ROS

REVIEW ARTICLES

MnSOD/SOD2 in Cancer: The Story of a Double Agent

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ABSTRACT | Manganese-dependent superoxide dismutase (MnSOD/SOD2) is one of the major antioxidant enzymes which scavenges the superoxide produced in the mitochondria, the main source of free radicals in non-pathological conditions. This first barrier is important to prevent early stages of cancer development. However, once the tumor is established, upregulation of MnSOD without a concomitant increase of scavengers of hydrogen peroxide (H_2O_2) plays a much complex role. It contributes to proliferation, angiogenesis and invasion, affecting glycolytic metabolism and preventing the mitochondria from inducing apoptosis favoring therapy resistance. The increase of H_2O_2 mediated by MnSOD is able to induce phenotypical changes in several tumor types, such as epithelia-mesenchymal transition increasing the motility of tumor cells, neuroendocrine differentiation and senescent secretory phenotypes which feed tumor progression by producing cytokines and growth factors.

KEYWORDS | Differentiation; Gene regulation; MnSOD; Senescence; Tumor suppressor; Tumorigenesis

ABBREVIATIONS | AMPK, AMP activated kinase; CSC, cancer stem cells; EMT, epithelial-mesenchymal transition; GPX, glutathione peroxidase; GSH, reduced form of glutathione; MMP, matrix metalloproteases; NTC, non-tumorigenic progeny cells; PTEN, phosphatase and tensin homolog; PTM, post-transcriptional modification; ROS/RNS, reactive oxygen/nitrogen species; SASP, senescence-associated secretory phenotype (SASP); SOD, superoxide dismutase

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1. INTRODUCTION

The study of the importance of superoxide dismutase (SOD)2/MnSOD in cancer has to be approached considering some premises. First, the study of SOD2/MnSOD has two main scopes: (a) the genetic one, the study of polymorphisms in SOD2 gene is a wide field of study. Single nucleotide changes in the gene have been shown as very important in the risk of developing cancer and its further evolution; (b) the phenotypic one, the study of tumor cell undergoing changes in SOD2/MnSOD protein expression, and importantly, SOD2 gene does not need to be mutated to act on tumor biology. This is due to the fact that SOD2 has a very wide range of expression levels depending on the induction stimuli. MnSOD protein changes are involved in the phenotypic adaptation of the tumor cell to a changing environment like tumor tissue. Second, the fact that MnSOD is not only an antioxidant protein, but a modulator of mitochondrial superoxide levels and of hydrogen peroxide (H_2O_2) in the rest of the cell, makes it a very complex actor to locate in any scenario. The product of MnSOD has to be subsequently scavenged; therefore, whether MnSOD will act as an antioxidant or a modulator of superoxide/peroxide concentration will depend on the presence of a subsequent peroxidase or catalase activity in the vicinity. Third, its role varies according to the tumor stage. Although the role of MnSOD as a barrier preventing tumor initiation by preventing oxidative damage was described in the early 1990s, increasing lines of evidence have arisen about its critical importance as a facilitator of survival, progression, and metastasis. Finally, MnSOD and its product have a well-proven, but not very well known, role in senescence and differentiation. Oxidative stress can induce senescence, and H_2O_2 levels can induce quiescence. On the other hand, MnSOD or H_2O_2 have been proven to induce differentiation in a range of both pathological and non-pathological cell types across different species.

2. GENERAL FEATURES OF SOD2/MnSOD GENE AND PROTEIN

Evolutionarily speaking, the importance of antioxidant enzymes arises from the use of O_2 to maintain the energy demand. Unfortunately there is a price to pay for all aerobic cells, in terms of the associated collateral damage, due to the formation of the so called reactive oxygen/nitrogen species (ROS/RNS) [1]. ROS production as a side product of the respiratory chain was actually a powerful selective force during evolution among aerobic cells and obviously those able to defend against ROS/RNS were positively selected. As a result, all cells with a normal (or high) oxygen consumption require a 'cleaning' mechanism of these free radicals, specifically superoxide, which is formed within the mitochondria at a relatively high rate at certain respiratory chain complexes, namely, complexes I and III [2] by one electron reduction of molecular O_2. Pioneering reports from Jensen [3] and Chance and colleagues [4] demonstrated in vivo ROS production and H_2O_2 formation in isolated mitochondria. According to accurate in vivo intra-mitochondrial measurements of both O_2 (3–30 μM) and superoxide (10–200 pM),

this 'one-electron reduction' is thermodynamically favored [5], but fortunately only a small proportion (<2%) occurs [1]. Respiratory complex I and, in a lesser extent, complex III would in turn release superoxide to the intermembrane space, which is either converted to non-radical or otherwise transported through voltage-dependent anion channels into the cytosol [6]. Even though mitochondria would be the major site for ROS formation, other enzymes, particularly NADPH oxidases (NOX) and dual oxidases (DUOX), xanthine oxidase, and cytochrome P450 enzymes are also common sources of superoxide, accounting for roughly 10% of all the superoxide, formed under certain physiological conditions [5, 7].

ROS/RNS are inevitable byproducts of oxygen metabolism in aerobic cells. In addition to a set of small antioxidant molecules (endogenous or exogenous), all aerobic cells are equipped with a battery of defensive enzymes. Among these enzymes are superoxide dismutases (SOD1–3), which dismutase, (that is, a simultaneous oxidizing and reducing reaction) superoxide thus converting it into a less reactive ROS, H_2O_2. H_2O_2, a rather stable ROS, can flow out of mitochondria and being further converted into water and O_2 by the action of different enzymes including catalase (CAT), glutathione peroxidases (GPX1–8) and peroxiredoxins (PRDX1–6).

2.1. Essential Antioxidant Enzymes for Life: The Case of SOD2/MnSOD

Knockout and transgenic mice models have proved that a few of antioxidant proteins are essential for life, including thioredoxin (TXN)1/2, thioredoxin reductase (TXNRD), GPX4, and SOD2 [8, 9]. Among antioxidant enzymes, SODs have received particular attention since they are the only set of enzymes governing superoxide, the main radical, in aerobic organisms by converting it into O_2 and H_2O_2. Prokaryotic and eukaryotic cells display different types of SODs that fall into 3 unrelated families that converged in evolution to utilize a redox-active metal to disproportionate superoxide. These are classified according to the type of metal cofactors used: (i) an uncommon family of Ni-containing SODs (NiSOD) [10]; (ii) a Cu^{2+}/Zn^{2+}SOD family, which uses copper for catalysis and also bind a structural zinc atom in the active site [11]; and (iii) a Mn^{2+}SOD/Fe^{2+}SOD family that uses manganese or iron for catalysis and are structurally related.

