

The Role of Reactive Oxygen Species in Mesenchymal Stem Cell Adipogenic and Osteogenic Differentiation: A Review

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Mesenchymal stromal cells (MSCs) are promising candidates for tissue engineering and regenerative medicine. The multipotent stem cell component of MSC isolates is able to differentiate into derivatives of the mesodermal lineage including adipocytes, osteocytes, chondrocytes, and myocytes. Many common pathways have been described in the regulation of adipogenesis and osteogenesis. However, stimulation of osteogenesis appears to suppress adipogenesis and vice-versa. Increasing evidence implicates a tight regulation of these processes by reactive oxygen species (ROS). ROS are short-lived oxygen-containing molecules that display high chemical reactivity toward DNA, RNA, proteins, and lipids. Mitochondrial complexes I and III, and the NADPH oxidase isoform NOX4 are major sources of ROS production during MSC differentiation. ROS are thought to interact with several pathways that affect the transcription machinery required for MSC differentiation including the Wnt, Hedgehog, and FOXO signaling cascades. On the other hand, elevated levels of ROS, defined as oxidative stress, lead to arrest of the MSC cell cycle and apoptosis. Tightly regulated levels of ROS are therefore critical for MSC terminal differentiation, although the precise sources, localization, levels and the exact species of ROS implicated remain to be determined. This review provides a detailed overview of the influence of ROS on adipogenic and osteogenic differentiation in MSCs.

Introduction

REACTIVE OXYGEN SPECIES (ROS) are oxygen-derived small molecules, which react readily with a variety of chemical structures such as proteins, lipids, sugars, and nucleic acids. Most ROS that have been described in living organisms include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), hydroxyl ion (OH^-), and nitric oxide (NO). ROS are often termed free radicals; this does not apply to H_2O_2 and $ONOO^-$, which are nonradical ROS.

Chemists first discovered free radicals and described their highly reactive nature. Biologists then investigated the role of free radicals in biological systems. In 1956 Harman, a radiation biologist, introduced his noteworthy observations on the role of ROS in the aging process that were similar to his findings on radiation damage [1]. Shortly afterward the concept that emerged was that ROS lead to cellular damage in aging [2].

Nowadays, it is increasingly recognized that ROS are involved in the regulation of cell function despite the fact

that for many years they were considered to be harmful elements in biological systems. Indeed high levels of ROS cause cell damage by oxidation and nitration of macromolecules including DNA, RNA, proteins, and lipids. The concept that ROS are harmful was confirmed by the discovery of ROS detoxifying enzymes (eg, superoxide dismutase SOD, catalase, etc.), scavengers (eg, vitamin C and E) and the bactericidal activity of neutrophils, which is strongly dependent on the generation of large amounts of ROS in the phagosome. However, this concept was challenged following the description of a family of enzymes called NADPH oxidases (NOX-es) at the beginning of the 20th century. NOX enzymes generate ROS by oxidizing intracellular NADPH to $NADP^+$ and the transfer of electrons through membranes to reduce molecular oxygen and generate the superoxide anion as a primary product.

It is currently believed that only unregulated levels of ROS are harmful, while regulated ROS production promotes essential signaling pathways, which regulate cell functions [3] such as cell proliferation, differentiation, survival, and

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apoptosis [4–6]. Redox regulation or controlled ROS generation is the net effect of a subtle balance between ROS generation and neutralization/utilization by cellular antioxidant systems. Thus oxidative stress represents an unbalanced situation in which ROS generation exceeds antioxidant systems leading to tissue damage.

The responses of adult human stem cells to different stress stimuli such as oxidative stress, heat shock, and γ -radiation have been widely studied in the context of tissue repair, tissue engineering, and transplantation [7]. Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells that can be isolated from the mesenchyme or stroma of several tissues including bone marrow and adipose tissue [8–10]. Within this heterogeneous population is a subpopulation of cells with self-renewal and multipotent differentiation capacity that can be qualified as stem cells. Other sources include dental pulp, umbilical cord (Warton's jelly), cord blood, placenta, peripheral blood, and amniotic fluid [11–14]. There is an ongoing and intense debate regarding the precise origin, nature, and therapeutic potential of MSCs [15]. However, from an experimental perspective, MSCs display the following features: (1) following isolation, primary cultures of MSCs are plastic adherent and remain plastic adherent during subsequent propagation; (2) depending on their source, MSCs express several cell surface markers such as CD73, CD90, CD105, and CD44, but not CD31 or CD45, although there are many other markers that may be considered and may also be used to differentiate MSCs from different sources; (3) MSCs have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro* [16,17]; and (4) confirmation of the existence of the stem cell subpopulation requires *in vitro* clonogenic assays and demonstration that the cells have the ability to differentiate along the desired lineage *in vivo* [18].

MSCs from several sources are being assessed in a large number of clinical trials in a wide range of settings based on the following assumptions: (1) MSCs have the ability to home to sites of injury and inflammation; (2) MSCs have the ability to differentiate into cells of the mesodermal lineage; (3) MSCs secrete trophic factors that promote proliferation and differentiation of local progenitor cells [19]; (4) MSCs induce or increase neovascularization; (5) MSCs have immunomodulatory properties [20]; and (6) MSCs produce survival factors in ischemic tissues and have antioxidant properties [21]. However, despite the enormous effort that has thus far been invested into clinical trials, very few if any therapies have become part of routine clinical practice.

MSCs are known to have low levels of intracellular ROS and high levels of glutathione, a key antioxidant. They also constitutively express high levels of enzymes required to manage oxidative stress. For example, when compared with the pancreatic beta cell line INS-1, expression of SOD1, SOD2, CAT, and GPX1 was significantly higher in MSCs. These enzymes are able to scavenge peroxide and peroxynitrite (ONOO⁻). Thus, it has been proposed that the high antioxidant capacity of MSCs makes them ideal for the treatment of pathologies in which tissue damage is linked to oxidative stress [21].

In terms of redox regulation, numerous recent reports describe the importance of oxidants on MSC differentiation into adipocytes [22], osteocytes [23], chondrocytes [24], and myocytes [25] through activation of signaling cascades in-

involved in differentiation [26–32]. Increased adipogenic fate suppresses the osteogenic lineage, while upregulation of osteogenic signaling attenuates adipogenic terminal differentiation. Many signaling pathways such as Wnt, FOXO, Hh, NEL-like protein 1 (NELL-1), insulin-like growth factor (IGF), and bone morphogenetic protein (BMP) determine MSC terminal fate. Among the pathways that favor either adipogenic or osteogenic differentiation via their activation or suppression, IGF and BMP have a dual effect. These pathways positively regulate both adipogenesis and osteogenesis [28,33–36].

