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Poly(ADP-ribose) in the bone: from oxidative stress signal to structural element

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Abstract

Contrary to common perception bone is a dynamic organ flexibly adapting to changes in mechanical loading by shifting the delicate balance between bone formation and bone resorption carried out by osteoblasts and osteoclasts, respectively. In the past decades numerous studies demonstrating production of reactive oxygen or nitrogen intermediates, effects of different antioxidants and involvement of prototypical redox control mechanisms (Nrf2-Keap1, Steap4, FoxO, PAMM, caspase-2) have proven the central role of redox regulation in the bone. Poly(ADP-ribosyl)ation (PARylation), a NAD-dependent protein modification carried out by poly(ADP-ribose) polymerase (PARP) enzymes recently emerged as a new regulatory mechanism finetuning osteoblast differentiation and mineralization. Interestingly PARylation doesn't simply serve as a signaling mechanism during osteoblast differentiation but also couples it to osteoblast death. Even more strikingly, the poly(ADP-ribose) polymer likely released from succumbed cells at the terminal stage of differentiation is incorporated into the bone matrix representing the first structural role of this versatile biopolymer. Moreover, this new paradigm explains why and how osteodifferentiation and death of cells entering this pathway are closely coupled to each other. Here we review the role of reactive oxygen and nitrogen intermediates as well as PARylation in osteoblast and osteoclast differentiation, function and cell death.

Keywords: reactive oxygen intermediates, reactive nitrogen intermediates, poly(ADP-ribose), poly(ADP-ribose) polymerase, poly(ADP-ribose) glycohydrolase, osteoblast, osteoclast, antioxidants, Nrf2, apoptosis, cell death, mineralization, bone, differentiation

Abbreviations: AIF – apoptosis inducing factor, ALP- alkaline phosphatase, BMPs - bone morphogenetic proteins, BMP2 - bone morphogenetic protein 2, ckb - brain type cytoplasmic creatin kinase, CREB - cAMP response element-binding protein, eNOS – endothelial nitric oxide synthase, Erk - Extracellular signal-regulated kinase, FOXO - Forkhead box protein, GSH/GSSG ratio – reduced/oxidized glutathione ratio, H₂O₂ – hydrogen peroxide, IL-1 β - interleukine-1 beta, iNOS – inducible nitric oxide synthase, IFN γ - interferone gamma, Keap1 - Kelch-like ECH-associated protein 1, MSC –mesenchymal stem cell, MAP kinase - mitogen-activated protein kinase, M-CSF - macrophage colony-stimulating factor, NF- κ B - nuclear factor kappa B, NO – nitric oxide, NOX1 - NADPH oxidase 1, NOX2 - NADPH oxidase 2, NOX4 - NADPH oxidase 4, NFATc1 - nuclear factor of activated T-cells, cytoplasmic 1, OC – osteoclast, Nrf2 - Nuclear factor (erythroid-derived 2)-like 2, PAMM - peroxiredoxin activated in M-CSF-activated monocytes, PAR – poly(ADP-ribose), PARG - poly(ADP-ribose) glycohydrolase, PARP1 - poly(ADP-ribose) polymerase-1, PARylation – poly(ADP-ribosyl)ation, PGC-1 β – Peroxisome proliferator-activated receptor gamma coactivator 1-beta, PKG - protein kinase G, RANK - receptor activator of nuclear factor κ B, RANKL - RANK ligand, ROS – reactive oxygen species, RNS – reactive nitrogen species, RunX2 - runt-related transcription factor 2, SOD – superoxide dismutase, Sod1- superoxide dismutase 1, Steap4 - six transmembrane epithelial antigen of prostate 4, TCIRG1 - V-type proton ATPase 116 kDa subunit a isoform 3, TNF α – tumor necrosis factor alpha, TRACP - tartrate resistant acid phosphatase

Highlights

1. Differentiation, function and death of both osteoclasts and osteoblasts are under control of complex redox regulatory networks.
2. Poly(ADP-ribosyl)ation regulates osteoblast differentiation, production and mineralization of bone matrix.
3. Basal production of hydrogen peroxide by differentiating osteoblast precursors is sufficient to induce DNA breaks and activation of poly(ADP-ribose) polymerase-1.
4. Poly(ADP-ribose) released from dying osteoblasts is incorporated into the bone matrix.

I. Cells in the bone

Bone is often stereotyped as a simple organ fulfilling mainly mechanical functions [1]: providing support for the body, protecting internal organs and together with skeletal muscles, ligaments, tendons and joints mediating locomotion. In fact bones play much more complex roles in various other local or systemic functions ranging from sound transduction in the ear to diverse metabolic functions. This latter include but are not limited to serving as reservoirs for calcium [2], phosphates [3], growth factors [4] and fatty acids [5], detoxifying heavy metals [6] and regulating acid-base homeostasis by releasing or absorbing alkaline salts [7].