In most chordates, there are two forms of CuZn-SOD, i.e., a mainly cytoplasmic form or SOD1 and an extracellular form or SOD3. SOD1 is a 32 kDa protein, codified by a gene located in chromosome 21 in *Homo sapiens*. The protein SOD1 works as a non-disulfide linked homodimer [12]. It is stably and widely expressed in almost all the cell types throughout the organism [11]. The promoter region shows putative binding sites for several transcription factors including NF1, Sp1, AP1, or NFκB [13]. In addition to the cytoplasm, it can be also found in the nucleus, lysosomes, and the mitochondrial intermembrane space. Structurally related to SOD1, SOD3 is however a 135 kDa homotetramer having its expression restricted to some cell types and/or tissues, most (99%) is anchored to heparan sulfate proteoglycans in the tissue interstitium while roughly 1% is located in the vasculature in equilibrium between the plasma and the endothelium [14].

The third widespread family of SOD mentioned includes FeSOD and MnSOD. They are thought to have evolved from a common ancestor prior to the divergence of eubacteria, archaebacterial, and eukaryotes 3 billion years ago [15]. Iron containing SOD is found in prokaryotes, mostly cyanobacteria, in plant chloroplasts, and in some unicellular organisms (e.g. *Plasmodium*). Interestingly, due to their high structural similarity, it is known that Fe^{2+} can bind Mn^{2+} site in MnSOD, but under this circumstance, it blocks the enzyme instead of substituting the Mn^{2+} for redox purposes.

SOD2/MnSOD is a tetramer found in prokaryotes as well as in the matrix mitochondria from eukaryotes where it plays a key role in the first-line defense detoxifying mitochondrial superoxide. The complete genomic sequence of this nuclear-encoded mitochondrial protein has been characterized for mouse [16] and rat [17], among others. These reports revealed a highly conserved sequence (over 90%) in the coding region. Human SOD2 gene is a single-copy gene located on chromosome 6q25.3 [18] (**Figure 1A**) which codes for two different transcripts without many differences in translation residues. Studies of molecular organization identified five exons interrupted by four introns, all probably with functional splicing sites. The sequences provided for all 5 exons by different groups are all identical with the exception of an exon II fragment (+394 and +401) and exon III fragment (+4,995) [19]. The gene codes for a monomer which is subsequently grouped in a

tetramer (**Figure 1B** and **1C**). Systemic deletion of SOD2 was performed by two independent laboratories [20, 21], and in both studies, MnSOD ablation caused mice to die early after birth, showing cardiomyopathy and mitochondrial metabolic alterations or neurodegeneration, severe anemia and hematopoietic loss, and motor disturbances. $SOD2^{-/-}$ mice show an obvious higher sensitivity to hyperoxia and massive indicatives of DNA oxidative damage. Interestingly, both studies reported liver lipid abnormalities at the histological level.

2.2. Transcriptional Regulation of SOD2

Even though SOD2 is a highly expressed gene in a variety of cell types and tissues, it is also a highly regulated gene by a variety of intracellular and environmental stimuli. Cytokines such as interleukin (IL)-1, IL-4, IL-6, and TNF-α, or lipopolysaccharide (LPS) and IFN-γ are potent inducers of SOD2 [20] while, on the contrary, methylation in specific intronic regions repress its expression [22]. Interestingly, early in the 1990s it had been reported that SOD2 basal promoter lacks TATA and CAAT boxes within the 708 bp of the putative transcription initiation site, but instead possesses a GC-rich (78%) region within 400 bp of the initiation site, features suggested to be characteristic of a housekeeping gene promoter. This region contains multiple consensus sequences for specific protein 1, (Sp1) as well as 3 copies of activator protein 2 (AP2) transcription factors binding sites (**Figure 1A**). Sp1 seems to be an important regulator for the basal expression of the human SOD2 gene [23]. Computer analysis of the SOD2 has provided additional binding sites for other transcription factors including NFκB, AP-1, or C/EBP [24], although not all have been yet proved to have a regulatory role on SOD2 expression (**Figure 1A**).

2.2.1. NFκB

The family of transcription factors NFκB/Rel has been considered as a major mediator of the innate and adaptive immune response and represents a link between inflammation and cancer [25]. This transcription factor regulates, and is regulated by, free radical levels. The cytokine TNF-α, a classical activator of NFκB, is enhanced by lowering glutathione (GSH) levels while antioxidants block NFκB activation in IL-1- and TNF-α-incubated cells [26]. ROS production appears essential for NFκB activation and direct regulation of this transcription factor by H_2O_2 has also been reported [27]. Thus, not surprisingly, oxidative stress-mediated induction of SOD gene has been well documented in different organisms, tissues, and cells under a variety of stimuli including radiation or proinflammatory cytokines [19, 28]. Furthermore, some of these studies have revealed NFκB as a crucial transcription factor for SOD2, and an NFκB binding site located in the second Sod2 intron appears functionally essential for its activating role [29]. Interestingly p50, another NFκB member, acts as a negative regulator [30].

2.2.2. SP1

Sp/Kruppel-like factors (SP/KLF) are three zinc-finger domains (Cys_2-His_2 units), DNA-binding transcription factors. SP factors display a hallmark feature of a highly conserved amino acid sequence, the Buttonhead box (CXCPXC), absent in the KLF subfamily. SP1 binds to the GC boxes (5′-GGGGCGGGG-3′) [31], recurring motifs in many gene promoters including Sod2. They are transcriptional regulators of a broad range of cellular processes and play a role in embryonic and early postnatal development. Their importance come from studies with transgenic and knockout murine models, showing that many Sp1-8 knockouts are lethal, embryonically in the case of Sp1 [32]. Among the family, Sp1 has been by far the most extensively investigated member, it is essential for SOD2 constitutive levels as well as for inducible expression [33, 34]. Sp1 can interact with a subset of the consensus sequences for NFκB and a DNA looping structure in the 5′ flanking region of Sod2 seems to be essential for interactions with other transcription factors important for SOD2 expression as p53 or nucleophosmin (NPM) [35].