There is evidence for the role for ROS in MSC survival, proliferation, and terminal differentiation, and ROS affect adipogenesis or osteogenesis by stimulating or inhibiting several MSC differentiation signaling cascades. Recently, the impact of ROS on MSC differentiation has generated a great deal of interest due to its potential application in the clinic (ie, diabetes [37], hypertension [38], atherosclerosis [39], carcinogenesis, and aging [40]). Understanding the impact of ROS on MSC terminal fate will increase our knowledge of the nature and behavior of these cells and how this may be harnessed for therapeutic purposes. This includes the use of ROS inhibitors/activators as pharmaceutical agents. This review focuses on the various potential sources of ROS in MSCs and how they might influence adipogenic and osteogenic differentiation. We will also summarize the studies that have applied exogenous ROS to MSCs and the studies that measure intra and extracellular ROS during MSC differentiation.

Cellular Sources of ROS

ROS generation can be physiological, pathological, and tissue specific and varies under different circumstances [41]. ROS can be generated in mitochondrial electron transport systems, by NADPH oxidases, xanthine oxidase, cytochrome P450, nitric oxide synthases, lipoxygenases, heme oxygenase, cyclooxygenases, myeloperoxidase, and monoamine oxidases [42,43]. ROS in mammalian cells can be localized in (1) mitochondria [44]; (2) peroxisomes [45]; (3) endoplasmic reticulum (ER) [46]; (4) cytosol (ie, NO synthases, lipoxygenases) [47,48]; (5) the plasma membrane (ie, NADPH oxidases, lipoxygenase) [49]; and (6) the extracellular space [50] (Fig. 1). It has been suggested that NADPH oxidases are localized on the ER and possibly mitochondria [51,52].

Mitochondria

Mitochondria are the main source of ROS and the mitochondrial electron transport machinery is thought to be a primary generator of ROS. A small fraction of oxygen escapes from mitochondria during the generation of adenosine triphosphate (ATP) and water. This fraction is then implicated in the formation of ROS. O₂^{•-} is the first ROS produced by mitochondria. The main sources of mitochondrial O₂^{•-} are complex I [53] and complex III [54]. Complex I (NADH ubiquinone oxidoreductase) produces O₂^{•-} in the matrix, whereas complex III (co-enzyme Q, bc1 complex, ubiquinone/cytochrome c reductase) induces O₂^{•-} production either in the matrix or the inter-membrane space (Fig. 2). O₂^{•-} is then transformed into a more stable form, H₂O₂, through the activity of Mn, Cu, and Zn-SOD in the inter-

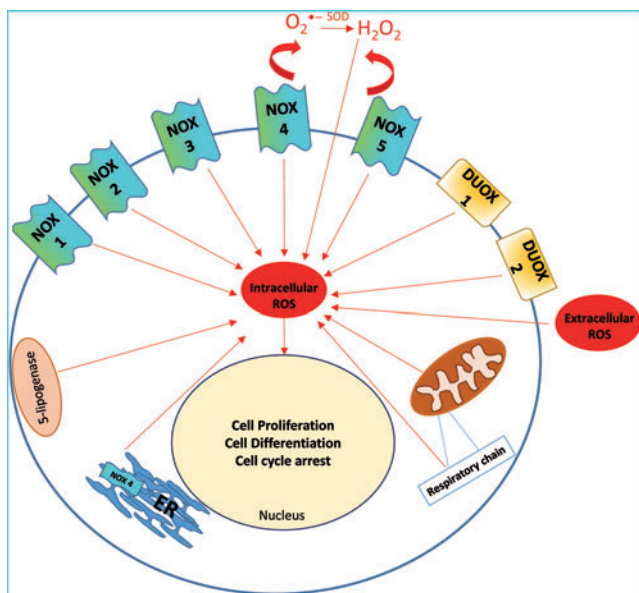


FIG. 1. Sources of reactive oxygen species (ROS). ROS can be intracellularly generated by mitochondria and diverse NOX isoforms, peroxisome, endoplasmic reticulum (ER), xanthine oxidase, and lipogenase. It can also be applied from exogenous sources. Irrespective of its source, it may cause cell proliferation, differentiation, and/or cell cycle arrest, and this effect appears to be concentration dependent. Color images available online at www.liebertpub.com/scd

membrane space [55]. H_2O_2 may have different fates. It can be removed by antioxidants such as catalase and peroxidases [56] or converted to H_2O and O_2 by glutathione peroxidase (GPX) or can act as a signaling molecule in the cytosol in several pathways including the stress response, cell cycle, energy metabolism, and redox balance [57] (Fig. 2). Inhibition of complex I and III by rotenone and antimycin

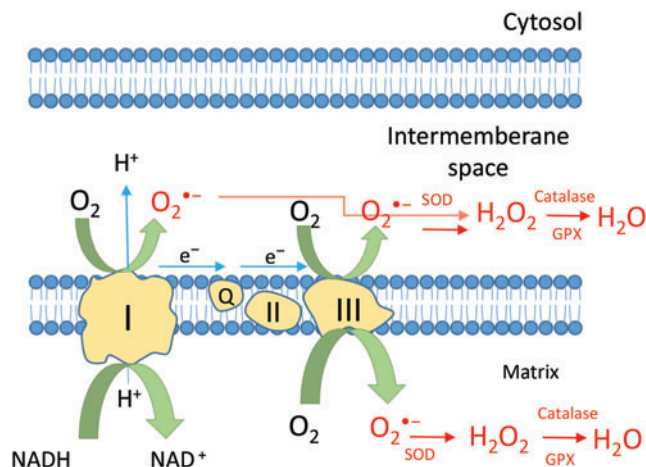


FIG. 2. Mitochondrial ROS production. The production of the superoxide anion, $O_2^{\bullet-}$, by complex I and complex III in the matrix or the inter-membrane space forms H_2O_2 through the activity of SOD catalase dismutation. H_2O_2 can then be converted to H_2O and O_2 by glutathione peroxidase (GPX) and catalase or may play a second messenger role in essential signaling pathways. Color images available online at www.liebertpub.com/scd

induces ROS generation by the respiratory chain machinery. Other potential sources of ROS within mitochondria could be α -ketoglutarate dehydrogenase [58] located in the matrix and monoamine oxidase [59] at the outer membrane. Recently, some investigators have suggested that NOX4 is localized in mitochondria, although this remains to be confirmed [60,61].