Quite remarkably a rather limited number of cell types appears to be sufficient to control all these diverse roles of the bones (Fig 1). Osteoblasts, osteocytes and osteoclasts are considered as the principal effector cell types of the bones [8] with various progenitor cells being also present [9], [10]. Osteoblasts are responsible for making bone tissue. The organic component of bone matrix (osteoid) is mainly composed of collagen [11]. Minerals start to crystallize around the collagen scaffold to form hydroxyapatite [12]. As osteoblasts form new bone tissue many become embedded within the new matrix and differentiate to osteocytes. The parent cell for osteoblasts and osteocytes is the mesenchymal progenitor cell [13], a multipotent stem cell with the potential to differentiate to adipocytes, chondrocytes, fibroblasts, myocytes and osteoblasts/osteocytes [14]. Upon receiving appropriate stimuli [15] Mesenchymal progenitor cells differentiate to pre-osteoblasts which can further differentiate to osteoblasts. Mature osteoblasts produce matrix proteins such as type I collagen [16] and bone matrix proteins (BMPs) [17], calcium binding proteins (osteocalcin [18] and osteonectin [19]), proteoglycans and alkaline phosphatase (ALP) [19]. The unmineralized matrix formed by these components is called the osteoid which in turn

undergoes mineralization. The fully differentiated form of osteoblasts which are embedded into the mineralized matrix they produced are osteocytes which serve primarily as mechanotransducers reacting to stimuli such as changes in the mechanical loading of bones [20]. Generation of osteocytes goes through the sequence of mesenchymal progenitor - preosteoblast – osteoblast –osteocyte. A critical event in this pathway is commitment of mesenchymal progenitors towards osteogenic differentiation. The stimuli responsible for this commitment are largely unknown. However, placing mesenchymal progenitors into a culture medium containing dexamethasone, beta-glycerophosphate and ascorbic acid is a widely used *in vitro* model [21] resulting in cells phenotypically and functionally very similar to osteoblasts: they express osteogenic marker genes (RunX2, osteopontin, osteocalcin) , produce and secrete type I collagen and alkaline phosphatase and deposit calcium minerals into the matrix they secrete.

Bone tissue is broken down by osteoclasts, multinucleated cells of hemopoietic origin [22]. While osteoblast-derived osteocytes maintain bone tissue, osteoclast (OC)-mediated bone resorption is also necessary for successful bone remodeling [23] (e.g. after fractures or when altered mechanical loading on bones requires an adaptation response). Secretion of cytokines (tumor necrosis factor alpha, TNF α [24]; interleukins; monocyte colony stimulating factor, mCSF [25]) by stromal cells of the bone marrow initiate the formation of OC precursors which express RANK (receptor activator of nuclear factor κ B), an essential cell surface molecule required for OC differentiation [26]. Stromal cells also produce RANK ligand (RANKL) [27] the binding of which to RANK gives the ultimate momentum for full OC differentiation.

*II. Reactive oxygen and nitrogen species in the bone**ROS/RNS in osteoblasts*

Many reactive species have been assigned regulatory roles in osteoblast differentiation, function or death [28]; [29]. However, their roles are quite controversial and depend on the context: physiological versus pathological conditions. Several lines of evidence suggest that in low physiological amounts, reactive species promote osteogenesis [30]. Exercise for example triggers ROS production known to be required for the beneficial health effects of regular training [31]; [32]. Exercise also helps maintain bone mineral density and strength in the elderly, although the role of ROS in this beneficial effect has not yet been confirmed [33]. Furthermore, Mandal et al [34] have demonstrated that bone morphogenetic protein 2 (BMP2) promotes differentiation of osteoblast precursor cells to mature osteoblasts via ROS production by NOX4 (NADPH oxidase 4). Moreover, Robaszkiewicz et al. [35] have shown that hydrogen peroxide is produced by differentiating osteoprogenitor cells and it is required for mineralization and expression of osteogenic marker genes.

Depending on the amount, intra-/extracellular location of production and their chemical nature, reactive species may also have detrimental effect on the bone. Evidence supporting this statement is also substantial. For example, in various pathological conditions such as osteoarthritis [36], osteoporosis [37] or as part of the physiological aging [38] process ROS have been shown to contribute to decreased viability, differentiating capacity and function of osteoblasts [39]; [29]. Moreover, Sod1-deficient mice exhibited lower bone mineral density and strength of femurs from both males and females compared with gender matched WT mice [40]. It is not known, however, whether increased tissue levels of superoxide or lower availability of H₂O₂ (the product of SOD) was responsible for these effects. ROS have also been shown to be crucial mediators of estrogen deficiency bone loss

[41]. In these experiments ovariectomy resulted in reduced levels of the thiol antioxidants (glutathione and thioredoxin) in the bone marrow which could be normalized by exogenous estrogen administration. In this model the antioxidants N-acetyl cysteine or ascorbate increased tissue glutathione levels and prevented bone loss, while inhibition of glutathione synthesis by buthionine sulphoximine caused bone loss [41]. In conclusion it seems that osteoblastogenesis and bone formation are strongly linked to ROS production, however, this relationship is rather complex and controversial.