2.2.3. AP-1 and AP-2

Activator protein 1 (AP-1) transcription factor is formed by basic leucine zipper (bZIP) proteins acting as homo- or hetero-dimer usually composed by Jun (JUN, JUNB, JUND), Fos (FOS, FRA1, FRA2), or ATF protein family members in mammalian cells. Binding of the complex to the cis 5′-TGA[CG]TCA-3′ consensus motif in turn regulates a variety of cellular processes including proliferation and survival, growth, differentiation, apoptosis, cell migration, and

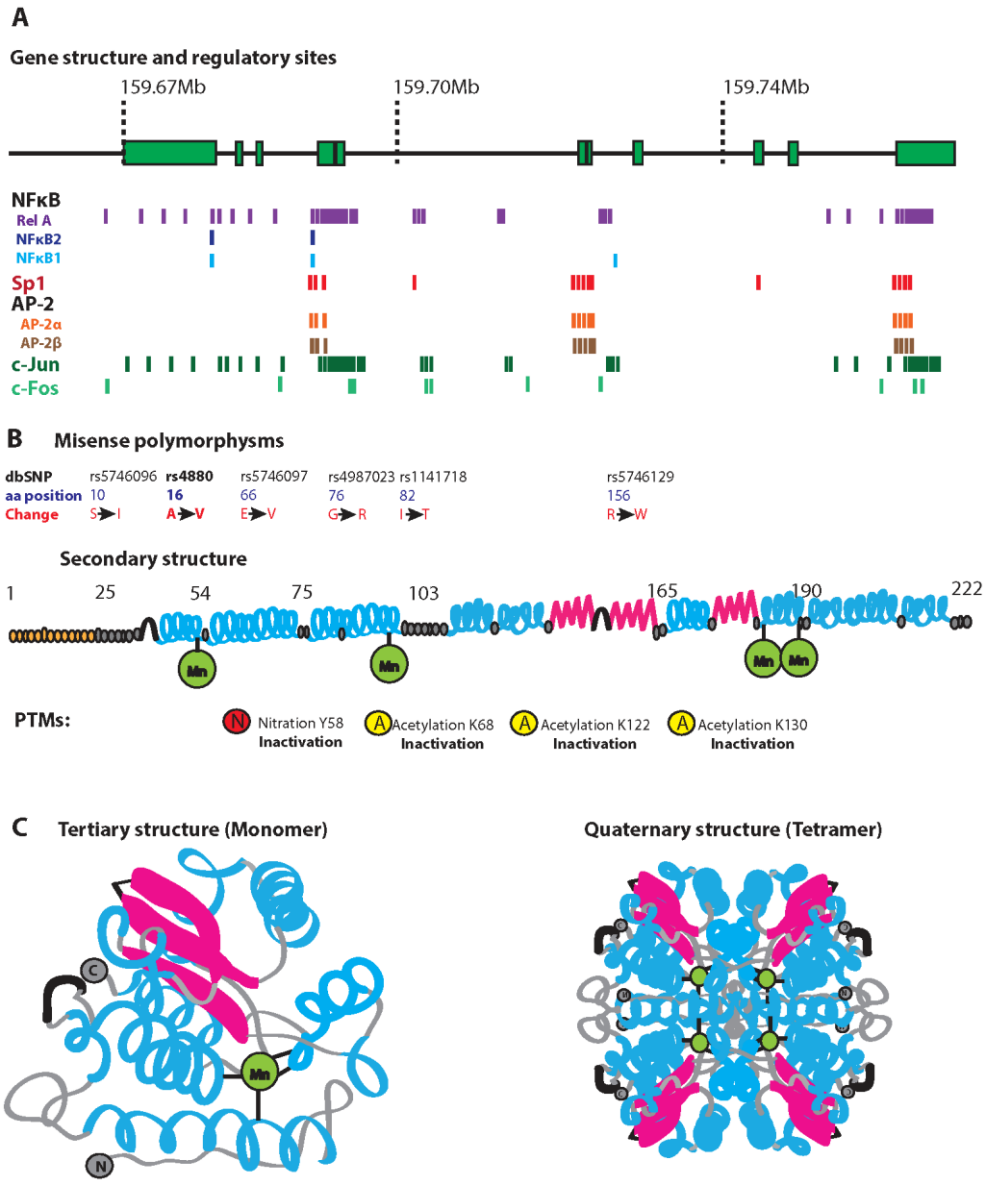

FIGURE 1. Structure of SOD2/MnSOD gene and protein and major regulatory events. (A) Gene structure of the human *SOD2* located in the chromosome 6, exons (coding and non-coding) are shown in rectangles (green) introns are shown as a line. The gene can produce 10 different protein-coding transcripts (source: https://www.ensembl.org) Location matched regulatory sites for the main transcription factors are shown below. Regulatory site locations were obtained from GTRD database (http://gtrd.biouml.org). (B) Missense polymorphisms indicating the aminoacid change, from those ones rs4880 is the most relevant with implications in different diseases. The secondary structure of the protein indicating: mitochondrial signalling peptide (orange), enzyme final sequence (grey) and secondary structures: turn (black), α-helix (blue) and β-sheet (pink). Manganese (Mn) binding points are indicated in green. The nitration and acetylation points are indicated below. (C) Tertiary and quaternary structure of an MnSOD monomer and tetramer respectively. Color coding follows (B). All panels, diagrams are representative, for clarity the scale of the distance may have been adjusted not being exactly proportional to the real distance.

transformation [36]. Additionally, redox regulation of AP-1 oncogenes have been also consistently reported [37].

The human/mice AP-2 transcription factors family consists in five members, AP-2α/β/γ/δ and ε, respectively, characterized by a highly conserved helix-span-helix dimerization motif at the carboxyl terminus, AP-2 homo- or heterodimers act as a negative regulator of sod2 by suppressing Sp1-dependent expression [38]. Several cancer cell lines display mutations in the basal sod2 promoter region accounting for an AP-2 binding increase concomitant with a decrease in the promoter transcription activity [39]. Other transcription factors involved in the regulation of sod2 expression include Nrf2 [40], Hif-1 [41], or the pluripotent stem cell transcription factors Nanog or Oct4 [42].

2.2.4. Post-Transcriptional Modification

Post-transcriptional modification (PTM) is one of the two levels (beside the mRNA splicing) of creating protein diversity; accordingly, SOD2 can also be regulated at post-transcriptional level by different mechanisms including nitration, acetylation or deacetylation (**Figure 1B**).

2.2.4.1. NITRATION

Even though nitration is a common marker for oxidative stress, it is as well one of the major oxidative post-transcriptional modifications, specifically at carbon 3 in certain tyrosine residues. 3-Nitro-Tyr is increased in diseased tissues, but it does not occur randomly but rather in certain proteins as it has been shown in animal models of sepsis or diabetes [43]. In SOD2 [44, 45], when 3-nitro-Tyr and further oxidation occur it may account for up to 50% of enzymatic inactivation of SOD2 [46].

2.2.4.2. ACETYLATION/DEACETYLATION

It is a key regulatory PTM mechanism for many proteins including several transcription factors. Acetylation at the ε-NH2 of lysine (i.e., lysine acetylation) is a very common, ancient and reversible PTM [47]. Several studies have reported that the decrease in SOD2 protein activity is dependent on sirtuin 3 (SIRT3), a type III mitochondria-targeted deacetylase [48].

