation. Thirty-two rats (n = 13 for CA and PD groups, resp., n = 6 for sham group) were used for the survival observation and neurological evaluation. The survival rate and neurologic deficit evaluation at 12, 24, 48, and 72 h were measured during survival observation without using pain relievers, anesthesia, and euthanasia after ROSC. The neurologic deficit scores (NDS) measures level of arousal, cranial nerve reflexes, motor function, and simple behavioral responses and has a range of 0–80 (Table 2). This experiment was defined as the 72 h NDS score. We prespecified the NDS cut-off for good (NDS ≥ 60) and poor (NDS < 60) outcome which represents a level of neurologic function required for independent function [22].

2.4. Tissue Preparation. Fifty-four rats (n = 24 for CA and PD groups, resp., n = 6 for sham group) were used for harvesting tissues preparation. Animals were sacrificed according to the time points of different groups. Therefore, animals in the sham group were sacrificed at 72 h after the sham operation. The rats were euthanized with intravenous injection of 2 mL of saturated potassium chloride solution and brains were immediately excised. The left brains were fixed in 4% buffered paraformaldehyde for immunostaining and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay (TUNEL). The right brains were used for ROS detection, oxidative stress detection, and western blot.

2.5. ROS Detection. Dihydroethidium (DHE) has been commonly used to detect cytosolic superoxide. DHE can bind irreversibly to the double-stranded DNA, causing amplification of a red fluorescent signal, and appears as punctate nuclear staining indicating ROS production [23]. Harvested brain samples were immediately frozen at −20°C and cut by CM1950 Cryostat Microtome (made in German Leica). Serial, coronal frozen sections (5 μm thick) were fixed on slices before being incubated with DHE (10 μmol/L) in PBS in a light-protected, humidified chamber (37°C, 30 min). Fluorescent images of five fields/section were obtained with Nikon A1 laser confocal microscope equipped with appropriate narrow band filter set with an excitation of 488 nm and an emission range of 574–595 nm. Fields were randomly chosen, avoiding the edges of the sections where autofluorescence was observed. Images were captured by the confocal laser microscope and were set by 20x objective lens at 1024 × 1024 pixels.

2.6. Determination of Indicators of Oxidative Stress. Following harvested brains, the tissues were immediately washed in chilled saline and then were homogenized in ice-cold saline for 20 min to prepare a 10% (w/v) homogenate. The

Table 1: Baseline parameters.

Group	n	BW (g)	HR (beats/min)	SP (mmHg)	DP (mmHg)	MAP (mmHg)	Stimulation duration (s)	CPR duration (s)
Sham	6	355.76 ± 46.21	424.60 ± 33.96	112.37 ± 10.47	91.31 ± 7.51	96.24 ± 7.39	—	—
CA	26	386.36 ± 44.53	412.48 ± 37.14	110.57 ± 9.17	90.74 ± 8.84	98.51 ± 8.87	80.16 ± 9.07	118.18 ± 50.30
PD	26	363.60 ± 47.14	418.58 ± 39.46	111.89 ± 10.66	91.88 ± 9.96	97.53 ± 9.66	82.57 ± 10.20	117.44 ± 48.77

homogenate was centrifuged at 2,500 rpm and 4°C for 15 min. The level of malondialdehyde (MDA), as well as the activities of superoxide dismutase (SOD), in the supernatant was investigated using a microplate reader (1510; Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions provided with the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The assay results were normalized to the protein concentration in each sample and expressed as U/mg protein or nmol/mg protein.

2.7. Western Blot. The prepared brain tissues were weighed and homogenized in 1 : 10 (w/v) ice-cold whole-cell lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) using a glass homogenizer. Soluble proteins were collected and centrifuged at 12,000 ×g for 10 min at 4°C. Tissue total protein concentrations were determined by a BCA Protein assay reagent kit (Beijing TransGen Biotech Co., Ltd.). Tissue total protein (50 μL; p-ERK1/2, ERK1/2, caspase-3, Bax, Bcl-2, and GAPDH) was separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The membrane was blocked with PBST containing 5% nonfat dry milk for 1 h and then incubated overnight at 4°C with the corresponding primary antibodies. The primary antibodies and dilutions were as follows: primary antibodies against phosphorylated ERK1/2 (number 9101, 1 : 1000), ERK 1/2 (number 4695, 1 : 1000), and GAPDH (number 5174, 1 : 1000) were purchased from Cell Signaling Technology (Danvers, MA), and caspase-3 (1 μg/mL, number ab32351, 1 : 1000), Bax (number ab7977; 1 : 1000), and Bcl-2 (number ab7973; 1 : 100) were purchased from Abcam Plc, Cambridge, UK. After washing 3 times with PBST, the membrane was incubated with secondary antibody (goat anti-rabbit IgG, 1 : 10,000; Licor). The membrane was quantified using a western blotting detection system with a Li-cor Odyssey Scanner imaging densitometer, and the results of detected bands were quantified with Multi-Analyst software (Bio-Rad Laboratories).

2.8. Cell Apoptosis Assay. For TUNEL staining, paraffin blocks were cut into 5 μm thickness coronal sections. To detect apoptotic cells, TUNEL staining was performed using an In Situ Cell Death Detection Kit (POD, ROCHE, cat.: 11684817910) according to the manufacturer's protocol. Briefly, the sections were deparaffinized in xylol, rehydrated by successive series of alcohol, washed in phosphate-buffered saline (PBS), and deproteinized (or permeabilized) by proteinase K (20 μg/mL) for 30 min at room temperature. Then, the sections were rinsed and incubated with 3% H_2O_2 for 10 min in the dark to block endogenous peroxidase and rinsed with PBS; then, the sections were incubated in the TUNEL reaction mixture for 60 min at 37°C in light-protected and humidified atmosphere and rinsed with PBS. After that, the sections were rinsed with PBS and 50 μL DAPI (4′,6-diamidino-2-phenylindole) was incubated for 5 min and rinsed with PBS and then all slides were mounted by cover slip and analyzed by fluorescence microscope. TUNEL labeling for cells death was normalized to DAPI staining for all cells. TUNEL controls were performed by incubating slides with 100 μL label solution.

2.9. Double Fluorescent Staining. To clarify the spatial relationship between phospho-ERK1/2 expression and DNA fragmentation, we performed double staining for phospho-ERK1/2 and terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) using a fluorescent method. After deparaffinization and hydration, the sections in 10-mmol/L sodium citrate (pH 6.0) were boiled for 5 minutes at 95°C. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 10 min in the dark. The sections were incubated with PBS containing 10% normal goat serum at room temperature to eliminate any nonspecific binding and were incubated overnight at 4°C with monoclonal antibodies against p-ERK1/2 (1 : 100). Then, the sections were incubated with goat anti-rabbit IgG H&L (Alexa Fluor 647, ab150079) for 60 min. After that, TUNEL reaction mix was applied and incubated for 1 h. Then, the sections were rinsed with PBS and all slides were mounted by cover slip and analyzed by fluorescence microscope. Some sections were single labeled with TUNEL and DAPI for quantification of TUNEL positive cells.

2.10. Statistical Analysis. Values are expressed as mean ± standard error of the mean. All data were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference post hoc test (two-tailed). All statistical analyses were performed using SPSS software (version 6.0). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline Characteristics. As Table 1 showed, there were no significantly different baseline parameters before CA induction among all groups, including body weights (BW), heart rate (HR), systolic pressure (SP), diastolic pressure (DP), and mean arterial pressure (MAP), the duration of

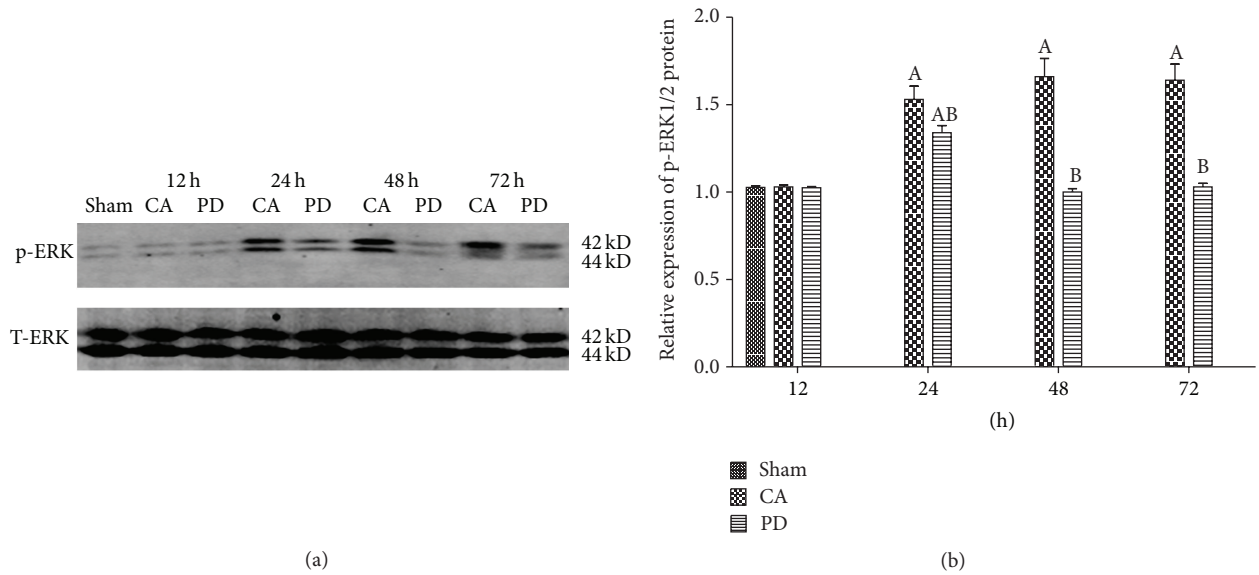

Figure 1: Time-course western blot analysis of p-ERK1/2 in cerebral ischemia reperfusion at 12 h, 24 h, 48 h, and 72 h. (a) Representative western blot. (b) Phosphorylation of ERK1/2 ratios (phosphorylated versus total protein). The phosphorylation of ERK1/2 increased significantly in all rats within 24 h after CA/PCR compared to sham group ($P < 0.05$). Compared to CA group, treatment with PD98059 significantly reduced phosphorylation of ERK1/2 from 48 h to 72 h of PCR, $P < 0.05$; mean ± standard deviation. $n = 6$ for each group (A = $P < 0.05$ versus the sham group; B = $P < 0.05$ versus the CA group).

transesophageal stimulation prior to CA (stimulation duration), and the duration of CPR prior to ROSC (CPR duration) ($P > 0.05$).

3.2. PD Improved Survival Rates and Neurologic Deficit Scores. Survival rates and neurologic deficit scores (NDS) of rats after ROSC were shown in Table 2. All sham rats lived to the observing end point of 72 h. Better survival rates of 12, 24, 48, and 72 h were seen in the PD groups compared with the CA group after ROSC ($P < 0.05$). Rats in the CA and PD groups exhibited a significant neurologic deficit and improvement over time, as compared with the sham group (no neuronal deficit; $P < 0.05$). Deficits were consistently less severe in PD group compared with CA group ($P < 0.05$ at 12, 24, and 48 h).

3.3. PD Decreased Brain ROS. To evaluate the effects of PD on ROS production induced by CA/CPR, the levels of ROS were measured by DHE staining after 12, 24, 48, and 72 h of reperfusion (Table 3). Compared to the sham-operated group, ROS were markedly upregulated ($P < 0.05$) in the CA group at 12, 24, 48, and 72 h of ROSC. Treatment of the rat with PD exerted antioxidant effects as evidenced by a decrease in the ROS levels ($P < 0.05$) after 12, 48, and 72 h of reperfusion while at 24 h of reperfusion treatment with PD showed no effects. Table 3 also indicated that the level of ROS increased at 12 h, reached peak levels at 24 h, and gradually decreased from 48 to 72 h of reperfusion in both CA and PD groups.

3.4. Antioxidant Activity of PD in Rat with Cerebral IR Injury. To evaluate the effects of PD on oxidative stress induced by CA/CPR, the levels of MDA and SOD activity were measured after 12, 24, 48, and 72 h of reperfusion (Table 4). Compared with the sham-operated group, SOD activity decreased significantly ($P < 0.01$) and the MDA level markedly increased ($P < 0.01$) in the CA group at 12, 24, 48, and 72 h of ROSC. However, treatment with PD restored an increase of SOD activity ($P < 0.01$) and a decrease in the MDA concentration ($P < 0.01$) after 12, 48, and 72 h of reperfusion while at 24 h of reperfusion treatment with PD showed no effects.

3.5. PD Decreased ERK1/2 Phosphorylation. We used western blot for analysis of phospho-ERK 1/2 and total ERK 1/2 expression. As shown in Figure 1(a), the bands of phospho-ERK1/2 and ERK1/2 were observed at 42 and 44 kDa in the whole-cell fraction from rat brains. There were no significant differences in p-ERK level between sham group and CA and PD groups at 12 h after ROSC. The phosphorylation of ERK1/2 increased significantly in rats subjected to CA/CPR at 24 h of reperfusion as compared to sham group ($P < 0.05$). The elevating of p-ERK 1/2 was sustained to 72 h in CA group. However, treatment with PD showed markedly decreased phosphorylation of ERK1/2 from 48 h to 72 h of reperfusion as compared to CA group ($P < 0.05$) (Figure 1(b)).

3.6. PD Decreased TUNEL Positive Neurons in Cortex. Figure 2 presented the fluorescent signal of TUNEL and DAPI staining in cortex: TUNEL staining to monitor DNA damage, and DAPI staining to monitor morphological changes of nuclei. Cell apoptotic rate was defined as the percentage of TUNEL staining cells to DAPI staining cells.

Table 2: The survival rate and neurologic deficit scores (NDS) after ROSC.

Group	12 h		24 h		48 h		72 h	
	Survival rate	NDS	Survival rate	NDS	Survival rate	NDS	Survival rate	NDS
Sham	6/6 (100%)	80.00 ± 0 ($n = 6$)	6/6 (100%)	80.00 ± 0 ($n = 6$)	6/6 (100%)	80.00 ± 0 ($n = 6$)	6/6 (100%)	80.00 ± 0 ($n = 6$)
CA	10/13 (76.9%)	47.94 ± 5.83* ($n = 10$)	6/13 (46.2%)*	53.26 ± 2.96* ($n = 6$)	4/13 (30.8%)*	58.62 ± 3.33* ($n = 4$)	3/13 (23.1%)*	73.55 ± 2.15 ($n = 3$)
PD	13/13 (100%)$^{\#}$	62.77 ± 357$^{*\#}$ ($n = 13$)	10/13 (76.9%)$^{\#}$	67.71 ± 3.23$^{*\#}$ ($n = 10$)	8/13 (61.5%)$^{*\#}$	72.46 ± 2.44$^{*\#}$ ($n = 8$)	5/13 (38.5%)*	75.25 ± 1.68 ($n = 5$)

$^{*}P < 0.05$ versus sham group; $^{\#}P < 0.05$ versus CA group ($n = 6$).

Table 3: The ROS level of rat brain after ROSC.