As for the role of reactive nitrogen species (RNS) in bone biology, nitric oxide and peroxynitrite are also recognized as an important regulator of bone metabolism [42]. The endothelial and inducible isoforms of nitric oxide synthases (eNOS and iNOS, respectively) are expressed in the bones with both of these NOS isoforms having been implicated in the regulation of bone formation [28]; [43]. While eNOS is constitutively expressed, iNOS expression is stimulated by inflammatory cytokines (TNF α and IL-1 β and IFN γ) [44]. Mice deficient in eNOS or in the eNOS downstream effector protein kinase G (PKG) show bone abnormalities [28], while abnormalities in osteogenesis and in bone healing have been detected in iNOS knockout mice [43]; [28]. While eNOS-derived NO promotes osteoblast differentiation and matrix formation, large concentrations of NO produced by iNOS suppress osteoblast activity [45]. Peroxynitrite formed in the diffusion-limited reaction between nitric oxide and superoxide [46] appears to be responsible for the inhibitory effects of iNOS-derived NO overproduction. Application of exogenous peroxynitrite or peroxynitrite-releasing agent SIN-1 reduced osteoblast proliferation and differentiation [47]. While this latter reaction may be relevant in inflammatory conditions where cytokines trigger the iNOS \rightarrow NO* \rightarrow ONOO $^-$ pathway culminating in osteoblast dysfunction and cell death [48], under physiological conditions low amounts of NO produced by eNOS promotes osteoblast

function and bone formation [28]. NO has also been implicated in the signal transduction cascade triggered by mechanical loading (Klein-Nulend et al. Osteoporos Int 2014) with osteocytes functioning as mechanical sensors in the bone. Increased mechanical loading changes hydrostatic pressure as well as alters canalicular flow of interstitial fluid (Burger & Klein-Nulend FASEB J 1999; Klein-Nulend et al. FASEB J 1995; Klein-Nulend et al. Osteoporos Int 2014) stimulating calcium-dependent NOS activity. In mechanotransduction NO stimulates canonical Wnt- β -catenine signaling (Santos, Bakker et al BBRC 2010), a key pathway in the adaptive response of bones to mechanical loading. In accordance with the above, several lines of evidence support the potential beneficial effects of NO donor compounds in the prevention of estrogen deficiency bone loss [49]; [50].

ROS/RNS in osteoclasts

Osteoclasts are multinucleated cells derived from the fusion of monocytes and macrophages. Their primary function is to mediate bone resorption [51]. The key inducer of OC differentiation is RANK ligand (RANKL) produced by osteoblasts [52]. Stimulation of osteoclast activity by RANKL is enhanced by costimulation by growth factors, TNF α and other cytokines [53]. ROS also appear to be crucial for OC differentiation. ROS produced by NADPH oxidases and the mitochondrial electron transport chain play a second messenger role in RANK signaling [54]. In line with this, several antioxidant compounds [55] [56] [57] (resveratrol, genistein, coenzym Q10, selenite, curcumin) and antioxidant enzymes [58] (SOD, catalase and heme oxygenase-1) have been shown to inhibit differentiation of monocytes and macrophages to osteoclasts. Below we highlight the roles of some endogenous redox regulatory proteins implicated in OC differentiation (Fig. 2).

a) *NADPH-oxidases*: Osteoclasts and their precursor cells express NOX1, NOX2 and NOX4 enzymes [59]. ROS-mediated OC differentiation of macrophages appeared to be unchanged in RANKL stimulated cells derived from the bone marrow of either Nox1 or Nox2 knockout mice. However silencing Nox1 in Nox2 knockout cells or silencing Nox2 in Nox1 knockout cells lead to decreased ROS production and suppressed differentiation of macrophages [60]. Moreover, the number of osteoclasts as well as the expression level of osteoclast markers were found to be reduced in Nox4 knockout mice. A lower RANKL-induced activation of NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1) and c-Jun have been identified as underlying mechanism in impaired osteoclastogenesis [61]. Collectively these data indicate, that NOX enzymes play important roles in OC development and maturation and these enzymes can compensate for reduced expression of their protein family members.

b) *Keap1/Nrf2*: Nrf2 [nuclear factor (erythroid-derived 2)-like 2] is a key transcriptional regulator of enzymes implicated in or associate with antioxidant defense: it regulates the expression of heme oxygenase-1, glucose-6-phosphate dehydrogenase, NAD(P)H dehydrogenase (quinine 1), gamma-glutamyl-cysteine synthase (the rate limiting enzyme in glutathione biosynthesis) [62]. Keap1 (kelch-like ECH-associated protein 1) is a negative regulator of Nrf2 as it inhibits the nuclear translocation and facilitates ubiquitination-mediated degradation of Nrf2 [63] [64].