3. FIRST STUDIES IN SOD2 GENE: SOD2 AS A TUMOR SUPPRESSOR GENE AND GENETIC VARIATIONS

3.1. SOD 2 as a Tumor Suppressor Gene

One of the theories to explain initiation and progression in tumor development is the accumulation of faults in the DNA due to ageing processes. These faults are caused in part by oxidative stress, which will eventually affect to those genes involved in cell cycle control, DNA reparation or cell death initiating an oncogenic process.

Based on this initial hypothesis, first studies performed in the 1990s by Oberley and colleagues showed some very innovative and promising results. They postulated that MnSOD levels were reduced in different cancer types compared with non-tumor tissue, and that the decrease in SOD2/MnSOD and the antioxidant defenses would facilitate the acquisition of more mutations leading to tumor progression (**Figure 2**) [49]. Consequently with these results, Oberley and colleagues described a decrease in SOD2/MnSOD during estrogen-induced tumorigenic transformation in kidney [50] and later St Clair and colleagues were able to block malignant transformation caused by radiation in a mouse embryonic cell lines by overexpressing human MnSOD [51].

The subsequent publications showed the drastic effect of MnSOD overexpression inhibiting growth and tumorigenesis in different cancer cell lines. In melanoma for example, overexpression of MnSOD reduced both tumorigenicity in nude mice and growth capacities in agar. However, no direct results in proliferation were reported in this article [52]. Also, in a skin cancer model, *Sod2*-overexpressin transgenic mice showed a reduced tumor incidence by suppressing AP-1 [53]. Another work using the estrogen-dependent breast cancer cell line MCF-7 described that MnSOD was able to almost arrest completely the cell cycle. Increased protein levels affected in vitro growth, colony and tumor formation capacities even in very pro-mitotic environments with serum and pyruvate concentrations up to 20% and 12 mM respectively [54]. A remarkable article, also product of the collaboration between St Clair and Oberley groups in glioma, was one of the first one taking into account the impact of the overexpression of MnSOD upon other antioxidant enzymes. This work shows the reduction in tumor growth and

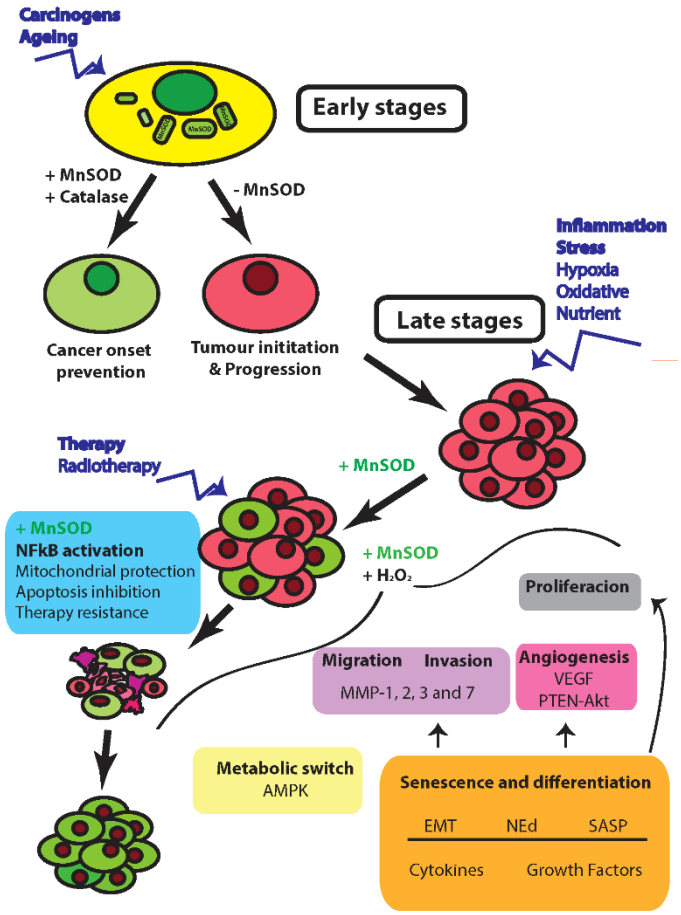

FIGURE 2. MnSOD/SOD2 changes in tumour development. In early stages normal cells with high levels of antioxidant enzymes such as MnSOD and Catalase can be more resistant to environmental carcinogens and age-related neoplasic processes. One of the major sources of oxidative stress is the mitochondria and MnSOD protein helps to scavenge the excess of reactive locally but also acts as a drain of free radicals balancing the redox state of the whole cell, alleviating exposure to external carcinogens. Tumour cells show lower levels of antioxidant enzymes compare with non-tumorigenic cells and normal cells shows higher levels than hyperplasic and neoplastic, low levels of antioxidant enzymes favour tumour transformation. During late stages of the tumour progression some stimuli such as inflammation or stress (hypoxic, oxidative or nutrient deprivation due to cell density for absence of vascularization) can induce an increase of MnSOD in some tumour cells, this increase can facilitate resistance to treatments such as radiotherapy. Anti-tumour therapy increases free radical levels and MnSOD helps to keep the radicals in the viability range improving mitochondrial protection, those radicals can also activate NF-κB inhibiting apoptosis and favouring therapy resistance. When the H_2O_2 produced by the MnSOD is not scavenge by catalases or peroxidases the subsequent increase has some effects in the tumour cells: (a) Increase of invasiveness and migration by producing matrix metalloproteinases (MMPs) that degrade the extracellular matrix and trans-differentiation into a mesenchymal phenotype (EMT) which increases the mobility. (b) Angiogenesis by inhibiting PTEN which activates Akt and promotes angiogenesis and transdifferentiating cells into neuroendocrine phenotype (NEd) or secretory senescent phenotype (SASP) which produce VEGF and cytokines which reinforces angiogenic signal. (c) Proliferation moderate levels of H_2O_2, Akt upregulation and the growth factors produced by NEd and SASP cells induce proliferation. (d) Metabolic switch, increase in glycolytic metabolism by activating AMPK.

volume in vivo, but a modest effect on proliferation in vitro at normal serum concentrations (10%) [55].

Although very promising, the inconsistent effect of MnSOD in some of the features, for example proliferation, reduced the impact of these works. The range of variation on the effect among different cell lines might be due to two reasons. First the basal levels of MnSOD in each cell line: U118 showed 3–4 times more basal activity than MCF-7 indicating a very different basal redox state. On the other hand, these first publications, although illustrated very carefully the increase in enzymatic activity, did not analyze neither the general redox state nor the specific redox state in different subcellular locations, nowadays known to be essential to understand the array of effects of MnSOD. Finally, later publications in tumor and non-tumor cell lines [56, 57] placed MnSOD as an anti-tumor protein, and for some years it was suggested as a tumor suppressor gene [58].