NADPH oxidases

Nonmitochondrial ROS production was first described in neutrophils and macrophages during phagocytosis [62]. NOX consists of a family of seven isoforms that catalyze the reduction of oxygen to superoxide using the pyridine nucleotide NADPH as an electron donor and molecular oxygen as electron acceptor, with the secondary production of other ROS (Fig. 3). In phagocytes, NOX activity requires the cytosolic regulators p47phox, p40phox, p67phox, and the small GTPase RAC. An active complex is formed when the catalytic (gp91phox) and the regulatory subunits are assembled [5].

While it has been known since the seventies [63] that phagocytes contain an NADPH oxidase activity, a number of studies performed in the nineties described low amounts of ROS in nonphagocytic tissues such as smooth muscle cells [64,65]. In 1999 the first nonphagocytic NOX open reading frame was described in the colon [66]. This isoform is nowadays called NOX1. Other NOX enzymes were then described in other tissues. Seven NOX isoforms are detected in most mammals: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. However, NOX5 is absent in mice and rats for reasons that are not understood. It has been reported that levels of NOX4 and NOX5 expression are higher than NOX1, NOX2, and NOX3 in adipose-derived MSCs [67]. Most NOX isoforms produce $O_2^{\bullet-}$ as a primary product. However, H_2O_2 is the dominant ROS detected for

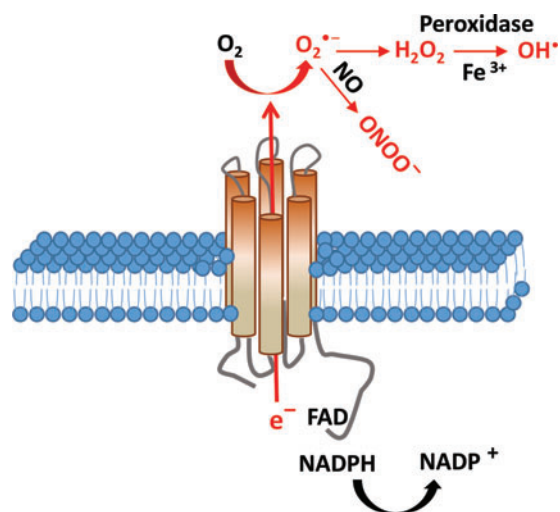


FIG. 3. NADPH oxidases. NOX enzymes reduce oxygen to $O_2^{\bullet-}$ by using pyridine nucleotide NADPH as an electron donor and molecular oxygen as an electron acceptor. ROS will be generated as the secondary product. A part of $O_2^{\bullet-}$ can directly react with nitric oxide (NO) to form a toxic peroxynitrite. It can also be dismutated by superoxide dismutase to form hydrogen peroxide to induce cell signaling cascades or directly react with Fe^{3+} to form hydroxyl radical. Color images available online at www.liebertpub.com/scd

NOX4, DUOX1, and DUOX2 [68,69]. This is generally explained by the rapid dismutation of $O_2^{\bullet-}$ into H_2O_2 .

Role of ROS in Regulating MSC Fate

There is a large difference in energy metabolism and cellular redox status between pluripotent stem cells and terminally differentiated (stem) cells. For example, in the proliferative phase (early passages), embryonic stem (ES) cells express high levels of glycolytic enzymes and mitochondria consume low amounts of oxygen. However, differentiated ES cells show a lower glycolytic flux, less than half of that predicted in proliferating ES cells [70]. In addition, the degree of stemness of adult stromal stem cells is linked to the intracellular distribution of mitochondria: stem cell differentiation competence could be defined by a perinuclear arrangement of mitochondria, a low ATP/cell content and a high rate of oxygen consumption, whereas lack of these characteristics was an indication of stem cell differentiation [71].

It is believed that MSCs derived from diverse sources (mostly from adipose tissue and bone marrow) implanted at a site of tissue injury are able to survive, proliferate, and differentiate into various cell types. However, several tissue regeneration-based studies have reported that the majority of engrafted MSCs die after several days and only a small percentage survive, which are hardly enough to replace lost tissue [72,73]. This low cell survival rate is due to local hypoxia. Eto et al. demonstrated that adipose-derived MSCs are very sensitive to oxygen concentrations and that only those cells implanted less than 300 μ m from an oxygen source would survive, the others undergo apoptosis [74]. Therefore, most transplanted MSCs experience oxidative stress and the excessive ROS produced either by host tissues or by MSCs themselves is believed to account for cell cycle arrest and cell death. ROS can induce the activation of MAPK pathways such as JNK and p38MAPK and ERK along with activation of apoptotic proteins and suppression of antiapoptotic pathways [75]. On the other hand, many investigations have claimed that mitochondrial metabolism and ROS generation regulate MSC differentiation into adipocytes, chondrocytes, osteocytes, and neuronal cells [23,52,75,76]. ROS induce micro RNA-210 (miR-21) ex-

pression via PDGFR-b, Akt, and ERK pathways. Micro RNAs act primarily at the post-transcriptional level. Consequently, MSC proliferation and migration increase as a result of miR-210 expression. Micro RNAs suppress mRNA translation and/or promote degradation [77]. Additionally, NADPH oxidase complex induced ROS was reported to induce cell survival cascades through activation of PI3K/Akt pathways and inhibition of p38 MAPK [78]. Therefore, based on previous studies, there is evidence for a role for ROS in MSC survival, proliferation, and terminal differentiation. The impact of the oxidative environment on the regulation of osteogenesis and adipogenesis is described in the following sections (Fig. 4).