Exposure of RAW264.7 macrophages to RANKL lowered Nrf2/Keap1 ratio and lead to a lack of increase in the expression of Nrf2-dependent enzymes thus favoring ROS signaling. Overexpression of Nrf2 enhanced RANKL-induced elevation in the level of antioxidant enzymes and suppressed OC differentiation. In the contrary, overexpression of Keap1 and knockdown of Nrf2 suppressed expression of antioxidant enzymes leading to increased osteoclastogenesis [65].

Data from *Nrf2*^{-/-} mice confirmed the role of ROS signaling in osteoclasts. Bone marrow-derived monocytes from *Nrf2*^{-/-} mice displayed lower glutathione levels, increased ROS production and resorption activity. These changes were not due to alterations in the NF-κB pathway but were suggested to be due to increased activity of p38 MAP kinase and NFATc1 induction [66].

c) *Steap4*: Osteoclasts express Steap4 (six transmembrane epithelial antigen of prostate 4), an endosomal ferrireductase [67] the deletion of which in macrophages results in limited availability of ferrous iron, lower mitochondrial ROS production and impaired osteoclastogenesis. Suppression of the CREB-PGC-1 β - mitochondrial biogenesis pathway has been proposed to be the mechanism underlying decreased ROS production in Steap4-deficient cells [68].

d) *Caspase-2*: The cysteine protease caspases-2 has also been implicated in the regulation of bone homeostasis [69]. Oxidative stress induces caspases-2 expression which mediates oxidative stress-induced osteoclast death. In line with the protection provided to osteoclasts by caspase-2 deficiency, an elevated osteoclast number and increased expression of the OC marker TRACP (tartarate resistant acid phosphatase) has been reported in caspase-2 knockout mice leading to bone loss. These data identify caspases-2 as an important mediator of OC number, maturation and death [70].

e) *FoxO (Forkhead box protein)*: Expression of antioxidant enzymes is under control of FoxO transcription factor and FoxO has been shown to operate in OCs [71]. FoxO stimulates catalase expression thus limiting hydrogen peroxide stress and bone resorption. RANKL-induced activation of “survival kinases” Akt and Erk (extracellular signal-regulated kinase) inactivate FoxO by phosphorylation. In line with this, deletion of FoxO in mice resulted in bone loss whereas its overexpression lead to increased bone mass [72].

f) *PAMM (peroxiredoxin activated in M-CSF-activated monocytes)*: PAMM is a CXXC motif-containing peroxiredoxin 2-like protein expressed by bone cells and in M-CSF and RANKL-stimulated monocytes. Expression of PAMM in RAW cells provided protection from hydrogen peroxide-induced damage by shifting the GSH/GSSG ratio towards the more reduced state. Altered redox state of the cells also resulted in defective activation of NF- κ B and c-Jun and consequently inhibited OC formation [73].

Reactive nitrogen species (nitric oxide and peroxynitrite) may also be involved in OC regulation [74];[75]. Osteoclasts express constitutive NOS and – especially after stimulation with IFN γ and/or LPS – also produce iNOS. Tonic production of low amounts of NO is required for proper OC function whereas in higher concentrations NO induces cell contraction and detachment of OCs from bone surfaces (MacIntyre, Zaidi et al. PNAS 1991). Peroxynitrite has been proposed to be responsible for this latter effect seen at elevated NO concentrations (Mancini L, Moradi-Bidhendi N, BBRC 1998). Although iNOS knockout mice do not have a bone phenotype under normal conditions, under inflammatory conditions, however, when inflammatory cytokines stimulate iNOS expression, iNOS-derived NO is a mediator of osteoclastic bone resorption (Van't Hof RJ, Armour KJ et al PNAS 2000). Osteoclastogenesis is also regulated by NO: while NOS inhibitors suppress, NO donor compounds stimulate osteoclastogenesis (Nilforoushan et al. Nitric oxide 2009). The NO sensitive step in this process appears to be the fusion of preosteoclastic cells (Nilforouskas Nitric Oxide 2009).

III. *PARylation in the bone*

III.1. *H₂O₂-induced PARylation in osteodifferentiation*

Production of various ROS/RNS species by different bone cells (detailed above) may trigger signaling events and may also cause damage in these cells as well as in neighboring cells [76]. It has previously been demonstrated that mesenchymal stem cells undergoing osteogenic differentiation produce hydrogen peroxide [35] and increased production of H₂O₂ is required for the successful completion of the differentiation program. The identity of the ROS species responsible for downstream effects is most likely hydrogen peroxide a common player in redox regulatory paradigms. The regulatory role of hydrogen peroxide in this model is supported by the effects of catalase which inhibited both mineralization and expression of marker genes [77] in differentiating cells. However, it seems that redox regulation by

hydrogen peroxide follows a path that is different from canonical redox mechanism and relies on poly(ADP-ribosylation) (PARylation) rather than on cysteine oxidation-based signaling events [78].