However, the concept of tumor suppressor was in clear conflict with previous and coetaneous works. The articles signed by Ishikawa and Kawamura reporting an increase of circulating levels of MnSOD in ovarian cancer [59] and neuroblastoma [60], were soon followed by a small but very interesting study in brain tumors by Cobbs. In this work the levels of MnSOD were assessed by immunohistochemistry in more than 7 different groups of central nervous system malignancies including distinct stages of astrocytoma, medulloblastoma and ependymoma, and compared with control or pre-neoplasic tissue. The results showed an increase of MnSOD in tumor samples. A more robust study of MnSOD in 163 colon adenocarcinoma human samples support these previous results, normal mucosa samples presented lower levels of MnSOD than carcinoma tissue. Moreover, the 5-year overall survival was smaller in those patients that showed higher levels of MnSOD [61].

These two opposite sets of results, especially those ones referred to nervous system tumors, in vitro results by St Clair group [55] and the preliminary results in patients by Cobbs, seemed to indicate a complex role for MnSOD beyond that first statement as tumor suppressor gene. Why these sets of data are showing an opposite trend could be explained by a main difference: the kind of data. Those results that support the tumor suppression gene role were mainly in vitro, and MnSOD expression levels were increased by genetic modification to very high levels, therefore, very unlikely to be reached in the physiological environment. On the contrary those results observed in patients seemed to support an active role of MnSOD as a mediator of tumor progression and invasion in the systemic biological context.

3.2. Genetic Predisposition to Cancer Due to SOD2 Polymorphisms

Different polymorphisms have been described for the SOD2 gene (**Figure 1B**), among them one has been widely studied. The polymorphism Ala16Val appears in the signaling peptide that leads the MnSOD protein from the cytoplasm to the mitochondria [62]. The presence of a triplet GCT or GTT produces Alanine, folding the initial part of the protein in α-helix, or Valine, folding in a β-sheet, respectively. This difference alters the abundance of the RNA and protein which is higher for the Ala form but also import of the protein in to the mitochondria due to an increase in the membrane retention of the protein with Val variant and a decrease in the net pool of active protein [63, 64].

The prevalence of the polymorphism in normal population is quite variable depending on the population group. Remarkable differences between races and ethnicities have been documented. The frequencies in control populations vary among different groups: the Asian population described by Ho et al. [65] showing Ala 3%, Ala/Val 22%, and Val 75%; the European populations from Czech Republic [66] showing Ala 21%, Ala/Val 50%, and Val 29% or UK showing Ala 17%, Ala/Val 60%, and Val 23% [67]. This indicates there is no prevalent allele in normal population.

During the late 1990s and the early 2000, some promising studies described a clear correlation between the genotype and the breast cancer incidence. For example, the presence of the Ala allele was shown to increase the risk to develop breast cancer in pre-menopausal women and post-menopausal with low vitamin intake [68]. Also other works postulated the Ala allele as a risk factor for breast cancer in post-menopausal women [69]. A protective effect of the Val/Val genotype was observed for prostate cancer incidence and progression [70]. However later meta-analysis failed to prove the consistency along different studies or several cancer types [44, 45]. The counterbalance effect of the antioxidant intake from the diet or the genetic background of other oxidative stress related genes might explain this inconsistency.

4. MnSOD IN ADVANCED STAGES OF CANCER

As we have mentioned in previous sections contradictory findings about the role of MnSOD in cancer have made this mitochondrial enzyme a matter of debate in the field of tumor biology. Thus, when reviewing literature, it can be found that some manuscripts claimed that MnSOD is a protein suppressing cancer progression while several others claimed that, on the contrary, MnSOD might participate in migration and invasion and metastasis. In fact, the pro-malignant potential of some antioxidants (enzymes or low molecular weight molecules) has been increasingly recognized. Consequently, with the latter, in vitro studies have demonstrated that a number of cancer cell types contain elevated levels of MnSOD and that this correlates with increased metastasis, proliferation, and resistance to apoptosis [71, 72] (**Figure 2**). In addition, several epidemiological studies have shown increased immunoreactivity of MnSOD in a variety of tumor types that are associated with poor patient outcome and more aggressive disease [61, 73, 74].

Perhaps the first experimental evidence showing that the accumulation of antioxidant proteins in cancer cells must represent an adaptive advantage for cancer cells was observed by Huang and collaborators [75]. They found that treatment of leukemia cells with 2-methoxyoestradriol (2-ME), an estrogen derivative that cannot bind estrogen receptor, substantially reduced cell survival. By using a human genome atlas to search for a candidate of its action, they found that CuZnSOD was 236% increased with respect to the control cells. In fact, 2-ME treatment caused a dose-dependent increment of intracellular H_2O_2. 2-ME specifically inhibited CuZnSOD and MnSOD, and this inhibition enhanced 2-ME toxicity. Collectively, these results indicated that SOD inhibition was responsible of cell death. Since superoxide accumulation was found, they proposed a combination of 2-ME treatment with chemo/radiotherapy as an effective mechanistic way to induce cell death in cancer cells. Independently, other groups have also reported elevated levels of MnSOD in gastric and colorectal adenocarcinomas with a clear correlation with aggressiveness and poor overall survival of patients [73, 76]. The activity of MnSOD was increased in the cancer tissue when compared with the normal adjacent non-cancer tissue in patients with colorectal carcinoma and particularly, in those patients who suffered from venous invasion [76].

4.1. Regulation of Matrix Metalloproteases

Connor et al. [77] nicely described that fibrosarcoma and bladder cancer cells enhance their invasive and migratory activity after MnSOD overexpression. The cells present at the leading edge of the wound in a wound healing assay expressed higher levels of the protein. Furthermore, migration and invasive properties of both cell lines were reversed by overexpression of catalase, thus indicating a key role for the H_2O_2 caused by MnSOD overexpression as the main driver. The in vivo metastatic potential, motility, and invasive properties of HT-1080 fibrosarcoma cells are higher in those cells with higher levels of MnSOD. These effects are partially mediated by the H_2O_2-mediated increases in matrix metalloprotease (MMP)-1 gene expression. Some years before, the same group had already demonstrated that MnSOD regulated not only MPP-1 but also MMP-2, -3, and -7 through an ERK1/2 activation mechanism [78]. By using a dominant negative construct, they elegantly demonstrated that H_2O_2 production by MnSOD overexpression mediated the increment of MMPs by Ras/ERK/AP-1 signaling pathway. In support of this hypothesis, it was also demonstrated that overexpression of MnSOD in dermal fibroblasts caused a profound increment in MMP-1 transcription when superoxide was administered to cells due to an increase in H_2O_2 levels by the dismutation function of MnSOD [79].