		The average fluorescence intensity of rat brain after ROSC			
Group	N	12 h	24 h	48 h	72 h
Sham	6	387.85 ± 35.54	387.85 ± 35.54	387.85 ± 35.54	387.85 ± 35.54
CA	24	681.31 ± 21.11*	722.27 ± 34.17*	697.38 ± 28.58*	655.33 ± 24.65*
PD	24	371.70 ± 10.30#	390.80 ± 33.50#	386.31 ± 26.24#	376.67 ± 37.71#

* $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to CA group ($n = 6$).

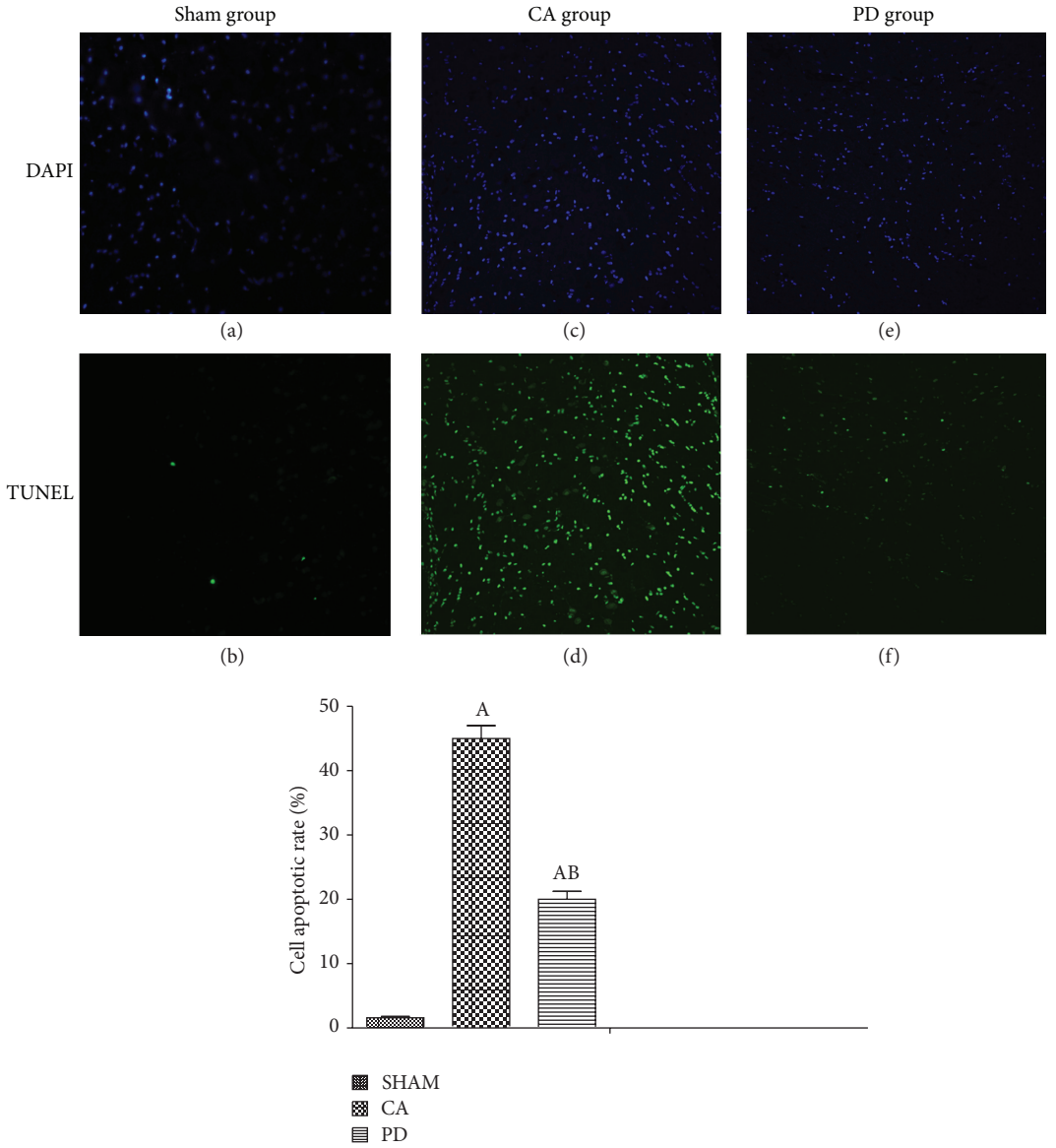

Figure 2: PD98059 decreased neuronal apoptosis in cortical brain. Apoptotic cells were induced after 72 h of CA/CPR. Quantitative analysis of TdT-mediated dUTP-biotin nick end labeling (TUNEL; green) positive cells or 4′,6-diamidino-2-phenylindole (DAPI; blue) to label all cells. Cells death increased significantly in the CA group and PD group as compared with sham group ($P < 0.01$). Treatment with PD98059 (PD group) induced a decreased apoptotic index ($P < 0.01$); mean ± standard deviation; $n = 6$ for each group.

As Figures 2(a) and 2(b) showed, in the sham-operated group, there was a small amount of apoptotic cells in the cortex. Compared to the sham-operated group, the neuronal apoptotic rate was significantly increased at 72 h in rat brain subjected to CA/CPR ($P < 0.01$; Figures 2(c)-2(d) and 2(e)-2(f)). However, the increase in neuronal apoptosis was markedly reduced in the groups treated with PD ($P < 0.01$). These results suggested that the treatment with PD effectively prevented expansion of apoptotic cell death in CA/CPR model.

Table 4: The MDA and SOD activity levels in brain after ROSC.

Group	N	12 h		24 h		48 h		72 h	
		MDA levels (U/mg prot)	SOD activity (nmol/mg prot)	MDA levels (U/mg prot)	SOD activity (nmol/mg prot)	MDA levels (U/mg prot)	SOD activity (nmol/mg prot)	MDA levels (U/mg prot)	SOD activity (nmol/mg prot)
Sham	6	4.67 ± 0.66	120.67 ± 8.96	4.67 ± 0.66	120.67 ± 8.96	4.67 ± 0.66	120.67 ± 8.96	4.67 ± 0.66	120.67 ± 8.96
CA	24	7.47 ± 6.01**	75.16 ± 4.85**	8.38 ± 0.75**	61.65 ± 8.56**	10.19 ± 0.70**	54.13 ± 5.81**	7.50 ± 0.729**	60.67 ± 7.17**
PD	24	5.82 ± 0.80$^{*\#\#}$	97.33 ± 7.02$^{**\#\#}$	8.18 ± 0.84**	70.60 ± 8.47**	5.19 ± 0.62$^{\#\#}$	84.12 ± 7.21$^{**\#\#}$	5.03 ± 0.71$^{\#\#}$	83.24 ± 7.03$^{**\#\#}$

$^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham group; $^{\#\#}P < 0.01$ compared to CA group ($n = 6$).

3.7. Double Labeling with Phospho-ERK Expression and DNA Fragmentation Detected by TUNEL Staining after CA/CPR. To corroborate p-ERK1/2 activation involved in cell death pathways, we examined the coexpression p-ERK1/2/TUNEL by double immunostaining with p-ERK1/2 and TUNEL. Figure 3 demonstrated that the coexpression of the p-ERK1/2 and TUNEL staining is mainly distributed in choroid plexus (Figures 3(a), 3(b), and 3(c)) and in cortex (Figures 3(g), 3(h), and 3(i)) in CA rats at 72 h after ROSC. The average p-ERK1/2/TUNEL copositive cell rate was $57 \pm 7.9\%$ of TUNEL cells. There was no fluorescent signal of p-ERK1/2/TUNEL copositive cell be detected in PD-treated rats in choroid plexus (Figures 3(d), 3(e), and 3(f)) as well as in cortex (Figures 3(j), 3(k), and 3(l)). These results suggested that the phosphorylation of ERK1/2 may be associated with neuronal death pathway in CA/CPR rat model.

3.8. PD Decreased Cleaved Caspase-3. The bands of cleaved caspase-3 were observed at 17 and 19 kDa in the whole-cell fraction from rat brains (Figure 4(a)). There was significant elevating of cleaved caspase-3 level between CA and PD group and sham-operation groups at 12 h after ROSC ($P < 0.05$). The increase of cleaved caspase-3 was sustained to 72 h in CA group. However, treatment with PD decreased significantly the cleaved caspase-3 from 24 h to 72 h of reperfusion as compared to CA group ($P < 0.05$) (Figure 4(b)).

3.9. PD Affects the Expression of Bax and Bcl-2 Protein. Compared with the sham-operated group, the CA group displayed a higher Bax protein level and a lower Bcl-2 protein at 12 h, 24 h, 48 h, and 72 h of ROSC (Figure 5, $P < 0.05$). However, treatment with PD resulted in a significant upregulation in Bcl-2 expression ($P < 0.05$) and a marked downregulation in Bax expression ($P < 0.05$) in rat subjected to CA/CPR. The Bcl-2/Bax ratio was significantly decreased ($P < 0.05$; Figure 5(c)) in CA group compared to the sham-operated group. However, the Bcl-2/Bax ratio increased from 12 to 48 h and returned to approximately normal levels at 72 h of reperfusion in the group treated with PD ($P < 0.05$; Figure 5(c)).

4. Discussion

In the present study, we revealed a neuroprotective effect of PD against cerebral global ischemia. Treatment with PD downregulated the percentage of apoptotic cells and improved survival rate and neurological deficits by decreasing of ROS and ERK as well as apoptotic protein. The mechanism underline PD neuroprotection may be involved in the suppression of the oxidative stress production through the regulation of the expression in ROS, MDA, and SOD activity as well as the suppression of p-ERK and apoptotic biomarkers as evidenced by decreased caspase-3 and Bax and increased Bcl-2 in brain of rat subjected to CA/CPR.

CA rat models that closely mimic patient cardiac arrest circumstances are considered reliable and less invasive to study neurological deficit and the development of pathology in the brain after CA [19, 20]. The survival rates, percentage of apoptotic cells, and NDS are useful to estimate the potency of cerebral drugs in the treatment of postresuscitation brain injury from CA. In the present study, treatment with PD significantly downregulated the percentage of apoptotic cells and improved survival rates and NDS following CA/CPR in a rat model.

Many evidences indicated that excessive ROS production and subsequent oxidative stress play harmful roles during cerebral I/R injury [6, 7]. Overproduction of ROS can inflict direct damage to cellular molecules such as lipid, proteins, and nucleic acids in the ischemic tissue, leading to membrane injury and cell death [8, 24]. Lipid peroxidation is known to be one of the primary pathophysiological mechanisms, which is also implicated in cerebral I/R injury [25]. Under physiological conditions, there is a low concentration of lipid peroxidation in brain tissue. By contrast, under oxidase stress, the cells may produce a high concentration of lipid peroxidation and ROS because the cells induce apoptosis or necrosis programmed cell death [26]. Moreover, the brain tissue is composed of high level of polyunsaturated fatty acids in membrane [27], which makes it very sensitive to lipid peroxidation [26]. As a final product of lipid peroxidation, MDA is one of the most preferred markers for oxidative stress which results in cytotoxic effects and neuronal death [28]. By contrast, SOD is an enzyme present in all oxygen-metabolizing cells, which plays an important role in the maintenance of low concentrations of oxidants and redox homeostasis in tissue through the scavenging of oxidants, preventing harmful ROS generation [29]. Our results indicated that, from 12 h to 72 h of ROSC, brain tissue markedly increased ROS and MDA and decreased SOD activity levels in rat subjected to CA/CPR. However, treatment with PD exerted antioxidant effects as evidenced by downregulation of ROS and MDA and restored upregulation of SOD expression.

Oxidative stress induced cell apoptosis through activation of some cellular signal pathways; for example, H_2O_2 can stimulate the Ras/Raf/ERK pathway by increasing the activation of tyrosine kinase receptors, such as platelet-defined growth factor receptor or EGF receptor [30, 31]. Consistent with this finding, in present study indicated that at 24 h of reperfusion, when the ROS at highest level may be associated with initial increase of ERK phosphorylation. The activation of ERK 1/2 signaling pathway also plays a significant role in endothelial cell injury after oxygen-glucose deprivation through vascular endothelial growth factor (VEGF) [32]. Persistent activation of ERK1/2 induced increased glutamate oxidative toxicity and H_2O_2 generation leads to cortical neuronal cells death [33, 34]. Our results indicate that prolonged activation of ERK from 24 h to 72 h may be involved in microvessel and cortical neuron cell death in rat subjected to CA/CPR (Figure 3). Moreover, coexpression of p-ERK/TUNEL showed that cell death of the microvessels mainly distributes in the choroid plexus of lateral ventricle. The choroid plexus is a free-floating organ located in the roof of the lateral ventricles and constitutes an essential part of the blood brain barrier (BBB) [35]. The BBB plays important roles in the maintenance of central

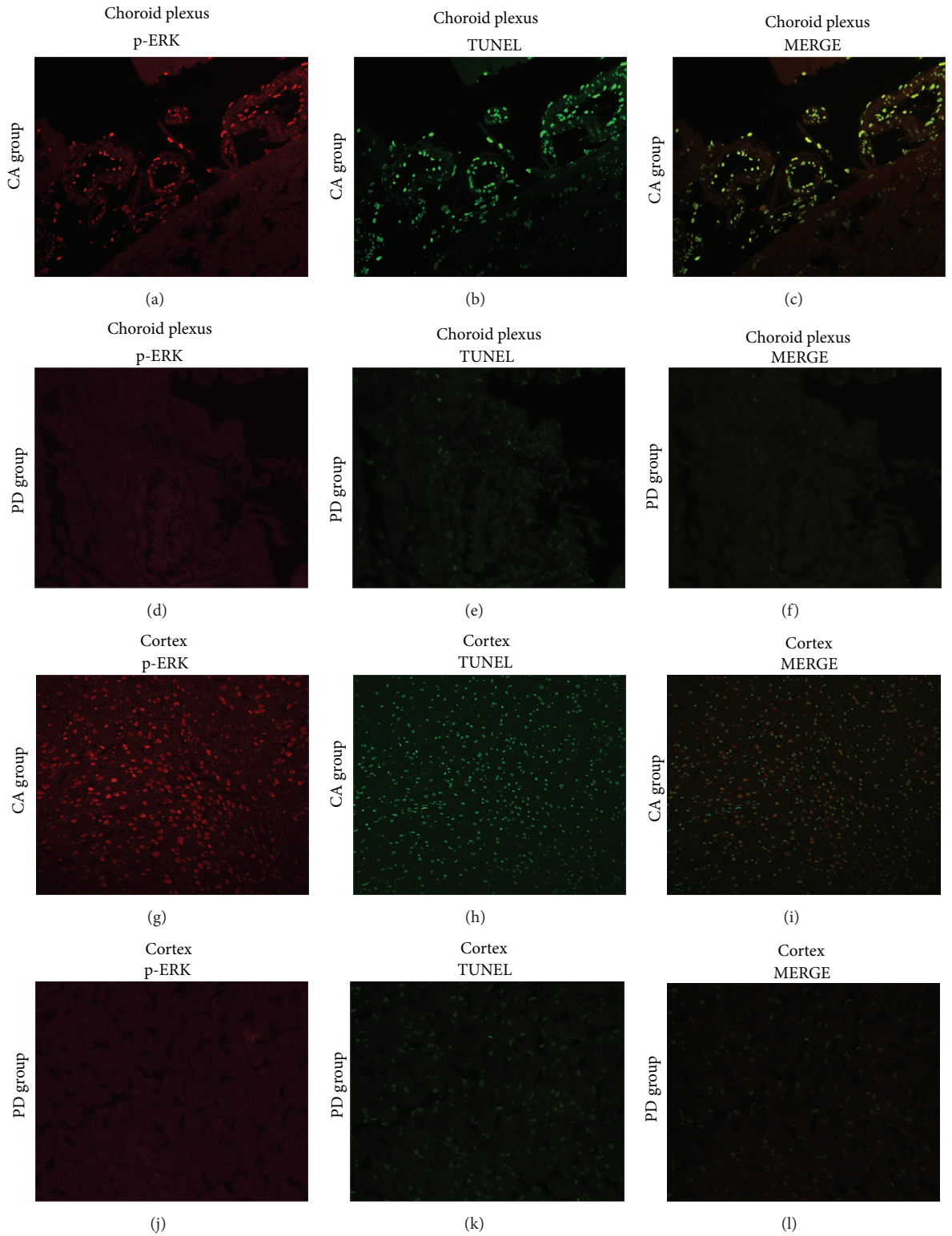

Figure 3: Representative photomicrographs show immunofluorescent staining for phospho-ERK and TUNEL. Phosphorylation of ERK1/2 (red) and TUNEL (green) in rat at 72 h induced CA/CPR. Sections were prepared from choroid plexus and cortex, magnification ×200; mean ± standard deviation; $n = 6$.