PARylation is a reversible protein modification carried out by some members of the poly(ADP-ribose) polymerase (PARP) family (also known as the ADPRT family) [79]. PARP-1 is responsible for the bulk of PARylation especially in cells that suffered DNA damage (Fig. 3). PARP-1 recognizes and binds to DNA breaks resulting in the activation of the enzyme [80]. Active PARP-1 uses NAD as substrate cleaving off nicotinamide from the energy metabolite. In turn PARP-1 attaches the remaining ADP-ribose moiety to suitable acceptor proteins and by switching to elongation mode it creates a branched poly(ADP-ribose) (PAR) polymer covalently attached to the target proteins [81]. The turnover of the polymer is usually rapid with poly(ADP-ribose) glycohydrolase and ADP-ribosyl hydrolase 3 (ARH-3) being responsible for PAR decomposition [82], [83], [84], [85]. PARylation regulates various cellular functions both in healthy and in injured cells. These include regulation of transcription [86], [87], metabolism [88], replication [89], [90], autophagy [91], and DNA repair [92]. Following severe DNA damage PARP-1 can also execute injured cells. Its active role in cell death involves inhibition of metabolic pathways and stimulation of the AIF-mediated necrotic cell death subroutine [93]. The oxidative stress – severe DNA damage – PARP activation – cell death pathway has been implicated in various oxidative stress-related diseases ranging from myocardial ischemia reperfusion injury, stroke, neurodegenerative diseases, sepsis and various forms of shock [94], [95]. Furthermore, PARP inhibition also effectively reduces inflammation [95], [94], [96]. Accordingly, PARP inhibitor therapy has emerged as a potentially effective treatment for these diseases, an option not yet tested in clinical trials in spite of very promising preclinical data.

The role of PARylation in osteodifferentiation is indicated by observations that PARP inhibitors or PARP-1 silencing inhibited the osteodifferentiation process (both mineralization and marker gene expression). The question arises as to whether H₂O₂ activates PARP-1 via DNA breakage or by any of the DNA break independent, “alternative” pathways [97]; [98]. Somewhat surprisingly, H₂O₂-induced DNA breakage appears to be the likely trigger for PARP-1 activation in this model. DNA breakage is not generally viewed as a typical step in signaling cascades but mounting evidence indicates that we should think twice before excluding this option. On the one hand, single cell gel electrophoresis assay [99] (also known as comet assay) identified DNA breaks in MSCs undergoing osteodifferentiation. Similarly, in a model of starvation-induced autophagy Oliver’s group also described a similar sequence of events: ROS production-DNA breakage-PARP activation with all of these steps required for the successful completion of autophagy [100]. Moreover, topoisomerase II β -induced promoter cleavage has also been shown to activate PARP-1 in estrogen-induced gene expression and this DNA break dependent PARylation was required for the transcription facilitating role of PARP-1 [101]. All these observations indicate that, however surprising it may sound, DNA breaks can be part of signal transduction cascades with DNA break sensors such as PARP-1 playing the role of signal transducer.

What are the molecular events acting downstream of H₂O₂ production-DNA breakage and PARP-1 activation in MSCs undergoing osteodifferentiation? Effects of PARylation could in theory be transmitted through various pathways: metabolic alterations, direct or indirect transcriptional effects, convergence on certain kinase-phosphatase pathways or impaired cell viability only to name a few. Robaszkiewicz et al. [102] have shown that p38 MAP kinase is one of these downstream mediators to consider. While PARP inhibition or PARP-1 silencing resulted in impaired p38 activation, the inhibition or silencing of p38 suppressed

osteodifferentiation. Moreover, the relationship between PARP-1 and p38 appear to be rather direct as the two proteins co-localize in and can be co-immunoprecipitated from differentiated cells [102]. These experiments identified p38 as a downstream mediator of the PARP-1-dependent regulatory pathway of osteodifferentiation.