Similarly, MnSOD rise was observed in highly metastatic bladder cancer cells 253J-BV when compared with the 253J parental cell line [80], concomitant with an increment of H_2O_2. Increased levels of MnSOD observed in 253J-BV cells were directly related to an increment in MPP-9 and VEGF production. In addition, highly bladder metastatic cells showed reduced levels of catalase without any changes in GPX or GSH, and overexpression of catalase resulted in a significant inhibitory effect on the metastatic cells by decreasing MMP-9 activity and suppressing the clonogenic activity. It seems that the development of the metastatic phenotype in bladder cancer could be associated with alterations in the redox state by the increment in the steady state H_2O_2 levels, derived from the increased MnSOD activity. Overall, it would account for the overexpression of a

number of metastasis-related growth factors and may enhance their metastatic behavior.

4.2. Inactivation of Phosphatase and Tensin Homolog

In addition to a protease activity increment, other important pathways closely related to cancer biology are similarly altered by MnSOD overexpression. In fact, the overexpression of MnSOD in fibrosarcoma cells modified phosphatase and tensin homolog (PTEN) activity. PTEN, a tumor suppressor protein, is sensitive to redox regulation through cysteine oxidation, and the formation of the corresponding disulfide results in its inactivation [81]. As a consequence, PTEN inactivation by MnSOD overexpression is responsible of an increment of phosphorylation of AKT at serine 473, which in turn increases *VEGF* gene transcription and therefore favoring angiogenesis in in vitro and in vivo models [82]. In that sense, MnSOD overexpression in tumors not only would favor migration of cancer cells from in situ tumors but also would favor the gain of vascularity, which may promote the dissemination and progression of the tumor in advances stages. This work also pointed out the important role of PTEN redox regulation through the PI3K signaling axis. The reversibility of PTEN oxidation and the downstream signaling pathways by cytosolic or mitochondrial catalase was evidence for the oxidative damage of PTEN function by mitochondria-derived H_2O_2 in a human tumor cell line, in the absence of DNA mutation.

Loss of tumor suppressor activity of PTEN has been associated with a number of tumors, including prostate cancer, and with poor prognosis [83] by promoting migration and invasiveness [84]. It can be deduced that prostate cancer must be also dependent on oxidative status, and several publications have focused on this type of tumor. In fact, overexpression of MnSOD inhibited androgen-dependent prostate cancer cells growth [85], and the expression and activity of CuZnSOD, MnSOD, and catalase were lower in prostatic intraepithelial neoplasia and prostate carcinoma than in benign epithelium [86]. However, our own group demonstrated that neuroendocrine prostate cancer cells showed elevated levels of MnSOD [87]. Moreover, the overexpression of MnSOD drove androgen-sensitive prostate adenocarcinoma cells into neuroendocrine differentiation without losing the proliferative state, displaying an androgen independence and increased survival, suggesting an association with increase in cancer progression [88]. More recently, it has also been reported that MnSOD levels were elevated at the invasive edge of primary prostate cancer [89]. Collectively, it appears that, on the one hand, MnSOD overexpression inhibits tumor cell growth, but on the other hand, it could promote metastasis even in the same tumor type. These differences may depend on the ability of cells to detoxify H_2O_2 maintaining their steady state levels. In that sense, we have recently demonstrated the critical role of MnSOD-catalase-GPX balance as a biomarker of cancer progression in prostate, lung, or colon cancer [90]. We found that not only an increment of MnSOD was associated with tumor progression, but also a decrease in catalase and GPX was observed thus resulting in an increment of H_2O_2 levels. Since neuroendocrine differentiation is associated with poor prognosis in patients suffering prostate, lung, and colon cancer, these data agree with previous work done by Melendez's group and others, proposing H_2O_2 as a critical metabolite in cancer progression.

4.3. Metabolic Switch

Probably related with its strategic role in the mitochondria, several links showed that MnSOD is both sensitive to the metabolic state and able to modulate it. Hyperglycemia-induced pancreatic cancer cell invasion upregulates SOD2 through H_2O_2 increase which in turn activates ERK/p38 MAPK pathways as well as transcription factors NFκB and AP-1 [91].

In addition, it has been shown that MnSOD overexpression activates AMP activated kinase (AMPK). MnSOD overexpression as well as H_2O_2 treatment significantly increased AMPK phosphorylation and consequently its activity in breast, colon, and prostate cancer cells [92]. The activating effect of MnSOD on AMPK shifts from cellular oxidative respiration to glycolysis, i.e., the so-called 'Warburg effect'. When persistently overexpressed, MnSOD contributes to the progressive deterioration of mitochondrial bioenergetics and the activation of glycolysis via mitochondrial H_2O_2. The MnSOD-H_2O_2-AMPK axis was postulated as a primary regulator of this metabolic switch in tumor cells.

The question that arises from all these experiments is how MnSOD is regulated in advanced stages of tumors to be overexpressed, but this issue is still

poorly understood. An increased expression of MnSOD would be responsible for the activity of redox sensitive transcription factors, such as NFκB, through a simple feedback regulation. Finkel's group proposed that the increase in metabolic flow in cancer cells augmented ROS production which in turn, through redox-regulated transcription factors such as AP-1 or NFκB, might increase the synthesis of MnSOD [93]. On the other hand, one of the master tumor suppressors, p53, regulates MnSOD by post-transcriptional mechanisms. Thus, low levels of p53 increased MnSOD activity to promote cell survival. Since p53 is inactivated or mutated in numerous types of tumors, this could easily explain the increment of MnSOD protein levels or its activity in advanced stages of cancer [94].

Cancer usually evolves to a more resistant phenotype and embraces adaptive advantages during progression. MnSOD, which is initially fundamental in keeping the integrity and health of normal cells, might be employed by cancer cells to battle exogenous insults and prolong their survival in adverse conditions such as anoxia, low glucose availability, or the increment of free radicals due to antitumor treatments. It is noteworthy to mention that, besides MnSOD, other important cellular antioxidants (e.g., thioredoxin, glutaredoxin, and peroxiredoxin) are similarly upregulated in certain tumors, apparently to adapt cancer cells to a higher ROS production and therefore the scenario of redox biology in tumor cells could be much more complicated than initially assumed.