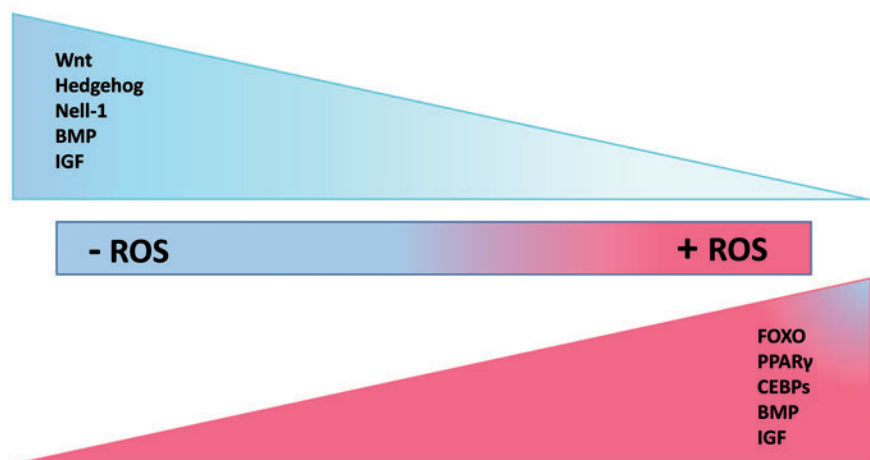
Oxidative Stress and Antioxidant Regulation During Osteogenesis

Several studies have suggested a link between oxidative stress, osteogenic differentiation and bone formation. It is known that oxidative stress impairs skeletal integrity, and reduces osteogenic differentiation of murine preosteoblastic (MC3T3-E1) and bone marrow-derived stromal (M2-10B4) cell lines [79]. On the other hand, by using antioxidants such as pyro-lidinedithiocarbamate, a thiol-containing antioxidant and Trolox, a hydrophilic vitamin E analogue, osteogenic differentiation could be restored [79] suggesting that antioxidants may play a role in preventing age-related osteoporosis [80,81].

A study on mitochondrial metabolism revealed that osteogenic induction in human bone marrow-derived MSCs *in vitro* is associated with an upregulation of mtDNA copy number, protein subunits of respiratory enzymes, oxygen consumption rate and antioxidant enzymes, but a reduction in the levels of intracellular ROS [51]. The authors reported a dramatic reduction in intracellular levels of H_2O_2 and $O_2^{\bullet-}$ on the second day of osteogenic induction. In addition, they reported that 14 days after induction the protein levels of the antioxidant enzymes Mn-SOD and catalase were upregulated three and four fold, respectively.

When compared to untreated cells, addition of exogenous H_2O_2 (125–500 μ M) to human bone marrow-derived MSCs reduced activity of alkaline phosphatase [82], a marker of

Osteogenesis



Adipogenesis

FIG. 4. ROS control signaling cascades involved in osteogenesis/adipogenesis. Wnt/ β -catenin, MAPK (NELL-1), and Hh signaling induce osteogenesis while FOXO, PPAR γ , and CEBPs signaling stimulate adipogenesis. BMP and IGF signaling have a dual effect in inducing both of these terminal fates. Induction of osteogenesis is optimal in the absence of ROS while induction of adipogenesis is optimal in the presence of ROS. Color images available online at www.liebertpub.com/scd

TABLE 1. ROLE OF REACTIVE OXYGEN SPECIES IN OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION

Source	Osteogenic differentiation	Adipogenic differentiation
Intracellular		
Mitochondria	mtDNA copy number, protein subunits of respiratory enzymes and oxygen consumption rate are upregulated while intracellular H ₂ O ₂ and O ₂ ^{•-} are reduced after osteogenic induction [51] Oxidative stress in aged mice results in reduced osteoblast and bone formation, increased osteoblast and osteocyte apoptosis and decreased bone density [84]	Inhibition of the mitochondrial electron transport chain suppresses MSC adipogenic differentiation [76] ROS produced by mitochondrial complex III are required for activation of adipogenesis. Intracellular ROS increase after exposure of MSCs to an adipogenic cocktail [139] NOX4 mRNA expression is decreased while NOX2 mRNA expression is unchanged during adipogenic differentiation [92]
NADPH oxidase	NOX4 knockout mice display higher bone density. NOX4 is involved in the transformation of osteoblasts to osteoclasts and is thus responsible for reduced bone density [112]	NOX4 increases lipid accumulation even in the absence of insulin. siRNA against NOX4 inhibit insulin-induced accumulation of lipid droplets in 3T3-L1 cells. Overexpression of NOX4 increases adipogenesis [22] Elevated oxidative stress and consequently elevated NADPH oxidase in accumulated fat is related to obesity-associated metabolic syndrome in humans and mice [85] NOX4 induces adipogenesis in adipose-derived MSCs [87] Knock down of NOX4 inhibits MSC adipogenic differentiation even in the presence of an adipogenic cocktail [92] Reduced expression of NOX4 is a hallmark of adipogenesis in 3T3-L1 cells [85,92,141]
Extracellular	H ₂ O ₂ reduces Alp activity in osteogenic induced hMSC [51] H ₂ O ₂ abolishes osteogenesis in osteoblast progenitors [84] H ₂ O ₂ induced oxidative stress reduces Gli protein levels thus preventing Hh signaling and reducing osteogenesis. The level of Alp mRNA expression is reduced [111] H ₂ O ₂ inhibits expression of osteogenic differentiation markers in MC3T3-E1 and M2-10B4 cell lines. Alp activity is also reduced [79]	H ₂ O ₂ induced oxidative stress induces MSC adipogenesis [22] Treating 3T3-L1 cells with H ₂ O ₂ results in adipogenesis even in the absence of insulin [85] H ₂ O ₂ increase adipogenesis in 3T3-L1 cells in a dose-dependent manner [86] H ₂ O ₂ diminishes expression of adipo-cytokines [87] eNOS rather than iNOS governs adipogenesis. NO stimulates rat preadipocyte differentiation [93]

eNOS, endothelial nitric oxide synthases; iNOS, inducible nitric oxide synthases; MSC, mesenchymal stromal cell; ROS, reactive oxygen species.

osteogenic differentiation. Thus, excessive amounts of ROS prevent MSC osteogenic differentiation in these cells [51]. Furthermore, differentiation of murine osteoblast precursors into osteoblasts was abolished by exogenous H₂O₂ (50 μM H₂O₂ for 1 h) [83].

Animals studies have demonstrated that reduced osteoblast and bone formation, increased osteoblast and osteocyte apoptosis and decreased bone mineral density are due to increased oxidative stress in aged mice. Loss of sex steroids expedites the effects of aging by reducing defence mechanisms against oxidative stress [84].

Taken together, these observations suggest that ROS suppress osteoblast differentiation, and antioxidants could

potentially rescue this phenomenon. Table 1 summarizes the effects of ROS on osteogenic differentiation.

Oxidative Stress and Antioxidant Regulation During Adipogenesis

Schröder et al. have suggested that stimulation of murine 3T3-L1 cells and human preadipocytes with exogenous H₂O₂ (30 μM, every other day) results in adipogenic differentiation even in the absence of insulin [85]. A dose-dependent role for H₂O₂ in regulating adipogenesis in 3T3-L1 preadipocytes has been observed, with higher doses of H₂O₂ (1 and 10 μM) increasing adipogenesis [86].