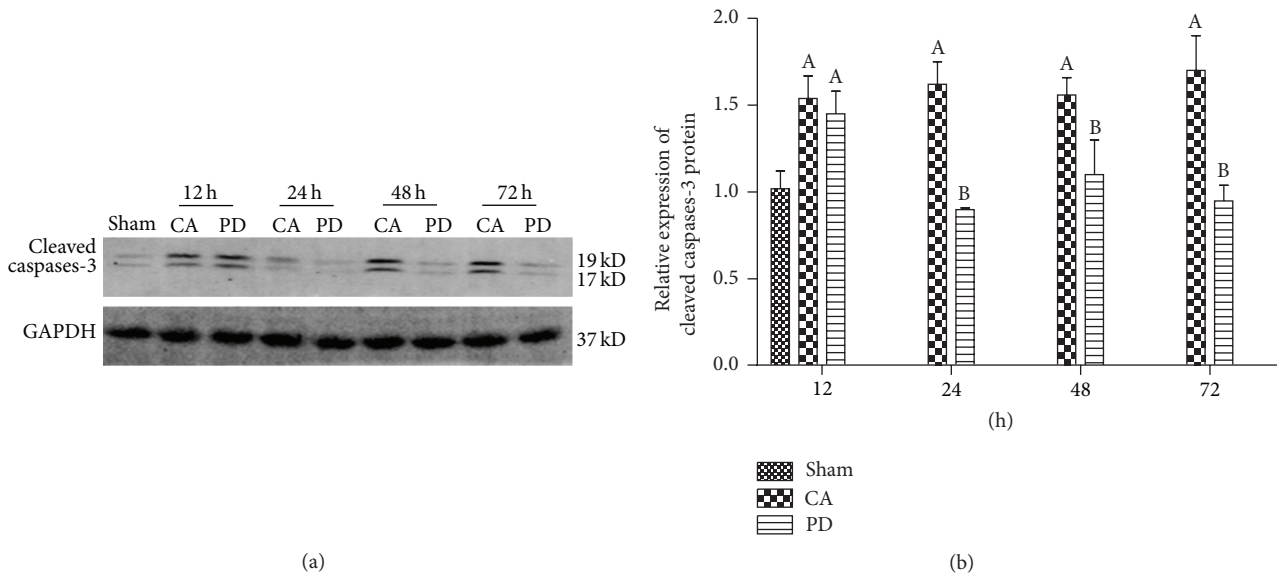

Figure 4: Effects of PD98059 (PD) on the expression of caspase-3. (a) Representative western blot of cleaved caspase-3 in brain of rat subjected to CA/CPR at 12, 24, 48, and 72 h following reperfusion. (b) Cleaved caspase-3 band intensity normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Representative western blot showing the cleaved caspase-3 significantly increased in CA group and PD at 12 h after CA/PCR compared to sham operation ($P < 0.05$). The increase of cleaved caspase-3 was sustained to 72 h of reperfusion in CA. In contrast, treatment with PD98059 decreased significantly caspase-3 from 24 h to 72 h; mean ± standard deviation. $n = 6$ for each group (A = $P < 0.05$ versus the sham group; B = $P < 0.05$ versus the CA group).

nervous system homeostasis; its disruption contributes to oxidative stress and neuronal damage [36]. The inhibition of ERK pathway with PD98096 or U0126 decreases ROS production by inhibition of glutamate toxicity, suggesting this effect is involved in protection of neurovascular system and neuron in cerebral I/R [32, 33]. In fact, the data of our study also indicate that treatment with ERK inhibition exerts neuroprotective effects by suppression of the increase of ROS/p-ERK as well as cells death.

ROS generation during cerebral ischemia reperfusion acts as upstream signaling molecules that initiate cell death [37, 38]. The increase of ROS generation is involved in increased intracellular Ca(2+) concentration and alterations of mitochondrial membrane potential [38]. The subsequent translocation of Bax to the mitochondria results in release of cytochrome c and activation of caspases [39]. Caspases are well-known drivers of apoptotic cell death, cleaving cellular proteins that provide critical links in cell regulatory networks controlling dying cell [40]. Active caspase-3 leads to DNA fragmentation, formation of apoptotic bodies, and neuronal cells death [41]. By contrast, as an antiapoptotic protein, the Bcl-2 expression is maintained at relatively high levels in neuron [42] and helps preserve the mitochondrial integrity by suppressing the release of cytochrome c [43]. Bcl-2 overexpression in neurons reduces caspase-3 activation and rescues cerebellar degeneration [44]. Moreover, high level of ROS can regulate cell death by decreasing expression of Bcl-2 [45]. Thus, inhibition of Bcl-2 expression was known to induce death of a variety of neuronal cell lines [42]. In this study, we found that brain tissue significantly increased ROS and Bax and decreased Bcl-2 and Bcl-2/Bax ratio in rat subjected to CA/CPR. The results which may explain the increased number of apoptotic neurons in rat induced CA/CPR. Treatment with PD reduced these effects. Taken together, we suggest that PD downregulated Bax and caspase-3 and upregulated Bcl-2 may be associated with reducing ROS generation.

5. Conclusions

In conclusion, the findings of present study show that ROS and ERK 1/2 signaling pathway play a significant role in cerebral injury after CA/CPR. ERK inhibitor can be considered to explore for the treatment of brain injury resulting from cardiac arrest.

Conflict of Interests

The authors have no conflict of interests to disclose.

Authors' Contribution

Phuong Anh Nguyen Thi and Meng-Hua Chen contributed equally to this work.

Acknowledgment

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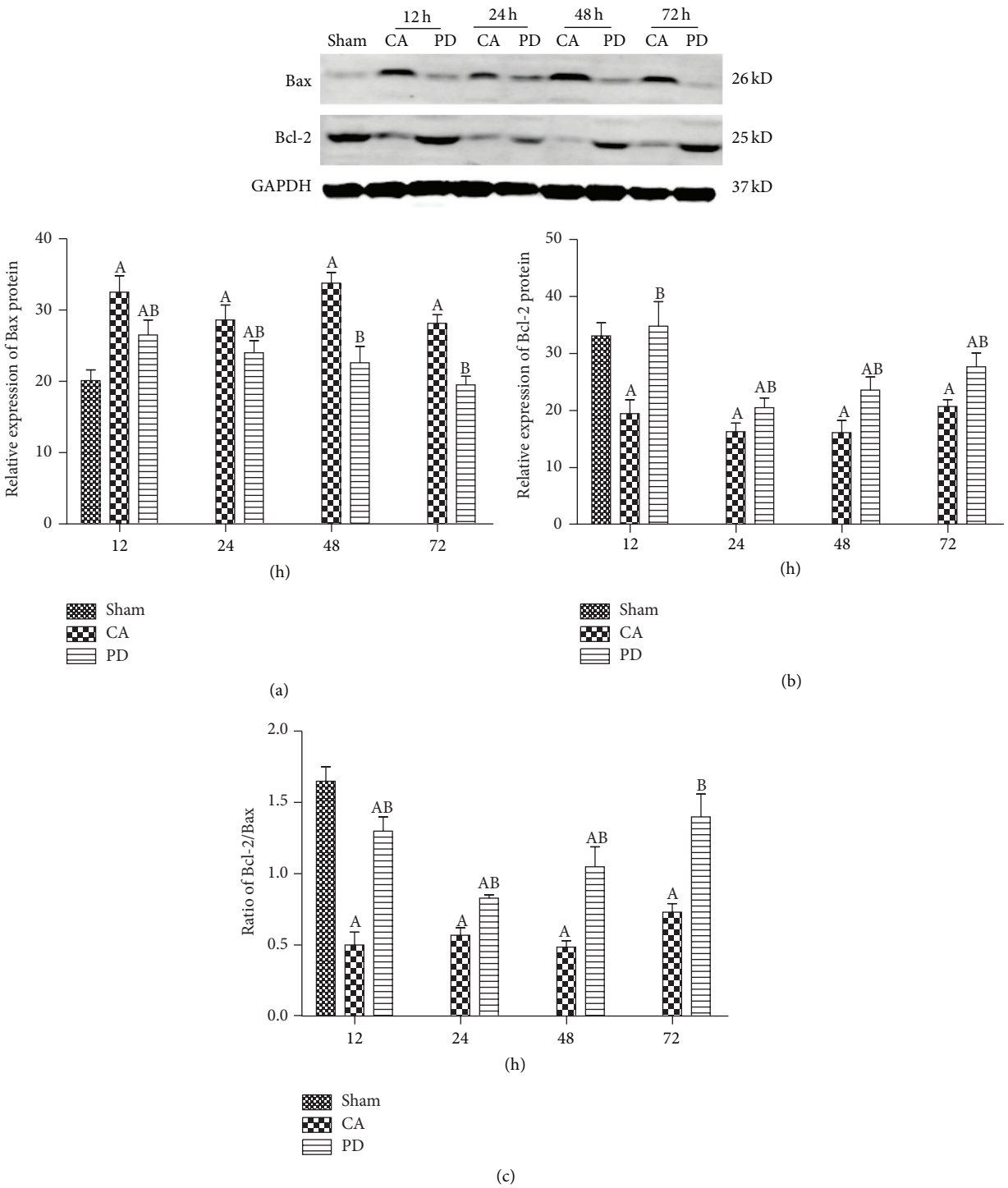

Figure 5: Effects of PD98059 (PD) on the expression of Bax and Bcl-2. (a) Representative western blot of Bax and Bcl-2 in brain of rat subjected to CA/CPR at 12, 24, 48, and 72 h following reperfusion. (b) Bax and Bcl-2 bands intensity normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH); mean ± standard deviation; $n = 6$ for each group (A = $P < 0.05$ versus the sham group; B = $P < 0.05$ versus the CA group).

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Research Article

Tanshinol Rescues the Impaired Bone Formation Elicited by Glucocorticoid Involved in KLF15 Pathway

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Decreased bone formation is responsible for the pathogenesis of glucocorticoid- (GC-) induced osteoporosis (GIO), while the mechanism remains to be elucidated. The aim was to investigate how natural antioxidant tanshinol attenuates oxidative stress and rescues impaired bone formation elicited by GC in Sprague-Dawley rats and in C2C12 cells and/or MC3T3-E1 cells. The results showed that tanshinol prevented bone loss and decreased biomechanical characteristics and suppressed reduction of biomarkers related to osteogenesis in GIO rats. Further study revealed that tanshinol reversed decrease of transcription activity of Osterix-luc and rescued impairment of osteoblastic differentiation and bone formation involved in induction of *KLF15* mRNA. Meanwhile, tanshinol diminished inhibition of protein expression of β-catenin and Tcf4 and transcription activity of Tcf4-luc induced by GC, especially under conditions of KLF siRNA *in vitro*. Additionally, tanshinol attenuated increase of reactive oxygen species (ROS) generation, phosphorylation of $p66^{Shc}$ expression, TUNEL-positive cells, and caspase-3 activity elicited by KLF15 under conditions of GC. Taken together, the present findings suggest that tanshinol attenuated the decrease of bone formation and bone mass and bone quality elicited by GC involved in KLF15/Wnt signaling transduction and counteracted GC-evoked oxidative stress and subsequent cell apoptosis involved in KLF15/$p66^{Shc}$ pathway cascade.

1. Introduction

It is well known that long-term administration of excessive glucocorticoid (GC) leads to glucocorticoid-induced osteoporosis (GIO), a vital risk factor of the increase in the incidence of bone fracture [1]. Bone metabolism disorder has been identified to play a significant vital role in the pathogenesis of GIO [2]. To date, the therapeutic strategy of GIO relies on clinical agents similar to those used for the treatment of postmenopausal osteoporosis, which is distinguished clinically from GIO characterized by the impairment of bone formation [3]. Consequently, most of these drugs for the treatment of GIO show diverse limitations and side effects. Urgently, raising focus on the new findings of bone metabolism related to GC may be beneficial for the development of a novel therapeutic approach of the prevention and treatment of GIO.

Increasing documents demonstrated that oxidative stress triggered by excessive reactive oxygen species (ROS) generation elicits a series of deleterious events in skeletal metabolism, ultimately contributing to the development and progression of osteoporosis [4, 5]. Profiles of genomics analysis showed that varied genes related to oxidative stress are changed in human's osteoblasts exposed to dexamethasone (Dex) [6]. Moreover, investigators have unraveled that Dex can directly or indirectly induce oxidative stress through either inhibition of antioxidant activities or induction of excessive production of ROS [5, 7]. Generally, bone formation needs the vast majority of mature osteoblasts differentiated from preosteoblasts for which Sp7/Osterix is required, while

multipotential mesenchymal progenitors differentiate into preosteoblasts for which Runx2/Cbfa1 is required [8]. However, osteoblast is susceptible to oxidative stress, which can cause inhibition of osteoblastic differentiation and increase of cell apoptosis, resulting in impairment of bone formation [9, 10]. Collectively, imbalance of bone metabolism may substantially contribute to bone loss under oxidative stress elicited by GC.