The above detailed observations also revealed a rather strong interrelationship between osteodifferentiation and cell death. A peculiar feature of the osteodifferentiation process is that it is accompanied by massive cell death. In fact it is estimated that more than 80% of cells undergoing osteo-differentiation commits cell suicide [103]. Osteodifferentiation and cell death appear to be very closely coupled in this model as indicated by the fact that all interventions that inhibited differentiation (H_2O_2 decomposition, inhibition or silencing of PARP-1 or p38) also suppressed cell death. These observations suggest that cell death somehow contributes to bone formation. Indeed, Fujita et al [104] have shown that necrotic and apoptotic cells serve as calcification nuclei at the end stage of mesenchymal stem cell differentiation. Moreover, Melinda Duer's laboratory recently identified an important molecular link between PARP-1 dependent osteoblast death and bone formation [105]. Duer's lab generated "heavy mice" by feeding the animals with a ^{13}C -rich diet to get the carbon of mice up from 1% to 20% ^{13}C . They pioneered the technique of solid state NMR and as part of a proof of concept study they have shown that poly(ADP-ribose) polymer (likely released from dying cells) forms integral part of the bone matrix. Deposition of PAR into the bone matrix may serve as a calcification core structure attracting calcium [105] (Fig. 4).

III.2. PAR in osteoclasts

Compared to the role of PARylation in osteoblasts much less is known about what this biopolymer may do in osteoclasts. From the three publications available on Pubmed it seems

that PARylation acts a transcriptional repressor in differentiating OCs. For example, it has been shown that silencing PARP-1 in OC precursors resulted in enhanced expression of OC marker genes TRACP and matrix metalloproteinase-9 [106]. The PARP-1 binding site has also been identified in the TRACP promoter [106]. Induction of OC differentiation of RAW monocytes has also been shown to liberate TCIRG1 (V-type proton ATPase 116 kDa subunit a isoform 3) gene from PARP-mediated repression [107]. Of note, this gene encodes the $\alpha 3$ isoform of the V-ATPase a subunit, mutations of which accounting for the majority of infantile malignant osteopetrosis cases. Furthermore, RANKL stimulation induces a massive upregulation in the expression of brain type cytoplasmic creatin kinase (ckb) [108]. Ckb expression also appears to be under PARylation control as indicated by observations that upon RANKL stimulation ckb induction is accompanied by reduced PARP-1 protein level [108]. These two molecular events are interrelated as PARP-1 has been demonstrated to bind to the consensus sequence TTCCA in the ckb promoter and silencing PARP-1 resulted in enhanced ckb expression [108]. These data collectively identify PARP-1 as a negative transcriptional regulator in differentiating OCs.

IV. Conclusion and future prospects

Data summarized in this review clearly point towards a central role of redox signaling in bone biology with reactive oxygen and nitrogen species regulating differentiation, vital functions and death of both osteoblasts and osteoclasts. Recently a new pathway of “hydrogen peroxide production-DNA breakage-PARP activation-metabolic dysfunction and cell death” has been described to accompany osteoblast differentiation. The most interesting new development in this field has been the identification of poly(ADP-ribose) as a structural element in the bone matrix. PARylation can no longer be considered as a transient

signaling event often described in various oxidative stress situations as the deposition of its product, poly(ADP-ribose) in the bone matrix has changed our view of this biopolymer. However, several open questions remain to keep PAR and bone enthusiasts busy in the next couple of years. These include the role of PAR degradation, the mode of PAR release (protein-bound or “naked” polymer, cell disruption versus regulated PAR release) and the role of the polymer in the bone matrix and in mineralization. Moreover, it remains to be seen whether or not animals deficient in PARP1 have any detectable abnormality in their bone structure and bone metabolism. Finally, probably the most intriguing question is if, similarly to the mineralization of the bone matrix, the polymer is also detectable in various soft tissue mineralization processes.

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Legends to figures**Fig. 1. Maturation of cells in the bone.**

The bone is home to progenitor (hematopoietic and mesenchymal lineage) and effector (osteoblast, osteocyte and osteoclast) cell types. The first notable representatives of the bone-forming lineage are the mesenchymal progenitor cells that differentiate into pre-osteoblasts then osteoblasts, finally lining cells and osteocytes are formed. Lining cells can be found both on the internal and the external bone surfaces (endosteal and periosteal cells, respectively). As for the development of cells in charge of bone resorption, the first step is the differentiation of hematopoietic progenitor cells to osteoclast precursors. The fusion of osteoclast precursors results in bone-resorbing, multi-nucleated osteoclasts.

Fig. 2. Redox signaling in the bone

Differentiation of osteoclasts is induced by RANK ligand (RANKL) produced by osteoblasts [52]. Stimulation is enhanced by costimulation by growth factors, TNF α and other cytokines [53]. Binding of RANKL to RANK results in the recruitment of TRAF-6 [109], which activates NF- κ B [109], [110], Akt [111], c-Jun and c-Fos (through p38) [112]. The key regulator of RANKL-induced osteoclast differentiation is NFATc1, which is regulated by NF- κ B, c-Jun and c-Fos [113]. ROS also seems to play a crucial role for OC differentiation, as a second messenger in RANK signaling [54]. Endogenous sources of superoxide are NADPH oxidases (NOX 1, 2 and 4) and the mitochondrial electron transport chain. In line with this, several antioxidant compounds [55] [56] [57] (resveratrol, genistein, coenzym Q10, selenite, curcumin) have been shown to inhibit OC differentiation. Nrf2 and FOXOs are responsible for the induction of different antioxidant enzymes which are also negative regulators of OC differentiation. The production of above mentioned antioxidant enzymes is inhibited by Akt

which facilitates differentiation also by the activation of NF- κ B pathway [111]. Dependent on the level of oxidative stress, caspase 2 decides about the fate of the cell. Low ROS results in the induction of FOXO-dependent antioxidant enzymes [114], while high amounts of reactive species switch caspase 2 to initiate apoptosis [70]. Caspase 2 is negatively regulated by NF- κ B.