Additionally, mimicking oxidative stress by addition of exogenous H₂O₂ (100 μM) for 8 days was shown to induce adipogenesis in human adipose-derived MSCs [22]. In contrast, one study has reported that incubation with H₂O₂ (0.1–0.5 mM) diminished expression of adipogenic cytokine mRNAs such as the fat-derived hormone adiponectin and the transcription factor PPARγ in murine 3T3-L1 cells in a dose-dependent manner [87].

Recently, several studies have focused on antioxidant levels during adipogenesis in an oxidative stress environment [86,88–91]. Application of an antioxidant such as N-acetyl-L-cysteine (NAC) inhibited the expression of transcription factors such as C/EBPα (days 2 and 4) and PPARγ (day 4) in rat bone marrow MSCs and murine 10T1/2 cells [92]. In agreement with these findings, increased levels of ROS production in adipose tissue are accompanied by decreased expression of antioxidant enzymes such as Cu, Zn-SOD, and catalase. ROS production was significantly decreased by the antioxidants apocynin or NAC in fully differentiated 3T3-L1 adipocytes [87]. Recently, it was demonstrated that a concomitant increase in the expression of SOD3 mRNA and protein occurs with the differentiation of human bone marrow-derived MSCs into adipocytes [90] and during the early stages of adipogenesis in 3T3-L1 cells [89]. Using siRNA interference to knock down Mn-SOD, it was observed that the expression of late adipogenesis markers such as adiponectin and fatty acid-binding protein 4 (FABP4) was reduced, demonstrating that Mn-SOD knockdown impairs adipogenesis in 3T3-L1 cells [88]. Similarly, upregulation of antioxidant enzymes such as SOD, catalase, and GPX have been observed during adipogenesis in human adipose-derived MSCs [22].

Several studies have looked at the role of other free radicals in adipogenesis. For example, a stimulatory role for endogenous nitric oxide (NO) on adipogenesis in preadipocytes derived from rat white adipose tissue has been reported [93]. A 50% increase in basal levels of NO was observed on the first two days after adipogenic differentiation. As inducible nitric oxide synthases (iNOS) inhibitors such as 1400W and aminoguanidine had a minor impact on differentiation and NO production [93], endothelial nitric oxide synthases (eNOS) rather than iNOS may be the major isoform of nitric oxide synthase that modulates adipogenesis [93].

Taken together, these findings demonstrate that an oxidized intracellular environment favors murine and human MSC and preadipocyte differentiation into mature adipocytes. ROS increases the expression of genes associated with adipogenesis. Additionally, adipocytes contain higher levels of intracellular ROS compared with progenitors. The adipogenic process increases the expression of antioxidants, an event that could become a hallmark of adipogenesis. Table 1 summarizes the effects of ROS on MSC adipogenic differentiation.

Regulation of Differentiation Toward an Osteogenic/Adipogenic Fate

Bone is produced by two mechanisms: (1) intramembranous ossification, the direct differentiation of mesenchymal progenitors into osteoblasts; and (2) endochondral ossification, bone formation via a cartilage anlagen, a mechanism initiated by the formation of MSC clusters [94]. Adipogenic differen-

tiation occurs via two phases: (1) commitment of MSCs to a preadipocyte stage; and (2) terminal differentiation of preadipocytes into mature adipocytes [95].

The interplay of several extracellular signals such as hormones (glucocorticoid and parathyroid hormones) and ligands of the wingless/invested1 (Wnt), BMP, fibroblast growth factor (FGF), transforming growth factor β (TGFβ), and hedgehog signaling pathways are required for osteogenic differentiation [96,97].

The main signaling pathways that determine MSC terminal fate are reviewed in the following sections.

Evidence for a Potential Role for ROS in Inhibiting Bone Formation

WNT signaling

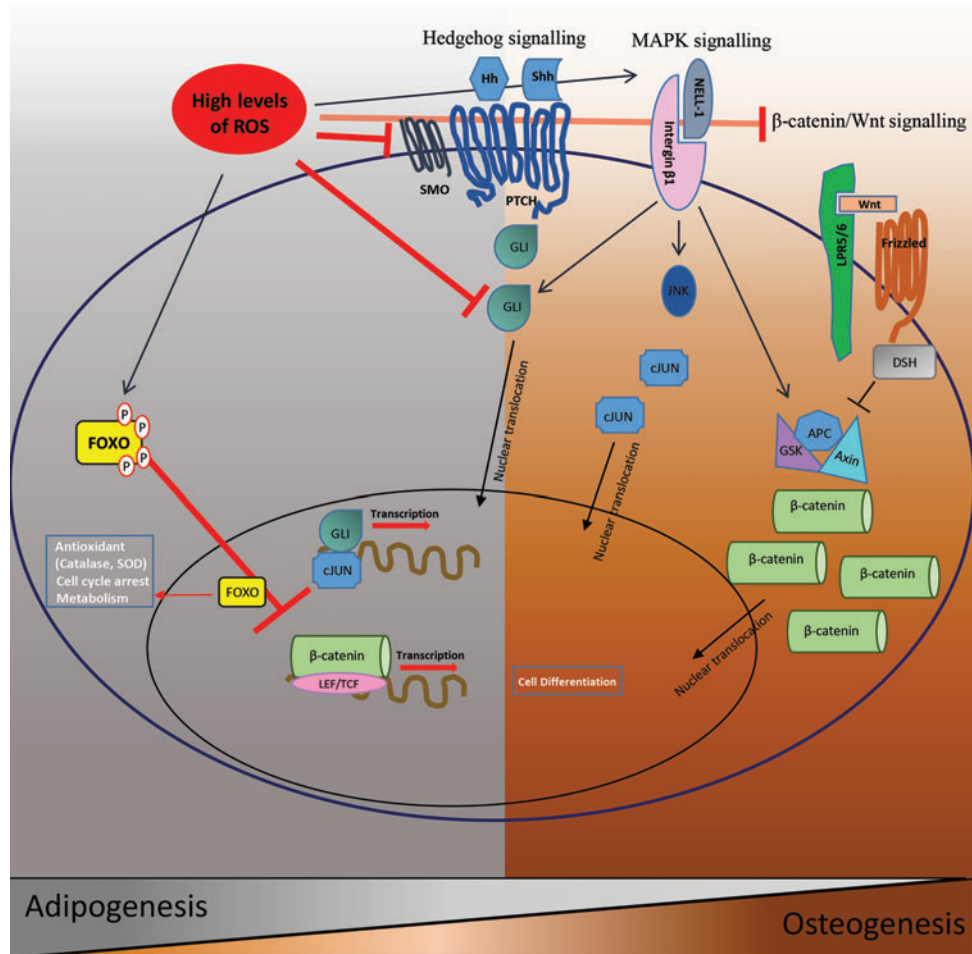
Wnt is a molecular switch for adipogenic/osteogenic differentiation. The Wnt canonical pathway (β-catenin dependent) is initiated through the binding of extracellular Wnt ligands to the frizzled seven pass transmembrane receptors (Frz). Consequently, intracellular signaling of the complex of axin, glycogen synthase kinase 3 (GSK3), and adenomatous polyposis scientifica 5 coli (APC) protein will be inhibited. Upon Wnt signaling, β-catenin degradation is inhibited by the Axin/APC/GSK3 complex, which results in the translocation of β-catenin from the cytoplasm to the nucleus [29]. Nuclear β-catenin binds to the T-cell factor/lymphoid-enhancing factor (Tcf/Lef), which then forms a transcriptional effector for activating Wnt target genes [32] (Fig. 5).