As one member of the shcA family, $p66^{Shc}$ can be activated by phosphorylation of serine 36 in response to a variety of stimuli, which further increases intracellular ROS [11]. The previous report in osteoblasts illustrated substantially that oxidative stress elicited by GC suppresses Wnt signaling, an essential stimulus for osteoblastogenesis, resulting in bone loss [5]. In addition, Kruppel-like factor (KLF) 15, a recently identified glucocorticoid receptor (GR) target gene, is one type of the family of zinc finger-containing transcription factors related to diverse cellular processes including regulation of cell differentiation, angiogenesis, and stem cell fate [12]. Interestingly, KLF15 as a central regulator of stress response interacts with components of the Wnt pathway, resulting in inhibition of β-catenin/Tcf-transcriptional activity in cardiac cells [13]. Although Lef/Tcfs mediate canonical Wnt/β-catenin signaling in various cell types, Tcf4 are mainly expressed in osteoblasts [14]. Recent evidence showed that expression of KLF15 is increased in osteoblasts exposed to Dex [15]. However, regulatory mechanisms in the downstream of KLF15 pathways in the process of bone metabolism are to be elucidated. Collectively, we ask whether activation of KLF15 elicited by GC causes $p66^{Sch}$-mediated oxidative stress and subsequently promotes cell apoptosis and simultaneously affects regulation of Wnt/Tcf4 signaling of skeletal tissue in the GIO model.

Generally, antioxidants have been considered to have beneficial influences on oxidative stress-associated diseases. Administration of antioxidant was ascertained to exhibit an inhibitory effect on ovariectomy-induced bone loss in rodent model [16]. Previous findings revealed that $D(+)\beta$-3,4-dihydroxyphenyl lactic acid (tanshinol, or named Danshensu) isolated from *Salvia miltiorrhiza* Bunge exerted inhibitory influence on oxidative stress [17]. The previous studies in our team indicated that tanshinol stimulates osteogenesis and depresses adipogenesis, exhibiting a protective action on bone formation in GC treated rats and on bone marrow stromal cells (MSC) exposed to excessive GC [18, 19]. Currently, our previous data confirmed that tanshinol attenuates suppression of osteoblastic differentiation induced by oxidative stress via Wnt/FoxO3a signaling pathway in C2C12 cells and MC3T3-E1 cells, in line with positive control resveratrol, a well-known antioxidant containing polyphenolic acid structure similar to tanshinol [20]. However, the exact signaling mechanism by which tanshinol attenuates impaired bone formation induced by GC has not yet been investigated. Additionally, varied preparation of complex prescription to prevent and treat cardiovascular diseases contains tanshinol, as principal active ingredient in Traditional Chinese Medicine [21]. Consequently, tanshinol may be developed as a potential candidate for prevention and/or treatment of GIO.

Based on the above lines of evidence, in this work presented herein, we will investigate *in vivo* and *in vitro* the notion that regulation of KLF15 pathway cascade may be a new understanding of the mechanisms involved in the pathogenesis of GIO. Meanwhile, we will confirm our hypothesis that tanshinol may exert a protective impact on bone mass and bone strength under oxidative stress elicited by GC and that tanshinol may stimulate regulation of KLF15 pathway cascade, contributing to suppression of oxidative stress and stimulation of bone formation.

2. Materials and Methods

2.1. Animal Experiments. Four-month-old female Sprague-Dawley rats (200–250 g, $n = 32$) were purchased from the Center of Experiment Animal of Sun Yat-Sen University Ltd., China. Certificate of quality was SCXK (YUE) 2012-0112. The animals were housed in Guangdong Medical College in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of Guangdong Laboratory Animal Monitoring Institute under the National Laboratory Animal Monitoring Institute of China. All experimental protocols were approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical College, Zhanjiang, China. Permit number was SYXK (YUE) 2008-0007. All animals were fed with standard chow and had free access to water at optimal temperature ranging from 24°C to 26°C with a humidity level of 70% and a 12-hour light-dark cycle. The animals were randomly assigned to the following four groups ($n = 8$ for each group): Con, standard chow and distilled water; GC, 5 mg prednisone acetate/kg·d; Tan, GC + 16 mg tanshinol/kg·d; Res, GC + 5 mg resveratrol/kg·d. The rats in every group were treated with prednisone acetate in the morning and with other drugs in the afternoon by intragastric administration once a day for 14 weeks. All rats were injected subcutaneously with calcein (10 mg/kg, Sigma Chemical Co., St. Louis, MO) on days 13 and 14 and days 3 and 4 before sacrifice.

2.2. Sample Collection and Applications. All rats were sacrificed by cardiac puncture under anesthesia with peritoneal injection of sodium pentobarbital (1.5 $mg \cdot kg^{-1}$ intraperitoneally, Sigma Chemical Co., St. Louis, MO) at the experimental endpoint. Serum was collected by centrifugation for biochemical assays. The right femur was evaluated for the measurements of bone biomechanical characteristics and bone microarchitecture. The proximal metaphysis of right tibia was subjected to undecalcified section for bone histomorphometry. The left femur was used to prepare decalcified section for TUNEL analysis. Bone marrow cells flushed from the left tibia were prepared to measure oxidative stress level as previous method [5]. The left tibia and the 6th lumbar vertebra (LV6) were collected to detect genes expression and proteins level.

2.3. Structural and Histological Bone Measurement. Bone trabecular microarchitecture was assessed in the right proximal femur by Micro-CT (SCANCO vivaCT40, Bassersdorf,

Switzerland). Briefly, the regions of cancellous bones to be scanned (18 μm/slice) were 1–4 mm distal to the growth plate-epiphyseal junctions. After reconstruction, the following parameters were measured: BV/TV, Tb.N, Tb.Sp, and Tb.Th. For the histomorphometric analysis, the proximal metaphysis of right tibia was fixed in 10% phosphate buffered formalin for 24 h, dehydrated in an ascending ethanol series, and embedded undecalcified in methyl methacrylate. The above tissues were cut into 5 mm sections for von Kossa staining to observe trabecular architectural property and 9 mm sections unstained for assessing the fluorescence labels to analyze bone formation indices such as %L.Pm, MAR, and BFR/TV using the two fluorescent labels. Histomorphometric analysis was performed with the Osteomeasure software (OsteoMetrics, Decatur, GA, USA).

2.4. Analysis of Serum Markers. BAP and OCN, as serum markers of bone formation, and OPG, sRANKL, TRAP5b, and OSCAR, as the markers of bone resorption, were measured in rats using commercially available ELISAs (Westang Bio-Tech, Shanghai, China).

2.5. Three-Point Bending Test. Mechanical strength of lone bone was determined by a three-point bending test using the testing machinery (MTS, Eden Prairie, Minnesota, USA). The right femurs removed from −20°C were thawed at room temperature and tested with a 1 mm indenter at a speed of 2 mm/minute with a 15 mm span (L). From the load-deformation curve, fracture load (N), elastic load (N), bending energy (N × mm), and stiffness coefficient (N × mm^2) were obtained by virtue of measurement and calculation.

2.6. Cell Culture and Osteoblastic Differentiation Assay. The pluripotent mesenchymal precursor C2C12 cells and pre-osteoblastic MC3T3-E1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). For osteoblastic differentiation, MC3T3-E1 calvarial cells were cultured in α-MEM (Gibco BRL, Carlsbad, CA, USA), whereas C2C12 cells were cultured in DMEM (Gibco BRL) containing BMP-2 (100 ng/mL). The osteogenic-induced culture medium was replaced every alternate day. MC3T3-E1 cells and C2C12 cells were maintained in α-MEM (Gibco BRL) or DMEM (Gibco BRL) supplemented with 10% FBS, respectively. For determination of ALP activity, cells were stained at day 7 using the BCIP/NBT color development substrate (Nanjing Jiancheng Biotech, China). The stained cellular images were acquired by Eclipse E800 microscope (Nikon, Tokyo, Japan). For analysis of activity of bone formation, MC3T3-E1 cells were stained at day 21 with 2% Alizarin Red S (ARS, pH = 4.2) (Sigma-Aldrich, St. Louis, USA). The images were photographed by microscope (Nikon). The bound ARS was dissolved in a 10% cetylpyridinium chloride monohydrate (CPC) solution (pH 7.0). Absorbance was measured at 545 nm using a microplate reader.

2.7. RNA Interference Experiments (si-KLF15) and Overexpression Assay (Ad-KLF15). C2C12 cells or MC3T3-E1 cells that reached 80–90% confluence were transfected with equal amounts of expression vectors encoding FITC-labeled scrambled sequence control (scrambled, negative control) or gene-specific siRNA of KLF15 (Genepharma, Shanghai, China) in Opti-MEM medium (Invitrogen) using 3 μL Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer's instruction. Cells were transfected with equal amounts of expression adenovirus vectors encoding exogenous KLF15 (Ad-KLF15), mock (empty vectors, negative control), or GFP (Genechem, Shanghai, China) using Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. After 48 h, transfected cells were induced to osteoblastic differentiation in DMEM containing 5% serum and the medium was replaced periodically as described above. For measurement of mRNA level or luciferase activity, transfected cells were treated with agents as indicated in the Results. Transfection efficiency was monitored on the next day by fluorescence microscopy in the cells transfected with the reporter GFP vectors in serum-free medium. When the transfection efficiency rate was >80%, cells could be used for the following experiments.

2.8. Luciferase Assay. The MC3T3-E1 cells and C2C12 cells were plated in 96-well plates at a density of 2×10^4 cells/cm^2 in triplicate. The cDNAs of Tcf4-luc and Osterix-luc were purchased from Qiagen (Frederick, MD, USA) and transfected using a dual firefly-renilla luciferase reporter assay. After 16 hours, transfected cells were refreshed with fresh media and cultured for an additional 8 hours. Subsequently, cells were serum-starved by culturing in the presence of 2% FBS for 4 hours and treated with or without tanshinol for 1 hour, followed by vehicle control (Con), Dex, and/or related reagents, at indicated concentrations for 24 hours. Then, the cells were lysed with lysis buffer (Promega, Madison, WI, USA) and firefly and renilla luciferase activity using the Dual-Glo Luciferase Assay kit (Promega). The RLU was determined by the ratio of renilla luciferase signal intensity to that of firefly luciferase for normalization.

2.9. Quantitative RT-PCR Detection. RNA was extracted from the left tibiae by crushing them in liquid nitrogen and collecting the bone powder in Trifast (Peqlab, Erlangen, Germany). The mononuclear cell fraction was lysed in Trifast (Peqlab). RNA from both C2C12 cells and MC3T3-E1 cells was isolated using Trifast (Peqlab) after washing twice with PBS. RNA isolation was performed according to the manufacturer's protocol. Five hundred nanograms (500 ng) of RNA was reverse-transcribed using Superscript II (Invitrogen, Darmstadt, Germany) and subsequently used for SYBR green-based real-time PCR reactions using a standard protocol (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are summarized in Table 1. Complementary DNA (cDNA) was synthesized, and qRT-PCR was performed on a Stratagene Mx3005P QPCR System (La Jolla, CA, USA). PCR results were analyzed using Opticon Monitor Analysis 2.0 software (Bio-Rad Laboratories, Hercules, CA, USA). Relative mRNA expression was quantified by subtracting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) threshold cycle (C_t) value from C_t value of the genes of

Table 1: Primers sequences for real-time PCR analyses of gene expression.

Genes	Species	Primer sequences	
		Forward primer (5′-3′)	Reverse primer (5′-3′)
Axin2	Rat	AGTCAGCAGAGGGACAGGA	CTTGGAGTGCGTGGACACTA
Col1α1	Rat	TGACCTCAAGATGTGCCACT	GGGAGTTTCCATGAAGCCAC
OSCAR	Rat	CTGGTCATCAGTTCCGAAGG	CTATGATGCCCAAGCAGATG
Tcf4	Mouse	CCAATCACGACAGGAGGATT	TGATGCTTTGAGCTGTGGAG
ALP	Mouse	AACCCAGACACAAGCATTCC	GCCTTTGAGGTTTTTGGTCA
OPN	Mouse	TCTCCTTGCGCCACAGAATG	TCGGTACTGGTGTACCTGCT
OCN	Mouse	CCATGAGGACCCTCTCTCTGC	AAACGGTGGTGCCATAGATGC
Runx2	Rat	ATTCCTGTAGATCCGAGCACCA	TACCTCTCCGAGGGCTACAACC
	Mouse	TACCAGCCACCGAGACCAA	AGAGGCTGTTTGACGCCATAG
Osterix	Rat	AGCTCTTCTGACTGCCTGCCTAGT	TTGGGCTTATAGACATCTTGGGGT
	Mouse	AGCGACCACTTGAGCAAACAT	GCGGCTGATTGGCTTCTTCT
KLF15	Rat	TCCTCCAACTTGAACCTGTC	CTTGGTGTACATCTTGCTGC
	Mouse	CAAGAGCAGCCACCTCAAG	GACACTGGTACTGCTTCACA
GAPDH	Rat	CCATCATGAAGTGTGACGTG	ACATCTGCTGGAAGGTGGAC
	Mouse	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGtcATGAGCC

interest and expressed as $2^{-\Delta\Delta C_t}$, as described by the protocol of the manufacturer.

2.10. Western Blotting Analysis. For Western blotting, cells were lysed in RIPA buffer containing complete protease inhibitor cocktail. The phosphorylation status of $p66^{Shc}$ was analyzed by immunoblotting in sixth lumbar vertebra or cultured cell lysates, as described previously [22], using a monoclonal antibody recognizing Ser36 phosphorylated $p66^{Shc}$ (Abcam). Protein levels of $p66^{Shc}$ were analyzed using a rabbit polyclonal antibody recognizing Ser36 phosphorylated $p66^{Shc}$ and $p66^{Shc}$ (Abcam). The antibodies recognizing β-catenin and Tcf4 were purchased from Cell Signaling Technology. The protein expression was monitored by the measurement of chemiluminescence alterations using Image Station 2000 MM (Eastman Kodak, Rochester, NY, USA).

2.11. Other Assays. Intracellular ROS were quantified with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) dye, using bone marrow cells flushed and washed with PBS from tibia, or cultured MC3T3-E1 cells as in previous publication [5]. Glutathione reductase activity (GSR) was assayed with a kit (Beyotime Biotech., Haimen, Jiangsu, China). Apoptosis in sections was measured by TUNEL staining, whereas apoptosis in cultured cells was determined by measuring caspase-3 activity by cleavage of the fluorogenic substrate Ac-DEVD-AFC (Beyotime Biotech., Haimen, Jiangsu, China), as described previously [5].

2.12. Statistical Analysis. ANOVA (SPSS 13.0) was used to detect effects of various treatments after establishing equivalency of variances and the notion that the data were normally distributed. Samples were considered normally distributed if $P > 0.05$. Heterogeneity of variance was accepted if $P > 0.05$, and LSD method was used to perform appropriate pairwise comparisons of treatment groups. Unless otherwise stated, the data are presented as the mean ± standard deviation (SD), and the values were considered statistically significant at $P < 0.05$.