5. MNSOD IN RESISTANCE TO THERAPY

Several studies have shown the critical role of MnSOD in cell survival, first against cytokines [95] and later on the resistance of tumor cells to radiation or chemotherapy. MnSOD prevents apoptosis induced by several stimuli, particularly those which trigger free radical production. Mostly, its role in preventing cell death is related to its particular location and the superoxide dismutation; nevertheless, other mechanisms have not been yet discarded. First, it was demonstrated that MnSOD prevented tissue damage caused by radiation in normal, non-tumoral, tissues and leukemia cells from radiation [96, 97]. Since chronic exposure to gamma-radiation increases survival signals that allow cancer cells to cope with ROS generated during the treatment, role of MnSOD in preventing radiation-induced apoptosis was initially demonstrated, and the induction of a subset of genes that participated in radioresistance in response to fractionated ionizing radiation was also described [98] (**Figure 2**).

5.1. NFκB

Ionizing radiation causes hydrolysis of water inducing formation of highly reactive hydroxyl radicals, long-term exposure to irradiation promotes mitochondrial dysfunction, thus increasing superoxide and H_2O_2 beyond the viability threshold causing cell, tissue, or organ damage. One of the principal regulators of adaptive responses to radiotherapy is the transcription factor NFκB which is in turn quickly activated after many types of stresses or insults, including radiation. Interestingly, stress-induced NFκB is then able to abrogate apoptosis and increase survival pathways, causing radiation or chemotherapy resistance [99]. As a yin-yang factor, NFκB would protect normal cells and tissues from gamma-radiation or chemotherapy-associated side effects, but eventually it would be equally responsible for the activation of adaptive responses in cancer cells. In fact, NFκB and its multiple regulating targets including MnSOD cooperate in inducing adaptive responses to ionizing radiation [99].

In addition to a reduced balance in the oxidative status, MnSOD might participate in adaptive responses to radiation by altering downstream pathways. Thus, in epithelial skin cells it has been demonstrated that MnSOD cooperates with NFκB in the activation of Cyclin B1 and 14-3-3 pro-survival proteins [100]. Both were found tightly regulated after low-dose irradiation in epithelial cells, with a positive correlation with MnSOD activity. Neuroblastoma, ovarian, hepatocellular, and oral squamous cancer cells overexpressing MnSOD have been consistently reported to be more resistant to radiation-induced cell killing compared to control cells [101].

Goswami's group has further demonstrated that, in pancreatic cancer cells, cells overexpressing MnSOD showed a direct correlation between MnSOD activity and survival fraction after low-dose irradiation. After irradiation, pancreatic cells showed an increase in ATM phosphorylation, which was prevented by MnSOD overexpression and correlated with a reduc-

tion in H2AX activity [102]. Inhibition of MnSOD increased the sensitivity of these cells to radiation-induced cell death. The authors proposed cell cycle checkpoints, and in particular, the G2 checkpoint as a potential target of MnSOD induced radioresistance and they further proposed MnSOD as a prognosis marker for cancer cell radiosensitivity.

More recently, new players in this intricate scenario have come out. Sirtuins, a subfamily of NAD^+-dependent histone deacetylases, play a role in stress-response and survival signals in normal and cancer cells. Specifically, mitochondrial SIRT3 enhances mitochondrial respiration and reduces oxidative stress by regulating several key metabolic proteins including MnSOD. SIRT3 is regulated in tumor cells under genotoxic conditions, and downregulation of SIRT3 sensitizes squamous carcinoma cells, mainly by regulating G2/M transition and MnSOD2 protein levels and activity [103]. By using human colon cancer HCT-116 cells, breast MDA-MB231, and glioblastoma U87 cells, Liu and coworkers found that SIRT3 was increased after 5-Gy irradiation. Chromatin immunoprecipitation (ChIP) assays and promoter mutation studies have shown that NFκB activation is responsible for SIRT3 increment. Moreover, increased MnSOD activity was found after irradiation in cells expressing wild-type SIRT3 but not in those expressing mutant SIRT3. Since SIRT3 activates MnSOD by deacetylation mechanisms and NFκB activity increases MnSOD levels by transcriptional regulation, there is a cooperative cycle among several adaptive mechanisms to protect cancer cells from radiotherapy-induced death by contributing to mitochondrial metabolic homeostasis and resistance to radiation [104].

5.2. Inhibition of Cell Death

MnSOD overexpression counteracts chemotherapy-induced cell death. In addition to the early evidence by Wong et al. [19, 95] demonstrating the role of MnSOD in protecting cells from cytokines-induced apoptosis, due to an increase in differentiation and an inhibition of apoptosis in fibrosarcoma cells [105]. In addition, it was found that MnSOD protected colorectal cancer cells by inhibiting mitochondrial apoptosis pathways. MnSOD overexpression impeded cytochrome c and SMAC/DIABLO release to the cytosol, resulting in protection against TRAIL-induced apoptosis [106]. These results confirmed previous report by Mantymaa et al., who demonstrated that etoposide-resistant subclones of myeloid leukemia cells show increased MnSOD levels, preventing mitochondrial membrane potential disruption and increasing BCL-2 protein levels [107]. More recently, Pervaiz and Clement have demonstrated similar results by using highly metastatic, estrogen receptor (ER)-negative breast cancer cells. The repression of MnSOD levels by siRNA in these breast cancer cells rendered them more sensitivity to doxorubicin and docetaxel, via a mechanism mediated by overproduction of mitochondrial ROS and peroxynitrite [108].

A combined effect upon metastatic abilities and prevention of cell death have been further proven in different scenarios. MnSOD overexpression inhibits anoikis in response to detachment from extracellular matrix (EM) [109]. Such detachment from EM is considered as one of the first key events in cancer progression and metastasis. After detachment, non-transformed cells should suffer a type of programmed cell death called anoikis, which occurs concomitantly with an increase in mitochondrial ROS. Upon detachment, an increase in MnSOD expression, mostly mediated by NFκB activation will impair anoikis by scavenging the superoxide while MnSOD depletion on the contrary will enhance it. Since resistance to anoikis is a prerequisite for cancer metastasis, it is feasible that MnSOD would be overexpressed during breast cancer metastasis. In fact, the same report demonstrated that high MnSOD expression correlates with advanced tumor grades and overlaps with other poor prognosis markers including p53 mutation and negative ER or progesterone receptor (PR) in human breast cancer samples. Since NFκB is a survival factor for cancer cells, the increment of NFκB-MnSOD as an inhibitor of cell death induce by detachment in non-transformed cells points out MnSOD as a mediator of NFκB pro-survival activity in suspended cells.

5.3. Cancer Stem Cells

Finally, the group of Clarke proposed an association between ROS and radioresistance in cancer stem cells. They found low ROS levels in several types of normal tissue stem cells, and that the ROS levels are lower in cancer stem cells (CSCs) than their non-tumorigenic progeny cells (NTCs). Using microarray data from human breast CSCs, they found that this low concentration of ROS was directly related to an

increase in antioxidant enzymes activity including MnSOD [110]. Since, as previously proved, radiation toxicity is caused by an increment of ROS, an increase in the expression of antioxidant enzymes found in CSCs is responsible for the radioresistance observed in these cells and supported by the lower levels of DNA damage after irradiation in CSCs as compared with NTCs. These data demonstrate, for the first time, the importance of low ROS levels and endogenous antioxidant defenses to CSC survival and radiosensitivity.