Currently available data suggest that Wnt/β-catenin positively regulate osteoblast and osteoclast activity. In line with this notion, disruption of the Wnt/β-catenin pathway impairs osteogenesis [29]. β-catenin induces essential signals for osteogenic initiation [98] and conditional inactivation converts osteoblasts into chondrocytes and thus delays skeletal mineralization. Wnt/β-catenin signaling suppresses adipogenesis and thus favors osteogenesis by reducing the expression of C/EBPα and PPARγ mRNAs; these molecules are key regulators of adipogenesis and suppressors of osteogenesis [32,99]. Recently, several studies have reported the adverse effect of oxidative stress on osteoblastogenesis [100]. Interestingly, the suppressive effect of H₂O₂ on Tcf-mediated transcription was abolished by overexpression of β-catenin. An *in vivo* study reported that ROS increases with increasing age, which in turn decreases the expression of Wnt target genes such as Axin2 and Opg in 31-month-old mice when compared with 4-month-old mice, and thus diminishes osteogenesis [83]. Taken together, these findings suggest that ROS inhibit the osteoinductive effect of Wnt signaling, although under normal circumstances this pathway positively stimulates osteogenesis.

FOXO signaling

Bone involution such as occurs during decreased bone formation and increased bone marrow adiposity is associated with increased oxidative stress and decreased growth factor production. This results in the activation of the FOXO family of transcription factors [101]. Indeed, the defence mechanism against oxidative stress is governed by the FOXO family of transcription factors [102–104]. The FOXO family consists of four members: FOXO1 (or Fkhr),

FIG. 5. ROS suppress important osteogenic signaling pathways while they promote adipogenic signaling pathways. Wnt/ β -catenin and Hh signaling cascades induce osteogenesis and this is inhibited in the presence of high levels of ROS, which favors adipogenesis. MAPK signaling induces osteogenesis and is stimulated by ROS. In response to oxidative stress, FOXOs are phosphorylated and translocate to the nucleus where they attenuate the transcription of osteogenic genes while inducing adipogenic differentiation. The active form FOXO also induces the regulation of antioxidant and cell cycle arrest genes. The expression of antioxidants also increases adipogenic differentiation. Color images available online at www.liebertpub.com/scd



FOXO3a (or Fkhr11), FOXO4 (or Afx), and FOXO6 [105]. β -catenin is also required for FOXO-mediated transcriptional downstream effectors of the Wnt/ β -catenin pathway [103]. In osteoblast progenitors and many other cell types, the association of β -catenin with FOXOs increases in the presence of oxidative stress [103]. In the absence of growth factors or in the presence of high levels of ROS, FOXO is activated. It then translocates to the nucleus and induces the transcription of a variety of target genes such as antioxidants (Fig. 5). It is known that FOXO represses osteogenic differentiation [101]. An *in vivo* investigation in both female and male C57BL/6 mice reported that FOXOs impair bone formation by antagonizing Wnt signaling [83].

Iyer et al. have demonstrated that mice deficient in FOXO1, -3, and -4 in osteoblast progenitors exhibit increased osteoblast number and a higher bone mass [101]. In line with this observation, treating murine osteoblastic cells with 100 μ M H_2O_2 for 1 h enhances β -catenin and FOXO3 association. β -catenin in turn is essential for FOXO target gene stimulation by H_2O_2 . FOXO transcription is promoted by H_2O_2 while Wnt/Tcf mediated transcription and osteoblast differentiation is reduced [83]. In response to oxidative stress, FOXOs induce cell cycle arrest and dormancy [104,106]. Under such conditions, FOXOs regulate transcription of antioxidant enzymes (eg, catalase, Mn-SOD) and also genes that play role in the cell cycle and cell longevity [102,107] (Fig. 5). Collectively, this

suggests that activation of FOXO signaling by oxidative stress attenuates the osteogenic process.

Hedgehog signaling

At least three members of the Hedgehog signaling, Hh, family have been described in vertebrates: sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh) [108]. Hedgehog signaling is activated by the binding of an Hh ligand to the receptor patched (PTCH), a 12 pass transmembrane protein that inhibits smoothed (Smo), a 7 pass transmembrane protein. This inhibition leads to translocation of the glioblastoma gene product [109] family of DNA-binding proteins to the nucleus where transcription of Hh target genes follows [29,110,111] (Fig. 5). Osteoinductive [111] and anti-adipogenic roles have been ascribed to Hh signaling (99, 135–140). Oxidative stress inhibits Hh-induced osteogenic differentiation in murine primary bone marrow-derived and other MSC cell lines [111] (Fig. 5). Addition of nonphysiological levels of H_2O_2 (0.5–1 mM) for 72 h suppressed Hh signaling thus inhibiting Hh-mediated osteogenic differentiation in bone marrow stromal cells [111]. The expression of osteogenic differentiation markers such as *Alp*, *OSX*, and *BSP* was significantly reduced, indicating that in MSCs, Shh-induced osteogenesis is inhibited by H_2O_2 -induced oxidative stress (0.5–1 mM H_2O_2 for

48 h). Moreover, H₂O₂-induced oxidative stress impaired the proliferation of MSCs. Taken together, these data suggest that under normal conditions, Hh signaling induces osteogenesis. However, ROS inhibits the osteoinductive effect of Hh signaling.