3. Results

3.1. Tanshinol Prevents Bone Loss and Decreased Biomechanical Characteristics in GIO Rats. In order to assess the influence of tanshinol on bone architecture and bone quality, we firstly measured structural parameters of trabecular bone using microcomputed tomography (Micro-CT) machine and reconstructed a 3D image of the trabeculae. As is shown in Figures 1(a) and 1(b), rats exposed to GC exhibited impaired bone architecture, as documented by a decrease in the bone volume/tissue volume (BV/TV) and the trabecular thickness (Tb.Th), and an increase of the trabecular separation (Tb.Sp). These data were confirmed by cancellous bone histomorphometric analyses (Figure 1(c)). Tanshinol, however, exerted a significant protective action on bone architecture in GIO rats. To further determine whether the treatment with tanshinol improved biomechanical properties of bone tissue, a three-point bending test was performed on femoral shaft samples. Compared to vehicle controls, the treatment of GC led to the significant reduction of fracture load and bending energy and a trend toward decline in elastic load and stiffness (Figure 1(d)). Expectedly, tanshinol attenuated the deleterious effects of GC on bone biomechanical characteristics. Similar results were obtained in rats exposed to resveratrol. Collectively, tanshinol exhibits a preventive action on bone mass and bone strength, in accordance with protective effect of resveratrol on bone tissue in GIO rats.

3.2. Tanshinol Reverses the Imbalance between Bone Formation and Bone Resorption. We next set out to investigate the

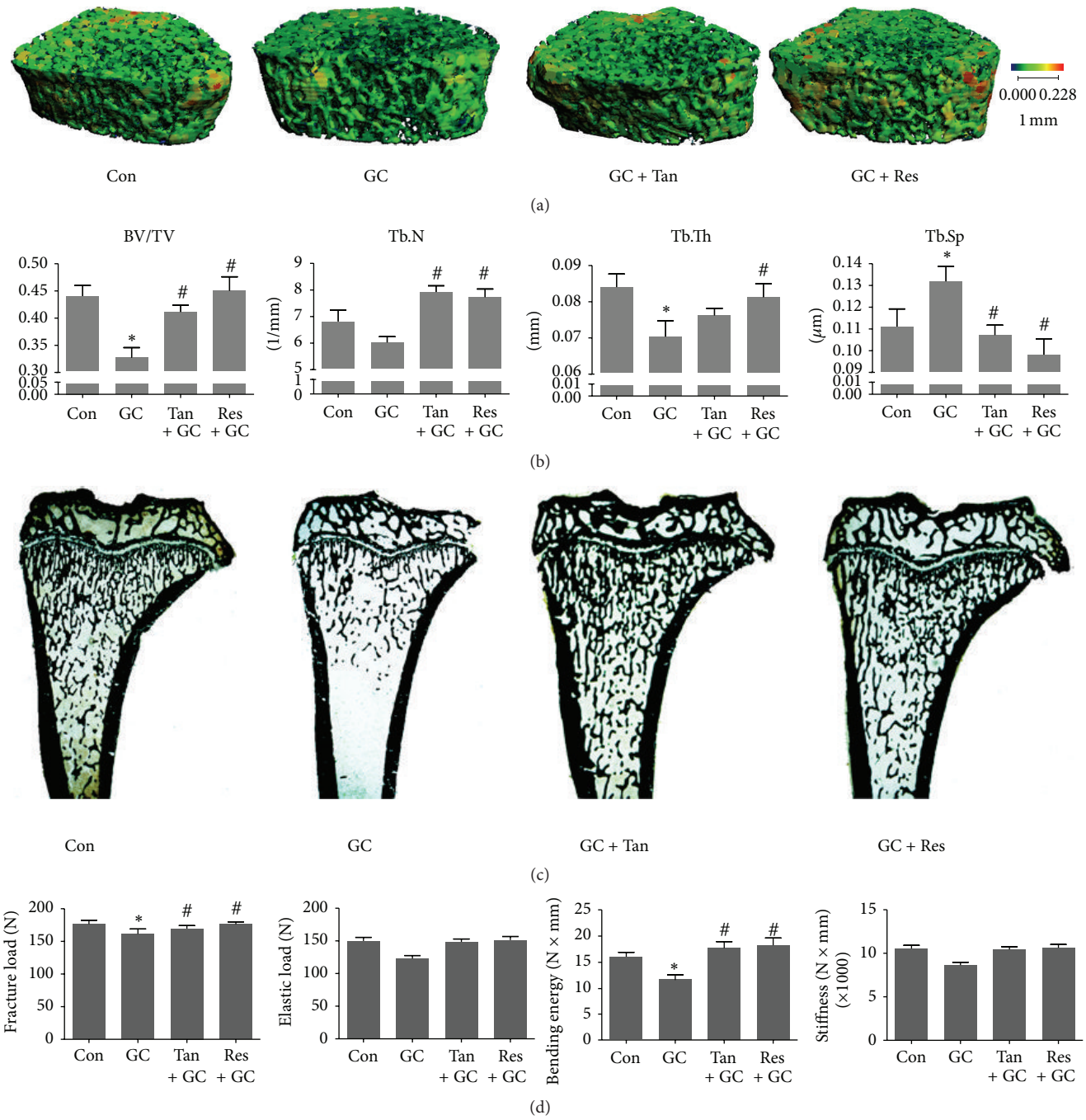

FIGURE 1: Tanshinol maintains bone microarchitecture and biomechanical properties. Rats were treated with distilled water (Con), prednisone (GC, 5 mg/kg·d), GC plus tanshinol (GC + Tan, 16 mg/kg·d), and GC plus resveratrol (GC + Res, 5 mg/kg·d) for 14 weeks. The following measurements were carried out. (a) Micro-CT reconstruction of the trabecular part of proximal femur of rats. (b) Microarchitectural parameters of proximal femoral spongiosa were measured by Micro-CT machine. (c) von Kossa staining of undecalcified sections of proximal tibia spongiosa of rats. (d) Biomechanics characteristics of femur were determined by three-point bending assay. Data are given as mean ± SD ($n = 8$). $^{*}P < 0.05$ versus normal control (Con); $^{\#}P < 0.05$ versus GC treatment (GC).

protective effect of tanshinol on bone tissue via improvement of metabolic imbalance between bone formation and bone resorption elicited by long-term excessive GC in rats. Dynamic alteration of bone formation was indicated by calcein double-labeled trabeculae in the distal femur. As was shown in the representative histologic images, larger space between the calcein labels and stronger fluorescence intensity were observed in bone sections of rats in Con, GC + Tan, and GC + Res groups than those of GC group (Figure 2(a)). The evidence of decreased bone formation was also demonstrated by a reduced percent labeled perimeter (%P-L.Pm) and the mineral apposition rate (MAR), as well as

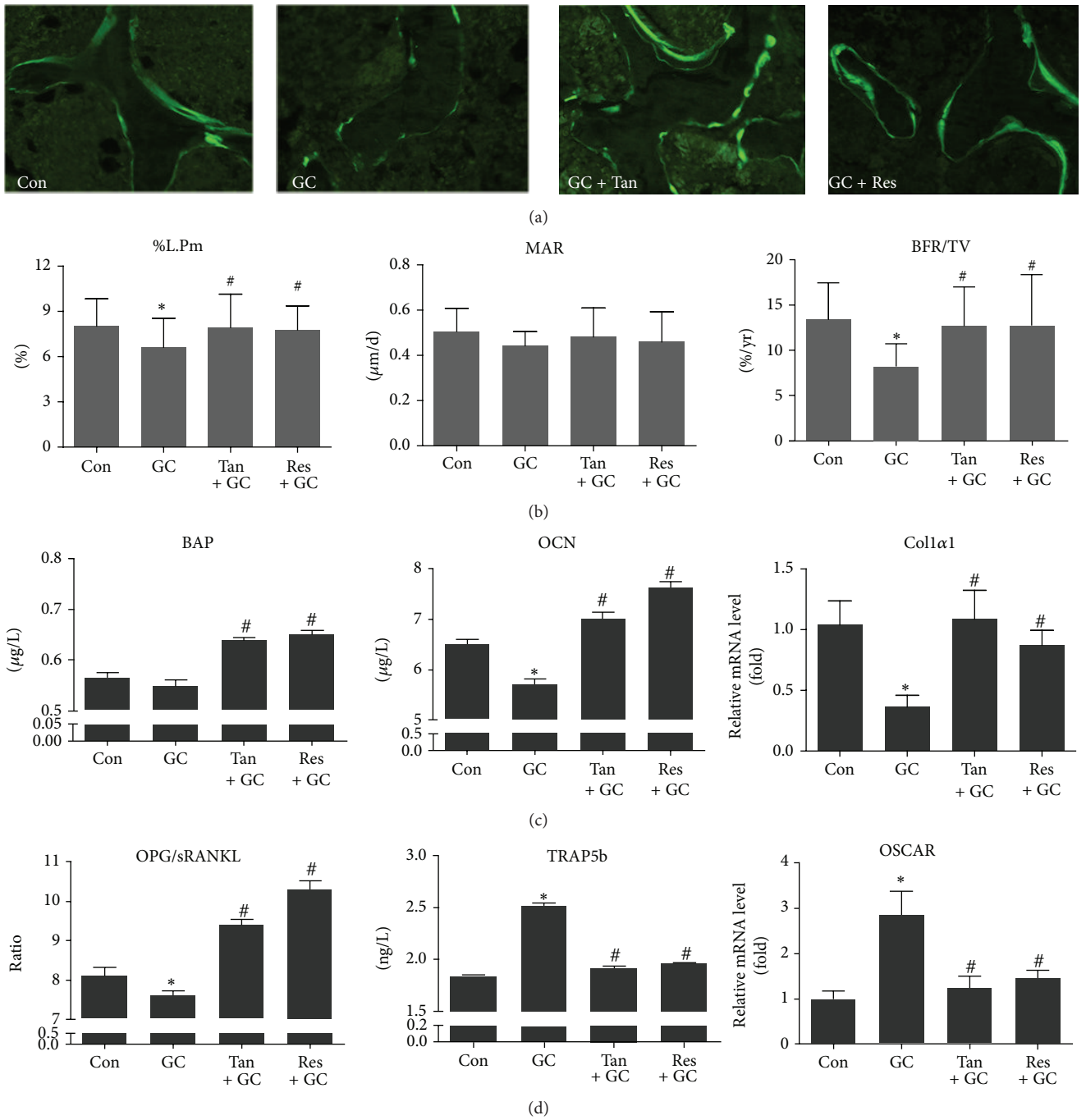

FIGURE 2: Tanshinol reverses the imbalance between bone formation and bone resorption. Procedures of treatment in rats were carried out as described in Figure 1, and determinations were executed as follows. (a) Fluorescent micrographs of dual calcein labeling in tibia of rats. (b) Histomorphometric quantitative analysis of dynamic parameters of %L.Pm, MAR, and BFR/TV used as key indicators of bone-forming capacity in tibia spongiosa of rats. (c) Biomarkers of the bone formation including serum BAP, serum OCN, and *Col1α1* mRNA level were measured using ELISA assay and qRT-PCR, respectively. (d) Ratio of serum OPG/RANKL reflecting changes of bone turnover and the biomarkers related to bone resorption including serum TRAP5b and *OSCAR* mRNA level of bone tissue. Data are given as mean ± SD ($n = 8$). $^{*}P < 0.05$ versus normal control (Con); $^{\#}P < 0.05$ versus GC treatment (GC).

the bone formation rate (BFR/TV). MAR as an index of the capacity of individual osteoblasts to form bone mineral was about 34% decreased in GIO rats compared with control rats. Meanwhile, BFR/TV determined by the number and function of osteoblasts exhibited an approximate 44% decrease in GIO rats compared with control rats (Figure 2(b)). In line with the histological data, the deleterious effects of GC on bone metabolism were also consolidated by the alterations

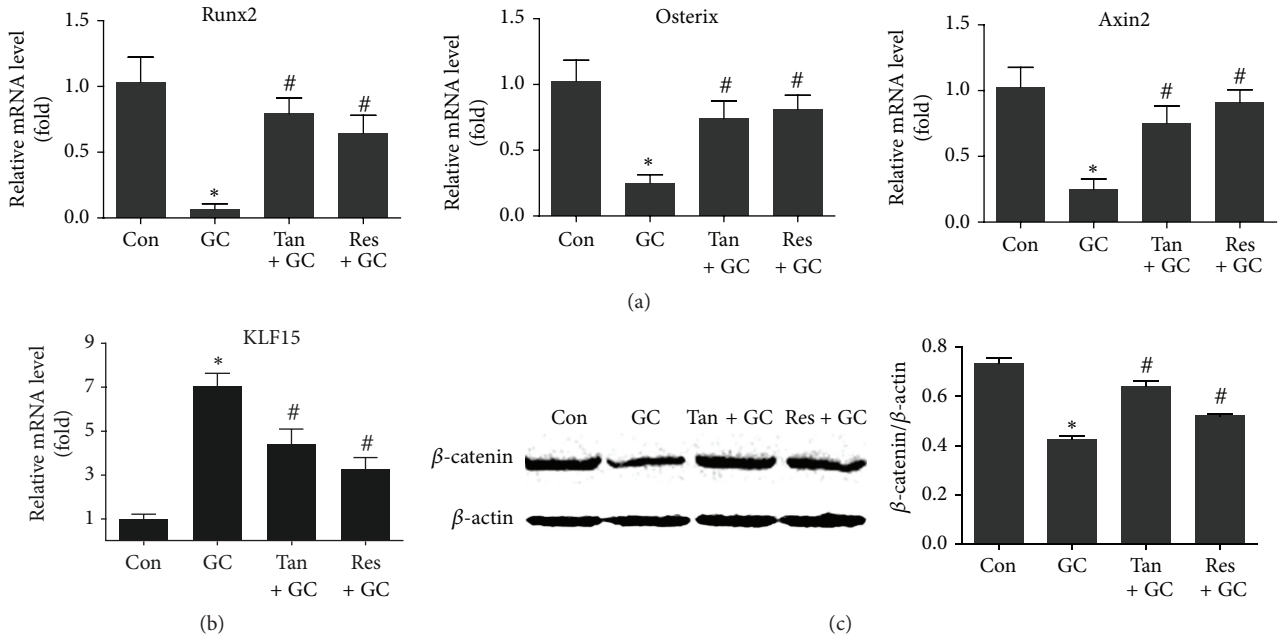

Figure 3: Tanshinol protects osteoblastic differentiation against GC involved in Wnt signaling and KLF15 transcriptional factor. Rats were treated as in Figure 1, and measurements were made as follows. (a) mRNA levels of *Runx2* gene and *Osterix* gene which contribute to osteoblast differentiation and of *Axin2* gene (an indicator of Wnt pathway) were determined by qRT-PCR assay in long bone of rats. (b) mRNA levels of *KLF15* gene were detected by qRT-PCR assay in long bone of rats. (c) Expression of β-catenin protein (a key molecule of canonical Wnt signaling) in the left tibia was measured by Western blot method. Representative figure was shown on the left panel, and quantification is shown on the right panel. Data are given as mean ± SD ($n = 3$). $^{*}P < 0.05$ versus normal control (Con); $^{\#}P < 0.05$ versus GC treatment (GC).

of biomarkers of bone turnover. We confirmed that GC resulted in the decrease of biomarkers of the bone formation, including serum bone specific alkaline phosphatase (BAP), serum osteocalcin (OCN), and *collagen I α1 (Col1α1)* mRNA level of bony tissue (Figure 2(c)). Contrarily, GC stimulated the increase of the biomarkers related to bone resorption including serum TRAP5b and *OSCAR* mRNA level of bone tissue. Furthermore, GC contributed to high bone turnover rate reflected by decreased serum OPG/RANKL ratio (Figure 2(d)). Encouragingly, tanshinol showed a capacity to reverse the deleterious impacts of bone turnover elicited by GC, as effectively as resveratrol (Figure 2). In brief, these lines of evidence confirm that tanshinol can promote the increase of bone formation and simultaneously prevent bone resorption.