6. MnSOD IN DIFFERENTIATION AND SENESCENCE

The levels of MnSOD protein and H_2O_2 have been proven as a differentiation trigger in a wide range of cell types, organisms, and pathological and non-pathological conditions. From fungi during conidia differentiation [111] to kidney embryonic development in rat [112] or oocyte differentiation in cow [113] upregulation of MnSOD gene (SOD2) seems to be a highly conserved feature among species. As it happens for other features that occur during development and differentiation, some pathological processes can reactivate these programs promoting the disease to aggravated stages. An example is the high concentration of H_2O_2 in aging-related pathologies such as vascular calcification [114, 115] and cancer. A summary of the effects of MnSOD in tumor cells is provided in **Figure 2**.

6.1. Neuroendocrine Differentiation

Several papers have shown this effect on differentiation over tumor cells, as mentioned before, Church et al. also reflected the clear increase in differentiation in the melanoma cells which overexpressed MnSOD [52]. Further publications have supported the link between MnSOD and differentiation in tumors by describing an increase in protein levels during phenotypic changes in cancer cell lines. The group led by St Clair described the increase of MnSOD due to NFκB activation during differentiation of murine fibrosarcoma cells into a myofibroblast-like phenotype [105]. Our own group described the link between an increase in MnSOD and neuroendocrine differentiation, a phenotypic change occurred during the progression of several types of adenocarcinoma related with therapy resistance, hormone-independence, and tumor progression. Interestingly, this phenomenon was rather common, and MnSOD levels were increased with any of the three stimuli used to induce the phenotype in prostate adenocarcinoma cell lines [87] or with two additional treatments. Later results confirmed similar findings in other types of adenocarcinoma (unpublished data). Whether MnSOD was enough to induce the phenotype was demonstrated in the androgen-dependent prostate adenocarcinoma cell line LNCaP in which overexpression of MnSOD was able to mimic the neuroendocrine phenotype including androgen-independence [88]. Other groups also reported this association on MnSOD in neuroblastoma cells SH-SY5Y, which expressed higher levels of MnSOD when differentiated into neuron-like phenotype by retinoic acid [116].

6.2. Epithelial-Mesenchymal Transition

Supporting the same main concept that MnSOD and H_2O_2 could regulate other differentiation programs, some studies have pointed out their role in epithelial-mesenchymal transition (EMT). The EMT is a trans-differentiation process observed in cancer and other diseases by which the epithelial cells loose part of their morphological and biochemical properties to express others typically associated with the mesenchymal lineage. Normal mesenchymal cells are pluripotent cells that can differentiate into osteoblast, osteoclasts, adipocytes, and muscle cells. In cancer, this trans-differentiation is associated with invasion and metastasis [117]. Physiological concentrations of H_2O_2 are able to induce EMT through TGFβ signaling pathway in lung adenocarcinoma cells [118]. More recently, MnSOD has been shown to induce EMT in colon cancer cell lines [119] and in esophageal cancer cells. A main criticism on this work is that it lacks partially a solid mechanistic demonstration, probably due to the massive alteration in basal redox state when MnSOD is interfered in these cell lines [120].

6.3. Cellular Senescence

Cellular senescence is a state of stable exit from the cell cycle [121]. Two main features are worth to be especially mentioned regarding senescence: (1) different stimuli can trigger senescence; (2) the senescent phenotype is very complex and can exhibit

different subtypes, and among them is senescence-associated secretory phenotype (SASP). Senescence has been demonstrated as an anti-tumor strategy, and damaged cells will undergo senescence induced by, for example, p53 [121]. However, it has been described that, analogously to neuroendocrine differentiation, SASP cells can secrete bioactive molecules promoting tumor growth, survival, and invasion. In fact, these two cell programs share several features. One of these features is that both are activated by changes in MnSOD and H_2O_2 levels.

First results from early 2000s showed NF-κB-mediated increase in MnSOD in senescent keratinocytes. The senescent phenotype could be partially rescued by addition of catalase to the media. The subsequent induction of senescence by physiological concentrations of H_2O_2 (30 μM) in keratinocyte culture proved that the induction of senescence in this cell culture model was likely due to an increase in H_2O_2 and MnSOD [122]. The same group demonstrated later also in keratinocytes that a higher but physiological concentrations of H_2O_2 (50 μM) or a long exposure to MnSOD overexpression could lead to autophagy [123], reinforcing the idea that redox regulation can have different effect depending on intensity and spatial-temporal exposure. Recently, another study has shown that an increase in H_2O_2 intracellular production in response to IL-1α is able to induce SASP in fibroblast, and that the conditioned media of these fibroblasts increases invasion and some features of EMT transition in breast and lung adenocarcinoma cell lines [124].

Although the fact that an increase in H_2O_2 causes senescence is widely accepted, whether MnSOD is the mediator of that increase remains unclear. Some papers have described a rise in MnSOD levels during impairment of senescence. In the article by Park et al., malignant transformation was induced by overexpressing the oncogene H-Ras. A further interference of the transcription factor FoxM1 was used to show its role in senescence. The inhibition of this transcription factor drastically decreased levels of MnSOD and catalase and altered the basal redox state of the cell lines used [125]. These changes might be beyond the physiological range of MnSOD/H_2O_2, and in fact, H_2O_2 treatment (used at a high concentration within the biological range, 100 μM) increased the percentage of cells upon senescence program. This may indicate that in this experimental model, cells are close to the viability threshold as the authors illustrated by showing a decrease of 20% in the viability in cells exposed to 100 μM of H_2O_2. Another work by Iglesias-Bartolome et al. also described MnSOD as the mediator of rapamycin to avoid senescence in normal culture and under radiation treatment. Although the authors claim the effect impairing senescence of rapamycin is mediated by MnSOD, they do not show the effect of SOD2 inhibition upon the number of senescent positive cells [126]. In addition, the effect of the drug is specific, i.e., increasing the MnSOD protein levels in non-tumor cells only.

As a summary, we can conclude that the articles discussed above indicate that the intracellular concentration of H_2O_2 within a tightly regulated range is a powerful and highly conserved tool to control cell cycle and differentiation. In addition, MnSOD probably due to its plasticity in terms of regulation of gene expression and post-translational control is the main tool to regulate H_2O_2 for this cell cycle and differentiation role in normal tissue, as well as also in several tumor types.

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