NADPH oxidase

The impact of NOX isoforms on MSC osteogenic differentiation has been less well studied. NOX induced ROS has been implicated in bone disease. NOX4, as a constitutively active source of ROS, is involved in osteoclastogenesis. It has recently been reported that NOX4 knockout mice display higher bone density. Interestingly, a specific single nucleotide polymorphism (SNP) in the human NOX4 gene has been shown to contribute to the greater expression of bone turnover markers and reduced bone density in women. NOX4 expression could be thus responsible for reduced bone density [112]. Understanding the role of NOX isoforms in osteoblast formation is therefore essential in studies on bone loss and regeneration. Future studies should determine the levels of ROS required for osteogenic attenuation and should also clarify the role of the different sources of ROS in osteogenic signaling pathways.

Evidence for a Potential Role for ROS in Promoting Adipogenesis

WNT signaling

A large body of experimental evidence, both *in vitro* and *in vivo*, has demonstrated the inhibitory role of Wnt molecules during adipogenic differentiation of mesenchymal or preadipocyte cells [99,113–115]. It is known that Wnt inhibits the early stages of adipogenesis. Inhibiting the Wnt pathway stimulates the generation of adipocytes in 3T3-L1 preadipocytes [99]. From a pathophysiological perspective, several genetic studies have revealed that polymorphisms in genes of the WNT signaling pathway are linked to the development of obesity and type 2 diabetes in humans [113]. It has also been shown that Wnt signaling promotes MSC osteogenic, myogenic, and chondrogenic differentiation and abrogates adipogenic differentiation [116–119]. β -catenin activation suppressed PPAR γ expression and impaired murine 3T3-L1 adipogenic differentiation [120]. Taken together, these findings indicate that Wnt has an adiporepressive effect. However, in an oxidative environment, β -catenin diverts to FOXO instead of Tcf to suppress Wnt, and thus favors adipogenesis [83]. Further studies should reveal the role of ROS, their optimum level, and the role of different ROS generators in regulating the Wnt pathway.

FOXO signaling

FOXO negatively regulates adipogenesis. The expression and transcriptional activity of PPAR γ , the master transcription factor for adipogenesis, is suppressed by FOXO1 [121]. Insulin induces phosphorylation of Akt, which then activates adipogenic transcription factors, specifically PPAR γ . Akt promotes PPAR γ expression by FOXO1 exclusion from the nucleus [122]. Akt tightly governs the function of FOXO proteins through Akt-mediated phosphorylation mechanisms [123,124]. FOXO plays a key role

in maintaining cellular redox homeostasis. FOXO1 limits oxidative stress in human adipose-derived MSCs by upregulating antioxidant enzymes [22]. Additionally, mice lacking FOXO1, FOXO3, and FOXO4 showed decreased adiposity in aged bone marrow, although osteoblast number was increased and these mice had a greater bone mass in old age [101]. Using siRNAs against FOXO1 in murine 3T3-L1 preadipocytes, decreased lipid droplet formation was observed after adipogenic induction. Adipogenesis was more severely inhibited when cells were exposed to FOXO1 siRNA before induction of adipogenic differentiation. Downregulation of FOXO1 in 3T3-L1 cells resulted in a decrease in expression of the adipogenic transcription factors, PPAR γ and C/EBP α [123]. However, another study in 3T3-F442A cells and murine embryonic fibroblasts suggested that FOXO1 prevents adipogenic differentiation [124]. Similarly, SIRT2, a cytoplasmic sirtuin, indirectly inhibits PPAR γ by reducing FOXO1 acetylation and phosphorylation; this increased the amount of FOXO1 in the nucleus and consequently PPAR γ transcription was repressed. These results propose a cell type and context-dependent role for FOXO expression in cellular signaling during adipogenesis [22]. Additionally, the exact role of FOXO, its regulation by phosphorylation and its effect on antioxidant expression requires further investigation.

Hedgehog signaling

An antiadipogenic role has been suggested for the Hh pathway upon its activation [31,125–130]. Hedgehog signaling inhibits adipogenic differentiation of murine 3T3-L1, NIH-3T3, and C3H10T1/2 cells, but when this pathway is inhibited adipogenesis is promoted. Hedgehog signaling induces antiadipogenic transcription factors (eg, Gata2 and Gilz) to repress adipogenesis. Consequently, the antiadipogenic factors downregulate PPAR γ expression [127]. Under normal conditions, Hh signaling induces osteogenesis while inhibiting adipogenesis. However, oxidative stress inhibits Hh-induced osteogenic differentiation, and may thus favour adipogenesis. To our knowledge, there is no study reporting the role of oxidative stress in the regulation of adipogenic differentiation via Hh signaling. It would therefore be of interest to assess this possible relationship in future investigations.

Transcription factors

Several transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs) and PPAR γ play a key role in adipogenesis [29]. Transcription factors such as C/EBP β and C/EBP δ are the major adipogenesis regulators during early phases of differentiation. Thus, C/EBP δ is expressed during the early phase of adipogenesis and disappears in the late phase [131]. C/EBP α and PPAR γ regulate terminal differentiation stages [132]. Coordinated activity between these two transcription factors functions as a positive feedback loop in which PPAR γ activates the promoter of the gene encoding C/EBP α and vice versa. This induces the expression of adipocyte specific genes such as glucose transporter GLUT4 (also known as SLC2A4), lipoprotein lipase, fatty acid-binding protein 4 (FABP4, also known as aP2), adiponectin, and leptin [133–135]. Many factors affect the ability of PPAR γ to influence the adipogenic process. Sirtuin 1 (SIRT1), a histone/protein deacetylase, directly

binds to PPAR γ and impairs adipogenesis [136]. It also acts as a PPAR γ co-repressor [137]. SIRT2, a cytoplasmic sirtuin, indirectly inhibits PPAR γ . It has been shown that SIRT2 is downregulated during adipogenesis in murine 3T3-L1 cells [138].