3.3. Tanshinol Stimulates Wnt-Mediated Osteoblast Differentiation Involved in KLF15. It is well known that skeletal structural fragility results from impaired osteoblastic differentiation and subsequent bone formation. According to the results of qRT-PCR, mRNA expression of runt-related transcription factor 2 (*Runx2*) and *Osterix* gene, which are characteristic early markers of osteogenesis, was hindered by GC treatment (Figure 3(a)). Strikingly, mRNA expression of KLF15 transcription factor, a direct target of GR, was increased in bone tissue of rats treated by GC (Figure 3(b)). Additionally, mRNA expression of *Axin2*, an indicator of Wnt pathway, was inhibited by GC treatment, in line with the evidence that expression level of β-catenin protein (a key molecule of canonical Wnt signal transduction) was inhibited by GC using Western blot assay (Figures 3(a) and 3(c)). Expectably, tanshinol blocked the decrease of the two vital biomarkers of osteogenesis and the two key proteins of Wnt signaling, while counteracting the increased expression of KLF15, just like resveratrol. Taken together, these findings suggest that tanshinol rescues the inhibition of Wnt/β-catenin signaling in charge of bone formation and exerts an inhibitory action on activation of KLF15 pathway elicited by GC.

3.4. Tanshinol Inhibits Oxidative Stress Mediated by p66^{Shc} in Response to Dex and Ameliorated Cell Apoptosis. Cellular redox status of skeletal tissue plays an important role in intracellular signaling pathways during the process of bone metabolism. To evaluate oxidative stress level, we measured the accumulation of intracellular reactive oxygen species (ROS) level and activity of intracellular GSR. As shown in Figures 4(a) and 4(b), the level of ROS generation was increased remarkably, and the activity of GSR was decreased in bone tissue of rats exposed to Dex. Next, we investigated the phosphorylation of p66^{Shc}, an adapter protein that amplifies mitochondrial ROS generation and stimulates apoptosis. Evidently, expression level of the phosphorylation of p66^{Shc} was elevated in vertebral lysates of rats treated with GC (Figure 4(c)). Additionally, apoptosis analysis showed that TUNEL-positive cells were increased in femoral cancellous bone from GIO rats (Figure 4(d)). Remarkably, tanshinol exerted an antioxidative stress effect to protect bone tissue

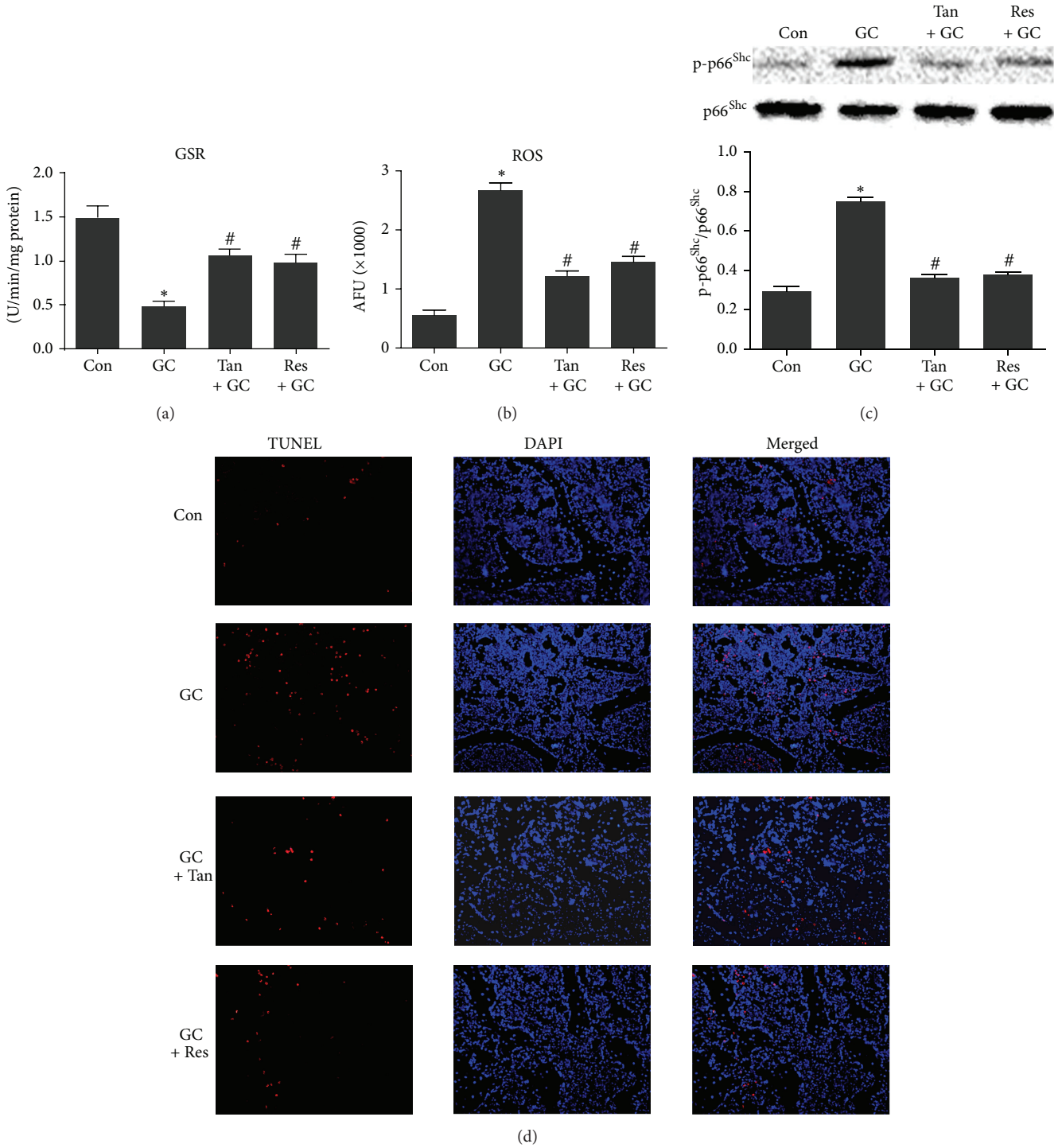

FIGURE 4: Tanshinol inhibits oxidative stress mediated by $p66^{Shc}$ and hampers cellular apoptosis in response to GC. Rats were treated as in Figure 1, and determinations were made as follows. (a) GSR (a critical endogenous antioxidant) activity in bone marrow cells flushed from tibia was assayed using colorimetric assay ($n = 4$). (b) Oxidative stress indicated as amount of ROS generation in bone marrow cells was estimated by DCFH-DA assay, and the fluorescence intensity of DCF was quantified using Image Station 2000 MM assay ($n = 4$). (c) Phosphorylated $p66^{Shc}$ (a significant mediator of amplification of oxidative stress) in lysates from vertebrae was detected by Western blot assay ($n = 3$). Representative figure was shown on the upper panel, and quantification is shown on the lower panel. (d) Apoptosis in sections was measured by TUNEL staining using fluorescent microscope ($n = 3$). Data are given as mean ± SD. $^{*}P < 0.05$ versus normal control (Con); $^{\#}P < 0.05$ versus GC treatment (GC).

against GC, as effectively as resveratrol. These lines of evidence revealed that tanshinol attenuates oxidative stress responsible for cell apoptosis elicited by GC via regulation of ROS/p66^{Shc} pathway in GIO rats.

3.5. Tanshinol Reversed Impaired Osteogenesis Linked to Inhibition of KLF15 in Response to GC. Encouragingly, our data *in vivo* indicated that tanshinol counteracted the activation of KLF15 transcription factor, a direct target of GR. To ask whether tanshinol hinders GC-induced negative regulatory role of KLF15 on bone formation *in vitro*, we further detected alterations of *KLF15* mRNA using qRT-PCR assay. The results showed that tanshinol could lead to downregulated expression of *KLF15* gene, while Dex could significantly induce expression of *KLF15* gene in C2C12 cells and MC3T3-E1 cells. However, the Dex-induced expression of KLF15 mRNA was hampered by RU486, a GR antagonist. Interestingly, tanshinol counteracted increase of KLF15 expression elicited by Dex in the two cells, especially in the presence of RU486 (Figure 5). The data may provide a clue to understanding the molecular mechanism of the protective effect of tanshinol on bone metabolism concerning regulation of KLF15.

Since little is known about whether GR-dependent KLF15 impairs osteogenesis, we next examined the effects of knockout of *KLF15* gene on the capacity of osteoblastic differentiation and bone formation in pluripotent mesenchymal precursor C2C12 cells and preosteoblastic MC3T3-E1 cells transfected transiently with siRNA oligonucleotides targeting KLF15. Based on the evidence of ALP staining, Dex-elicited decreased capacity of osteoblastic differentiation was blocked by KLF15 siRNA in the two cells (Figures 6(a) and 6(b)), as well as activity of bone formation measured by Alizarin Red S staining (Figure 6(c)). More importantly, the two cells exposed to tanshinol alone or in association with KLF15 siRNA could maintain good capacity of osteogenesis under conditions of Dex (Figure 6).

Next, we investigated the alteration in the gene expression profile related to osteogenesis in MC3T3-E1 cells transfected with the adenovirus-mediated exogenous expression of KLF15 or KLF15 siRNA. Surprisingly, the exogenous expression of KLF15 weakened induction of mRNA expression of *ALP*, *OPN*, *OCN*, *Runx2*, *Osterix* (a transcription factor required for osteoblast differentiation and bone formation), and *Tcf4* (an effector of downstream of Wnt signaling), while promoting increase of KLF15 mRNA level by approximately 7-fold (Figure 7(a)). In clear contrast, KLF15 siRNA caused induction of *ALP*, *OCN*, *Osterix*, and *Tcf4* expression, while it resulted in reduction of KLF15 mRNA level (Figure 7(b)). We then focused on Osterix and asked whether Dex-elicited reduction of osteogenesis was mediated by KLF15 using Osterix-luc reporter plasmid in MC3T3-E1 cells exposed to exogenous KLF15 or knocking down the expression of KLF15. Overexpression of KLF15 could repress the relative luminescence units (RLU) of Osterix-luc but Dex showed no influence on RLU of Osterix-luc. Meanwhile, Dex contributes to decrease of RLU of Osterix-luc and tanshinol could attenuate the inhibitory effect of Dex on RLU of Osterix-luc in cells treated with mock (empty vector) (Figure 7(c)).

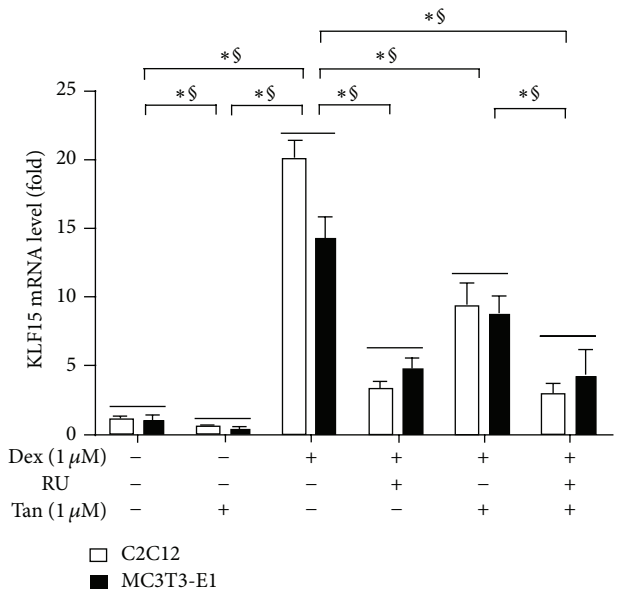

Figure 5: Tanshinol regulates expression of *KLF15* gene under condition of Dex involving glucocorticoid receptor. C2C12 cells and MC3T3-E1 cells were treated with Dex and/or RU486 (RU, a direct target of glucocorticoid receptor) in the presence or absence of Tan for 12 h; mRNA expression of *KLF15* gene was measured by qRT-PCR. Values are means ± SD of at least three independent experiments. $^{*}P < 0.05$ versus indicated group in C2C12 cells; $^{\S}P < 0.05$ versus indicated group in MC3T3-E1 cells.

Reversibly, KLF15 siRNA contributed to increase of RLU of Osterix-luc. Moreover, tanshinol counteracted decreased RLU of Osterix-luc elicited by Dex in MC3T3-E1 cells treated with or without KLF15 siRNA (Figure 7(d)). Taken together, these data revealed that transcription activity of Osterix-luc is negatively regulated by KLF15 in response to GC, which may contribute to impaired capacity of osteoblastic differentiation and the following bone formation.