Mitochondrial ROS

Mitochondrial ROS production appears to be critical in promoting the differentiation of human bone marrow-derived MSCs and an increase in ROS levels supports an unrestricted oxidative environment for launching signaling events leading to adipogenic differentiation [139]. Not only do ROS modulate adipogenic differentiation, but they also impact on MSC proliferation. Mitochondrial ROS was suggested to inhibit proliferation of murine 3T3-L1 preadipocytes [140]. Similarly, it was demonstrated that ROS produced by mitochondrial complex III is required for activation of adipogenic gene transcription in human bone marrow-derived MSCs. Intracellular H₂O₂ was increased after two days upon exposure of MSCs to adipogenic induction medium (containing indomethacin, dexamethasone, isobutylmethylxanthine, and insulin). However, mitochondrial targeted antioxidants (500 nM MitoCP) attenuated the amount of intracellular H₂O₂ and consequently impaired lipid accumulation during adipogenesis. In relation to this observation, protein levels of major adipogenic transcription factors such as C/EBP α and PPAR γ 2 were dramatically decreased. However, adipogenesis was rescued when cells were treated with D-galactose (0.5 mM) to deliberately generate exogenous H₂O₂ [139]. In agreement with these findings, it has recently been suggested that elevated mitochondrial activity is an essential requirement for human MSC adipogenic differentiation. siRNA-based knockdown of the mitochondrial transcription factor A (TFAM), which suppresses mitochondrial activity, inhibited adipogenic differentiation. Under hypoxic conditions or by inhibition of the mitochondrial electron transport chain, mitochondrial respiration was reduced. As a result, adipogenic differentiation was significantly suppressed [76]. Taken together, these findings indicate that superoxide generated by the mitochondrial electron transport chain is converted to H₂O₂, which initiates the PPAR γ transcriptional machinery that regulates adipocyte differentiation.

NADPH oxidase

NADPH oxidase was demonstrated to be the central source of ROS in adipocyte precursors [87,141]. NOX4, for instance, is highly expressed in preadipocytes and is emerging as a hallmark of preadipocyte proliferation and differentiation [85,92,141]. Recently, Schröder et al. reported that the effect of NOX4 expression on proliferation and differentiation of murine 3T3-L1 and human adipose-derived MSCs is mediated by the MEK/ERK pathway. They suggested that NOX4 controls proliferation by activating the ROS-dependent phosphorylation of ERK1/2. In addition, both knockout and siRNA studies demonstrate a role for NOX4 in governing MSCs differentiation. siRNA directed against NOX4 resulted in inhibition of ERK1/2 thus promoting proliferation and impairing adipogenic differentiation of 3T3-L1 preadipocytes. siRNA against NOX4 resulted in inhibition of insulin-induced accumulation of

lipid droplets in 3T3-L1 cells [85]. Similarly, RNA interference knockdown of NOX4 inhibited the adipogenic differentiation of rat bone marrow-derived MSCs and murine 10T1/2 cells, even in the presence of an adipogenic cocktail. NOX4 expression was decreased; NOX2 expression was, however, constant after adipogenic differentiation [92]. On the other hand, in the experiments reported by Schröder et al., overexpression of NOX4 increased the accumulation of lipid droplets even in the absence of insulin, demonstrating that NOX4 is a direct mediator of insulin-induced differentiation in human preadipocytes [85]. Similarly, overexpression of NOX4 was shown to induce adipogenesis in human adipose-derived MSCs [22]. A further study demonstrated that the level of production of ROS such as H₂O₂ increased in adipose tissue of obese mice when compared to control mice and was accompanied by increased expression of NADPH oxidase (gp91phox and p22phox, and cytosolic components p47phox, p67phox, and p40phox) and a reduction in the levels of antioxidant enzymes. ROS production was significantly decreased by the NOX inhibitor diphenyleneiodonium in murine 3T3-L1 preadipocytes [87]. Collectively, these data suggest a positive role for the NOX4 isoform of NADPH oxidase in the differentiation of adipogenic progenitors. Generation of ROS by NADPH oxidase appears to be necessary for positive regulation of MSC proliferation and adipogenic differentiation. Targeting various NOX isoforms may elucidate their role in the effect of ROS on MSC differentiation.

Therapeutic Role of ROS

Oxidative stress plays a key role in the pathogenesis of many diseases [87] such as diabetes [37], hypertension [38], atherosclerosis [39], carcinogenesis, metabolic, cardiovascular, pulmonary, and neurological diseases [142]. Recently, NOX inhibitors that target NOX1 and NOX4 enzymes have been used in patients with diabetic nephropathy [142]. The role of exogenous or endogenous ROS on osteogenesis and adipogenesis in the clinical setting has been less intensely investigated. However, ROS is known to be a critical factor in aging [40]. Increased oxidative stress, mainly associated with ageing, has been implicated in the pathogenesis of age-related bone loss in humans and mice [83]. Recently, it was reported that NOX4 is involved in osteoclastogenesis. Subsequently increased human bone resorption has been linked to NOX4, as a constitutively producer of H₂O₂ [112]. SNP analysis of NOX4 in middle-aged woman has revealed a link to altered bone density and plasma markers for bone turnover. In addition, NOX4 is highly expressed in osteoporotic bone in humans [112]. Thus, application of NOX inhibitors should be considered in the context of osteoporosis treatment and possibly bone-related disorders.

Studies in humans have revealed that accumulation of adipose tissue in obese patients is associated with increased systemic oxidative stress, which might therefore present an interesting target for the development of new therapies for obesity-associated metabolic syndrome [87]. In addition, several studies have reported elevated systemic oxidative stress in obesity [143]. A greater understanding of ROS signaling and the consequences thereof might therefore open new therapeutic avenues for the treatment of obesity and its comorbid entities.

Conclusion

ROS have for many years been regarded as having a negative effect on cell function and survival. However, it is becoming increasingly recognised that ROS also mediate important physiological functions. The findings reviewed here demonstrate a pivotal role for ROS in MSC differentiation. Regardless of the sources of ROS, it has been shown that osteogenic and adipogenic differentiation is partly ROS dependent. We conclude that osteogenesis is blunted by elevated ROS while ROS positively induces adipogenesis in MSCs and other adipogenic progenitors. The activity of the ROS generating NOX4 isoform most likely increases adipogenesis. However, mitochondrial ROS also appears to be necessary for MSC adipogenic differentiation. Taken together, these findings highlight the need to further investigate the role of ROS in regulating MSC differentiation. Further studies should clarify the role of ROS in each signaling cascade, the role of different sources of ROS and their concentration, the period required for treating cells with exogenous ROS and finally the impact of various antioxidants exogenously applied and/or produced in stem cell-based studies and also in the pathogenesis and treatment of relevant diseases.

Acknowledgments

The authors wish to thank Dr. Vincent Jaquet, Prof. Karl-Heinz Krause, and Prof. Jean-Claude Martinou for editorial assistance. Fatemeh Atashi and Ali Modarressi are funded by Swiss National Science Foundation (SNF) (grant #310030-120751). Michal Pepper is funded by the South African Medical Research Council in terms of the MRC's Flagships Awards Project SAMRC-RFA-UFSP-01-2013/STEM CELLS.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication October 10, 2014

Accepted after revision January 20, 2015

Prepublished on Liebert Instant Online January 20, 2015