3.6. Tanshinol Counteracts GC-Elicited Oxidative Stress and Cell Apoptosis Involved in KLF15/p66^{Shc} Pathway Cascade. Concerning the evidence that phosphorylation of p66^{Shc} in bone is associated with increased cell apoptosis *in vivo*, as well as evidence that p66^{Shc} amplifies ROS generation in mitochondria [5] and thereby promotes apoptosis, we investigated whether tanshinol could protect osteoblasts from oxidative stress and subsequent apoptosis elicited by GC involved in regulation of KLF15/p66^{Shc} pathway cascade. In MC3T3-E1 cells exposed to overexpression of KLF15 alone or KLF15 siRNA alone, level of ROS generation and cleavage activity of caspase-3 kept unchanged, as well as expression of p-p66^{Shc} protein. However, Dex provoked excessive ROS generation, higher cleavage activity of caspase-3, and more expression of p-p66^{Shc} protein, in various degrees which were strengthened by treatment with overexpression of KLF15; however, ROS generation and caspase-3 activity induced by Dex could be weakened by KLF15 siRNA. Additionally, tanshinol attenuated ROS generation, caspase-3 activity, and p-p66^{Shc}

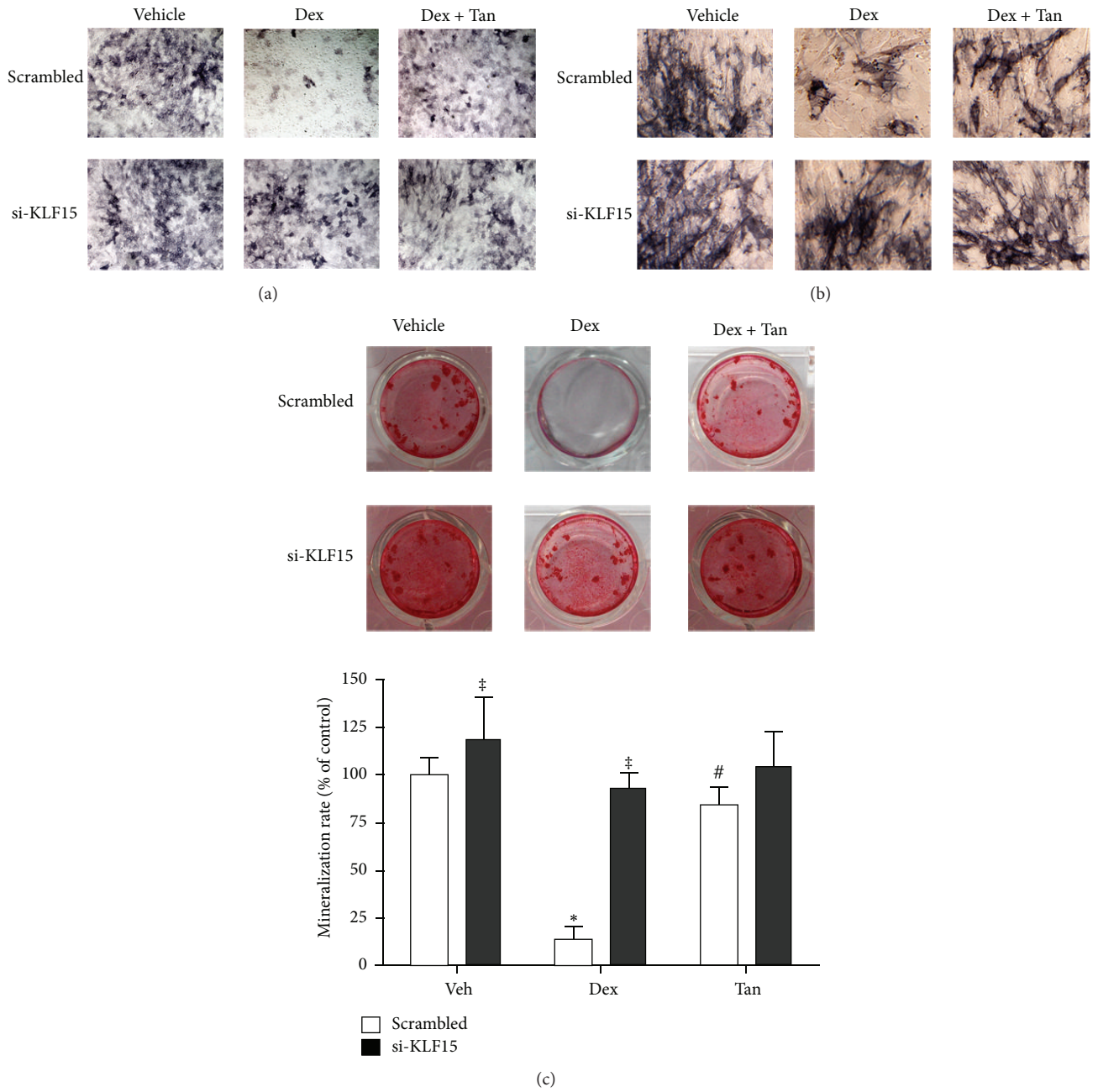

Figure 6: Tanshinol counteracts inhibition of osteoblastic differentiation and bone formation elicited by Dex in connection with downregulation of KLF15. C2C12 cells and MC3T3-E1 cells were transfected with KLF15 siRNA for 18 h, followed by DMEM medium supplemented with Dex in the presence or absence of tanshinol for 7 days. (a) Capacity of osteoblastic differentiation in C2C12 cells was determined by using ALP staining. (b) Capacity of osteoblastic differentiation in MC3T3-E1 cells. Original magnification (×100) in representative microscopic images. (c) Effects of knockdown of KLF15 on activity of bone formation. MC3T3-E1 cells were treated with KLF15 siRNA for 18 h, followed by Dex treatment with or without tanshinol for 21 days. Mineralization activity with the indicated treatments was stained using Alizarin Red S at day 21. Original magnification (×100) in representative microscopic images (upper panel). Quantitative determination was carried out by CPC solution (pH 7.0) (lower panel). Vehicle: vehicle control (Veh). Values are means ± SD of at least three independent experiments. $^{*}P < 0.05$ versus vehicle control; $^{\#}P < 0.05$ versus GC treatment; $^{\ddagger}P < 0.05$ versus corresponding scrambled control.

expression elicited by Dex in MC3T3-E1 cells, especially in the presence of KLF15 siRNA (Figure 8). Therefore, it is likely that tanshinol protects osteoblasts against Dex in connection with suppression of induction of KLF15 which may cause phosphorylation of $p66^{Shc}$ contributing to accumulation of ROS generation and subsequent cell apoptosis.

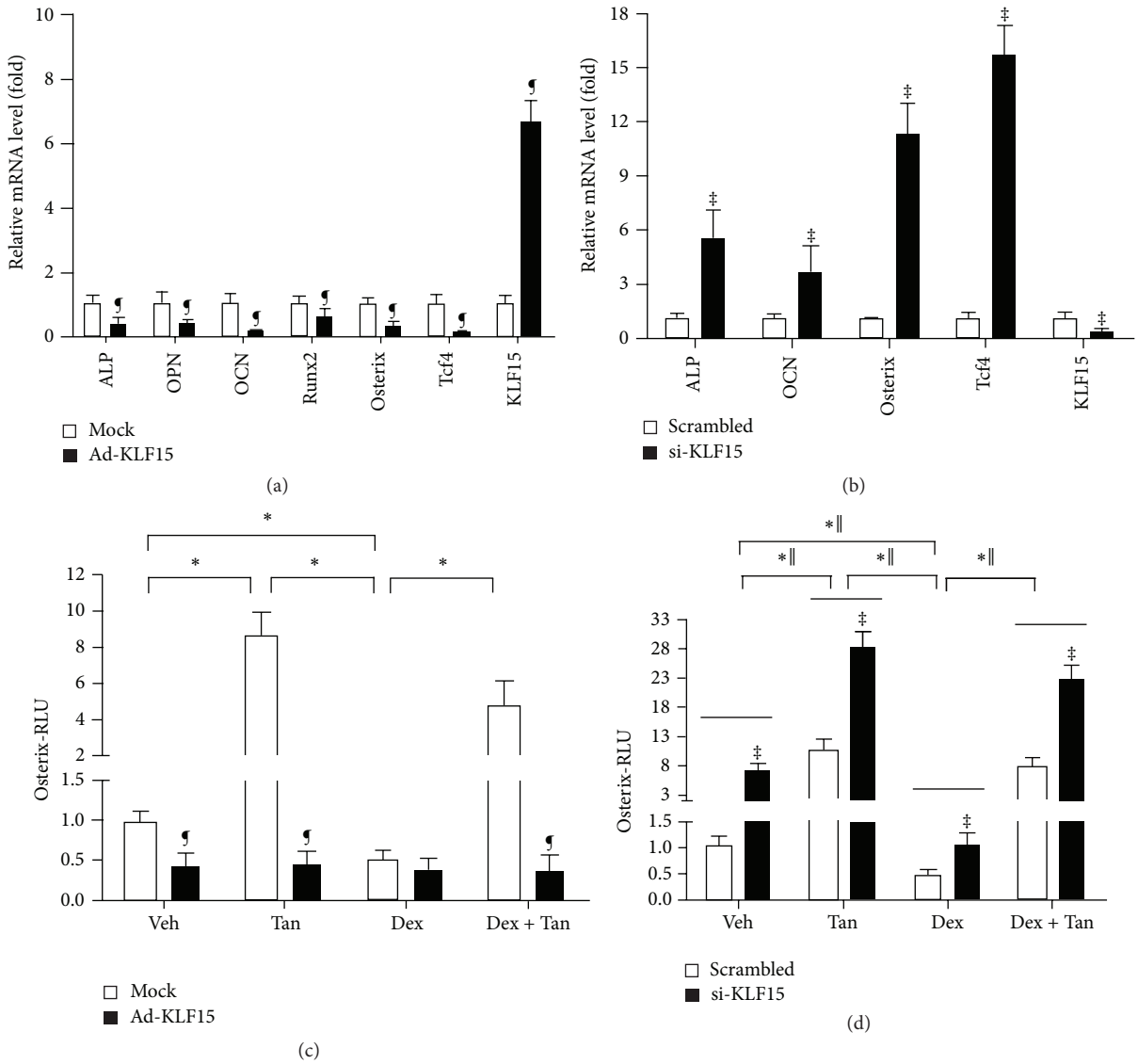

Figure 7: Regulation of differential genes by KLF15 and involvement of Osterix in protective effect of tanshinol on bone formation. (a) MC3T3-E1 cells were infected with recombinant adenovirus Ad-KLF15 for 4 days. mRNA expression of bone formation-related genes and KLF15 gene was measured by qRT-PCR. (b) MC3T3-E1 cells transfected with KLF15 siRNA for 18 h. mRNA expression of bone formation-related genes and KLF15 gene was measured by qRT-PCR. (c) MC3T3-E1 cells were infected with Osterix-luc reporter plasmid in combination with recombinant adenovirus Ad-KLF15 or mock (noninfection). (d) MC3T3-E1 cells were cotransfected with the Osterix-luc reporter plasmid in combination with KLF15 siRNA or the scrambled sequence. Luciferase activity assays were explored using the Dual-Luciferase Reporter Assay System as described under Section 2.8 in Materials and Methods. The data represent mean ± SD of luciferase relative luminescence units (RLU) normalized to corresponding renilla luciferase activity (triplicates). $^{*}P < 0.05$ versus indicated group in cells exposed to empty vector (mock) or scrambled control; $^{\|}P < 0.05$ versus indicated group in cells exposed to KLF15 siRNA or recombinant adenovirus Ad-KLF15; $^{¶}P < 0.05$ versus corresponding mock; $^{‡}P < 0.05$ versus corresponding scrambled control.

3.7. Tanshinol Counteracts Negative Regulation of Wnt Signaling by Dex Linked to KLF15/Tcf4 Pathway. To further elucidate the underlying mechanism for tanshinol to counteract KLF15-mediated reduction of osteogenesis under conditions of GC, we observed transcription activity of Tcf4-Luc and expression of Tcf4 protein in MC3T3-E1 cells and/or C2C12 cells treated with KLF15 siRNA or exogenous KLF15. The results showed that the RLU of Tcf-luc and expression of Tcf4 protein were evaluated by virtue of KLF15 siRNA in the two cell lines, while they were declined owing to overexpression of KLF15. Interestingly, the promoting role of tanshinol on Tcf-luc seemed to be more significant in the two cells transfected with KLF15 siRNA than those treated with tanshinol alone, whereas overexpression of KLF15 partly neutralized this promoting effect of tanshinol on Tcf-luc and expression of Tcf4 protein (Figures 7(a) and 7(c)). Moreover, tanshinol ameliorated the decreased RLU of Osterix-luc elicited by Dex in the two cells, especially in the presence of

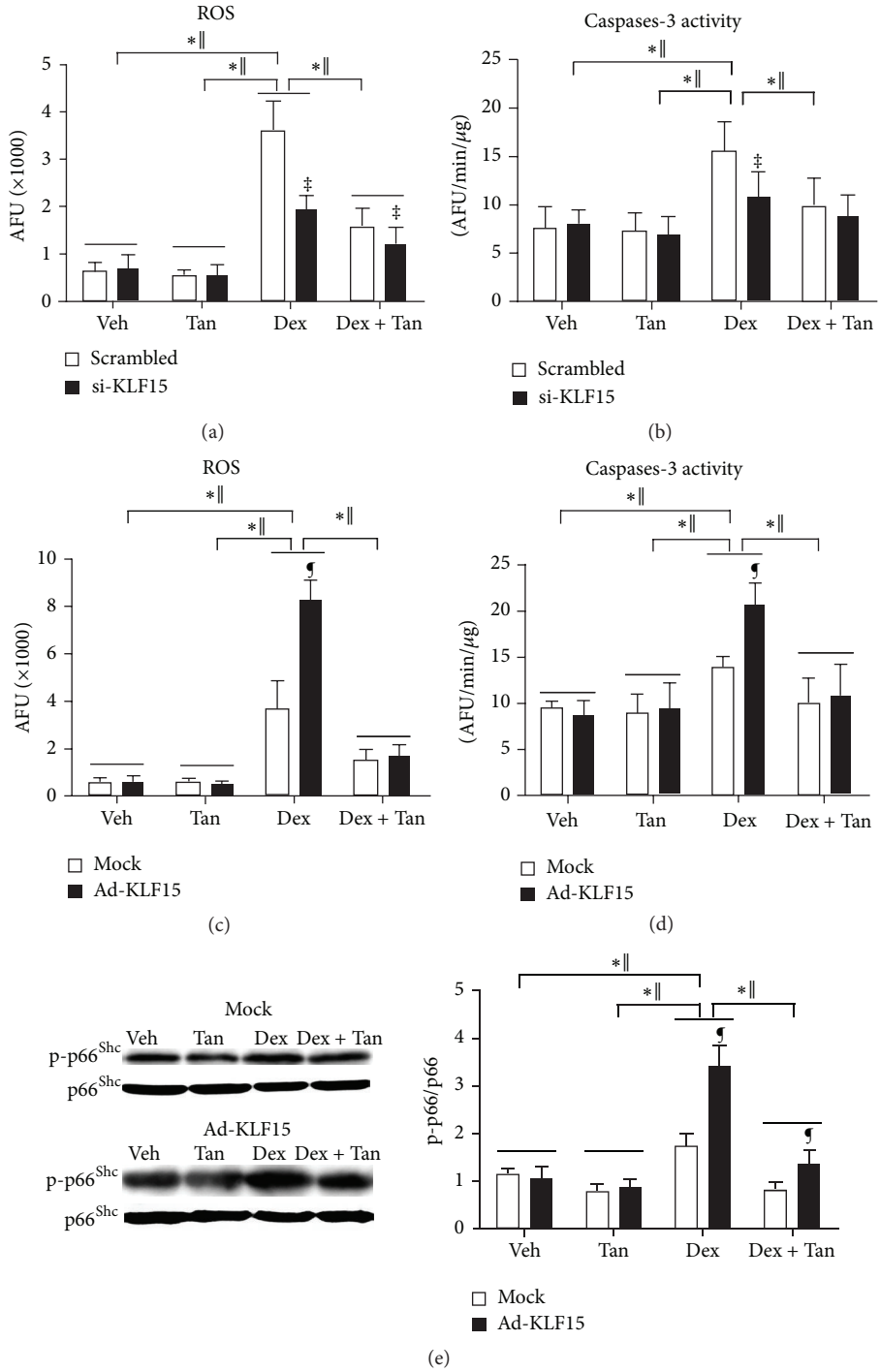

Figure 8: Tanshinol counteracts GC-induced oxidative stress and caspase-3-dependent apoptosis linked to phosphorylation of $p66^{Shc}$. MC3T3-E1 cells were transfected with KLF15 siRNA or recombinant adenovirus Ad-KLF15 in the presence or absence of Dex and/or tanshinol, and measurements were explored as follows. ((a) and (c)) ROS level indicated oxidative stress status was analyzed by DCFH-DA probe. ((b) and (d)) Cellular apoptosis was detected by caspase-3 activity. (e) Phosphorylated $p66^{Shc}$ in MC3T3-E1 cells exposed to Ad-KLF15 and mock was detected by Western blot assay (left panel). Representative figure was shown on the left panel, and quantification is shown on the right panel. Bars indicate mean ± SD of triplicate determinations. $^{*}P < 0.05$ versus indicated group in cells exposed to empty vector (mock) or scrambled control; $^{\|}P < 0.05$ versus indicated group in cells exposed to KLF15 siRNA or recombinant adenovirus Ad-KLF15; $^{¶}P < 0.05$ versus corresponding mock; $^{‡}P < 0.05$ versus corresponding scrambled control.

KLF15 siRNA (Figure 9). Therefore, the data suggested that tanshinol arrests downregulation of Wnt pathway responsible for bone formation under conditions of Dex involved in KLF15/Tcf4 pathway.

4. Discussion

Increasing well-documented evidence highlights the role of oxidative stress in the development and progression of osteoporosis [23–25]. We confirmed herein that oxidative stress elicited by GC contributes to bone loss and impaired bone strength and this might be hampered by antioxidants. Previous evidence in our team demonstrated that tanshinol as a natural antioxidant exhibited the potential to promote osteoblastic differentiation and bone formation, contributing to a strongly preventive effect on GIO [18, 20], as efficiently as resveratrol [26, 27]. In the present work, tanshinol counteracts reduction of trabecular parameters, impaired biomechanical characteristics, and imbalance of bone turnover parameters in the experimental model of GIO, respectively, just like resveratrol in light of the previous evidence [26, 27]. The findings herein reveal that tanshinol diminishes the deleterious effects of GC on bone quality involved in inhibiting downregulation of Wnt signaling responsible for osteogenic differentiation and bone formation and suppressing activation of ROS/$p66^{Shc}$ pathway cascade for oxidative stress and cell apoptosis under conditions of GC. Particularly, based on the evidence from siRNA interference and overexpression methods, we firstly elucidated in this study that tanshinol ameliorates induction of KLF15 transcription factor elicited by GC which may give rise to activation of $p66^{Shc}$ pathway and lead to arrest of Wnt signaling during osteoblastic differentiation and bone formation.

Tanshinol, consisting of polyphenolic hydroxyl groups similar to resveratrol, exhibits the inhibitory action on oxidative stress *in vitro* [20, 28, 29]. In the present paper, we found that tanshinol exerted a serial of antioxidative stress actions in bone tissue of GIO rats and/or in MC3T3-E1 cells exposed to Dex, including reduction of ROS generation, increase of GSR activity, and inhibition of phosphorylated $p66^{Shc}$ in GIO rats, as effectively as antioxidants like resveratrol [30]. Similarly, tanshinol could counteract increase of cell apoptosis measured by TUNEL assay *in vivo* and determined by cleavage activity of caspase-3 *in vitro*. Interestingly, the indexes of oxidative stress and cell apoptosis, including expression of phosphorylated $p66^{Shc}$, ROS generation, and caspase-3 activity, showed at low level under normal conditions in MC3T3-E1 cells in the absence of Dex, and tanshinol showed no effects in cells treated with either overexpression of KLF15 or KLF15 siRNA. Surprisingly, the indexes of oxidative stress and cell apoptosis mentioned above are strongly induced and activated in response to Dex in MC3T3-E1 cells, especially treated with overexpression of KLF15, and tanshinol could attenuate the activation of oxidative stress and cell apoptosis, in synergy with KLF15 siRNA. It is a plausible mechanism that ROS generation as a cellular metabolic activity maintains redox balance between oxidants and antioxidants for homoeostasis in general, but it can be overwhelmingly increased under conditions of oxidative stress elicited by Dex [31], and tanshinol as a natural antioxidant can exert a significant inhibitory effect on excessive ROS generation and can delay a series of deleterious events to damage molecule, tissues, and organs. $p66^{Shc}$ is a robust marker of oxidative stress, known as a sensor as well as amplifier of oxidative stress, because $p66^{Shc}$ can promote ROS generation by virtue of activation and further increase of intracellular ROS [32]. Furthermore, $p66^{Shc}$ activated by phosphorylation on serine 36 (S36) is an event on which the proapoptotic function of $p66^{Shc}$ depends [33]. The $p66^{Shc}$ protein mediates oxidative stress-related injury in multiple tissues [32]. In the present study, bone tissue of GIO rats showed significant increase of expression of $p66^{Shc}$ phosphorylation and ROS level, as well as cell apoptosis, which could be hampered by tanshinol and its implications for inhibition of the prevention and treatment of osteoporosis elicited by oxidative stress under conditions of GC.

Oxidative stress significantly leads to harmful consequence of increased osteoblastic apoptosis seen in GIO rats, as illustrated by the finding of increased osteoblast survival following the administration of tanshinol or resveratrol. The current findings are in agreement with our previous *in vitro* findings, in which tanshinol hindered inhibition of proliferation, cell cycle arrest, and increase of apoptosis in C2C12 cells under oxidative stress [20]. Meanwhile, oxidative stress hampers osteogenesis by virtue of inhibition of Wnt signaling [4, 23]. In skeletal tissue, suppression of Wnt signaling by ROS may lead to reduction of the differentiation and survival of osteoblasts and ultimately decreased bone formation [23]. As indispensable signaling of osteoblastic differentiation and bone formation, Wnt pathway regulates expressions of target genes related to osteogenesis (including ALP, OCN, and Runx2) via Tcf4 transcription factor which can be activated by association with β-catenin [34, 35]. Moreover, Osterix is an osteoblast-specific transcription factor required for osteoblast differentiation, acts as downstream of Runx2 to induce mature osteoblasts, and attenuates osteoblast proliferation via inhibition of Wnt pathway owing to feedback during osteoblastic differentiation and bone formation [36]. In the present study, both Osterix-mediated and Tcf4-mediated transcription were hindered by Dex *in vitro*, in accordance with previous publications [5, 37]. Additionally, tanshinol could attenuate decrease of transcriptional activity of Osterix and Tcf4 elicited by GC, as well as expression of Tcf4 protein, indicating that tanshinol can suppress inhibition of osteogenesis involved in downregulation of Wnt signaling. Thus, the increased anabolic efficacy of tanshinol may be at least in part due to suppression of the oxidative stress that obstructs osteogenesis and osteoblastic survival in GIO rats, in line with our previous *in vitro* finding [20].

Recently, findings suggest a regulatory impact of KLF15 transcription factor on downregulation of Wnt/β-catenin pathway in cardiac homeostasis [13]. KLF15 transcription factor as a direct GR target gene exhibits an extensive role in pathophysiologic progression of diverse disease in varied organs, such as heart fibrosis [38], cardiac lipid metabolism [39], hepatic gluconeogenesis [40], chronic kidney disease

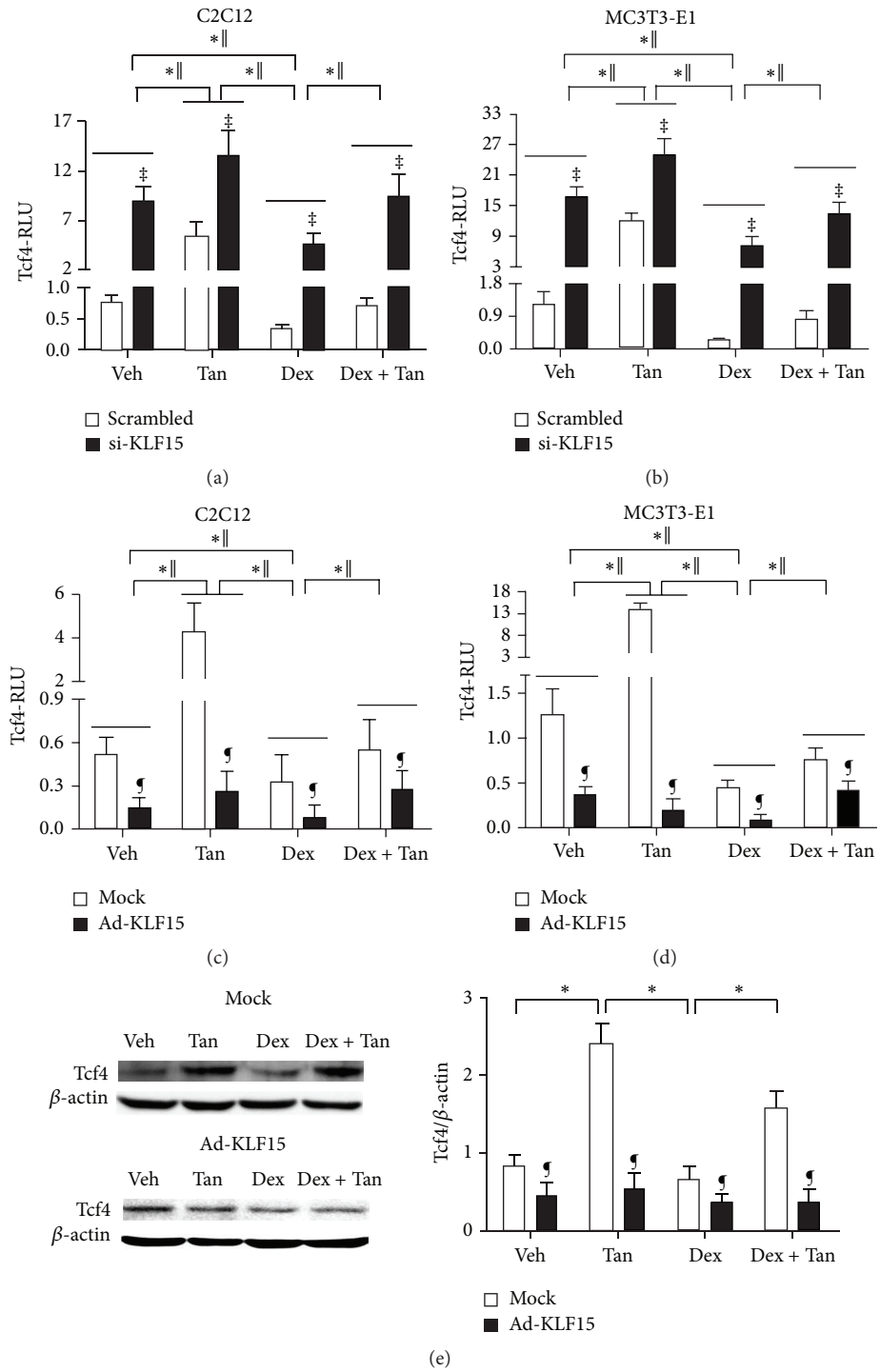

Figure 9: Tanshinol attenuates downregulation of canonical Wnt signaling elicited by Dex associated with regulation KLF15. ((a) and (b)) C2C12 cells or MC3T3-E1 cells were cotransfected with the Tcf4-luc or FoxO3a-luc reporter plasmid in combination with KLF15 siRNA or the scrambled sequence. ((c) and (d)) C2C12 cells or MC3T3-E1 cells were infected with the FoxO3a-luc or Tcf4-luc reporter plasmid in combination with recombinant adenovirus Ad-KLF15 or mock (noninfection). Luciferase activity assays were explored using the Dual-Luciferase Reporter Assay System as described under Section 2.8 in Materials and Methods. The data represent mean ± SD of luciferase relative luminescence units (RLU) normalized to corresponding renilla luciferase activity (triplicates). (e) Tcf4 (a requisite mediator for downstream effector Tcf of canonical Wnt pathway contributing to bone formation) in MC3T3-E1 cells exposed to Ad-KLF15 and mock was detected by Western blot assay. Representative figure was shown on the left panel, and quantification is shown on the right panel. Bars indicate mean ± SD of triplicate determinations. $^{*}P < 0.05$ versus indicated group in cells exposed to empty vector (mock) or scrambled control; $^{\|}P < 0.05$ versus indicated group in cells exposed to KLF15 siRNA or recombinant adenovirus Ad-KLF15; $^{¶}P < 0.05$ versus corresponding mock; $^{‡}P < 0.05$ versus corresponding scrambled control.

[41], muscle wasting [42], and airway hyperresponsiveness [43]. Strikingly, we provide the first evidence herein that induction of KLF15 expression elicited by GC attenuates osteogenesis involved in suppression of Wnt/β-catenin pathway and the following Tcf4-dependent transcriptional activation in C2C12 cells and MC3T3-E1 cells. Firstly, the levels of *KLF15* mRNA increase in the GIO rats, and induction of *KLF15* mRNA is observed in C2C12 cells and MC3T3-E1 cells exposed to Dex, in accordance with the previous evidence that Dex promotes expression level of *KLF15* mRNA in primary osteoblasts [15]. Moreover, our data also illustrated a phenomenon of downregulation of *KLF15* mRNA in C2C12 cells and MC3T3-E1 cells treated with RU486, an antagonist of GR, indicating that GC is responsible for the activation of KLF15 transcription factor. In addition, KLF15 is likely to be implicated in the regulation of multiple genes, including those participating in glucose transport [44], energy homeostasis [42], podocyte differentiation [45], adipogenesis [46, 47], and so forth. In a previous study, *KLF15* mRNA was found to increase during the process of osteoblastic differentiation in MSC, but the mechanism remains unclear [48]. However, further detection under conditions of siRNA interference or overexpression of KLF15 may help to clarify the role of KLF15 transcription factor in regulating signaling transduction associated with osteogenesis. Notably, we show here that overexpression of KLF15 results in significantly decreased genes related to osteogenic differentiation and the components of Wnt pathway, which were reversely upregulated by virtue of siRNA interference of KLF15, suggesting that KLF15 may be a vital protein to regulate osteogenesis. Taken together, our study on C2C12 cells and/or MC3T3-E1 cells treated with knockout of KLF15 gene or overexpression of KLF5 further confirmed that tanshinol may protect against GC-elicited repression of osteogenesis involved in KLF15 pathway.

In conclusion, our findings provide evidence for a novel target of KLF15 transcription factor for skeletal niche in GIO model and reveal a preventive effect of tanshinol on bone tissue involved in inhibition of oxidative stress and subsequent osteoblastic apoptosis via ROS/$p66^{Shc}$ pathway cascade and reduction of impaired bone formation via Wnt/β-catenin/Tcf4 signaling transduction. Furthermore, notwithstanding the beneficial influence of resveratrol on bone, it has not been developed as a therapeutic agent in clinical use, only as an extensive tool for the purpose of research by virtue of severe defects in poor solubility and pharmaceutical preparation property [49]. Contrarily, as a water-soluble compound, tanshinol is commonly used as an indicator of diverse complex prescription for quality control in Traditional Chinese Medicine; thus the clinical use of tanshinol will hold promise for an effective and safe candidate for the prevention and treatment of GIO. Our results strongly suggest that tanshinol with optimized pharmacological properties could be developed for therapeutic use in human.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Yajun Yang, Dongtao Wang, Yanjie Su, Yahui Chen, Yuyu Liu, and Shiying Luo performed the research. Liao Cui, Yajun Yang, and Dongtao Wang designed the research study. Liao Cui, Yajun Yang, and Tie Wu analyzed the data. Yajun Yang and Liao Cui wrote the paper.

Acknowledgments

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