Fig. 3. The PARylation cycle

PARP enzymes cleave NAD into nicotinamide and ADP-ribose and covalently attach the latter to suitable acceptor proteins. By cleaving further NAD molecules the enzymes attach more ADP-ribose units to the protein proximal first ADP-ribose residue and build a branched PAR polymer which is decomposed mainly by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl hydrolase (ARH) enzymes.

Fig. 4. Role of PARylation in bone formation

Mesenchymal stem cells undergoing osteogenic differentiation produce H_2O_2 . H_2O_2 causes single stranded DNA breaks, which lead to the activation of PARP-1. Osteogenic differentiation signals also trigger the activation and nuclear translocation of p38 with both steps being dependent on H_2O_2 production and PARP-1 activity. The H_2O_2 -DNA breakage-PARP-1-p38 pathway mediates not only the differentiation but also cell death accompanying osteodifferentiation. PAR released from dead cells is incorporated into the bone matrix where the negatively charged pyrophosphate groups of the polymer likely contribute to calcium binding and mineralization of the matrix.

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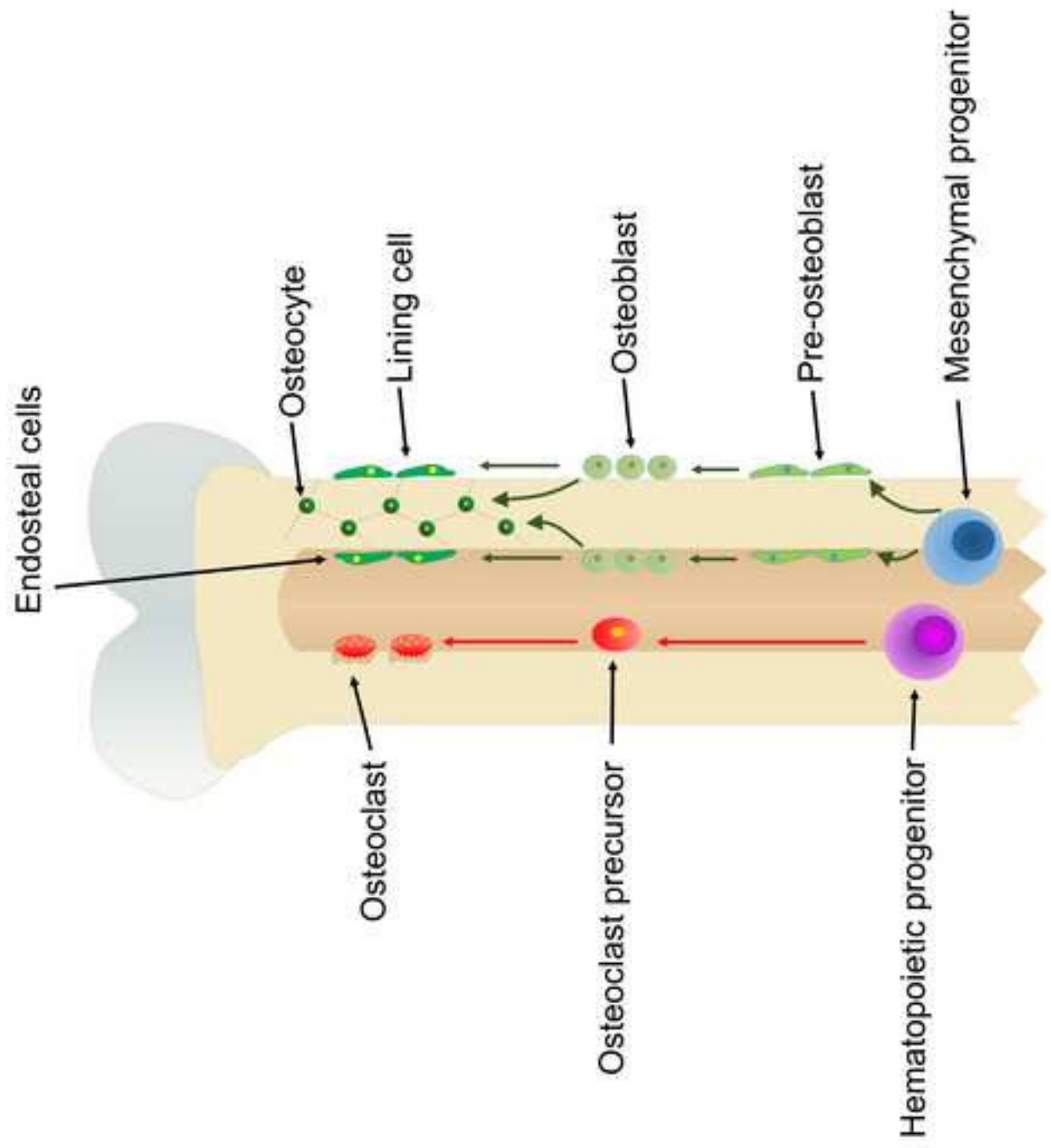
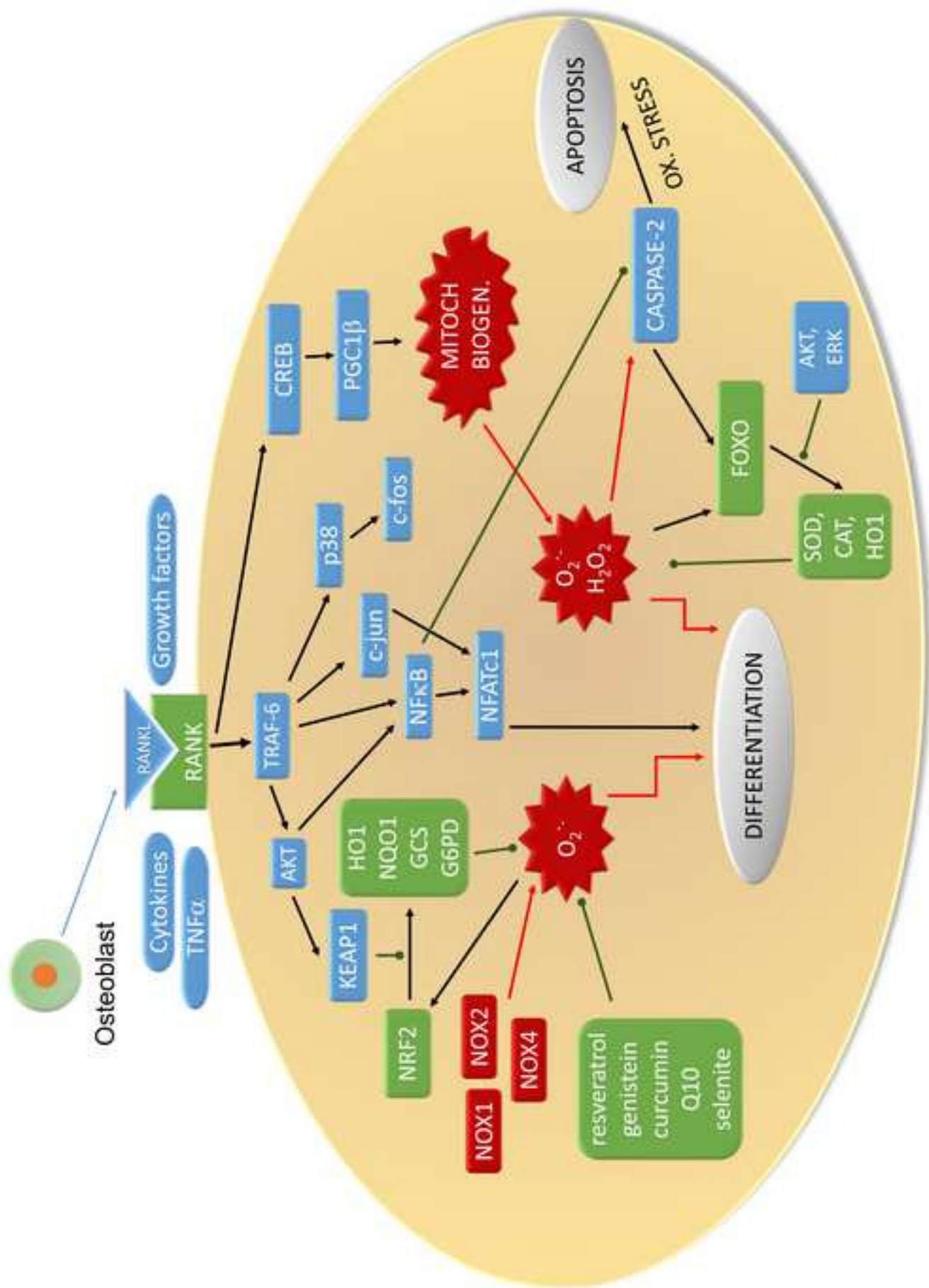
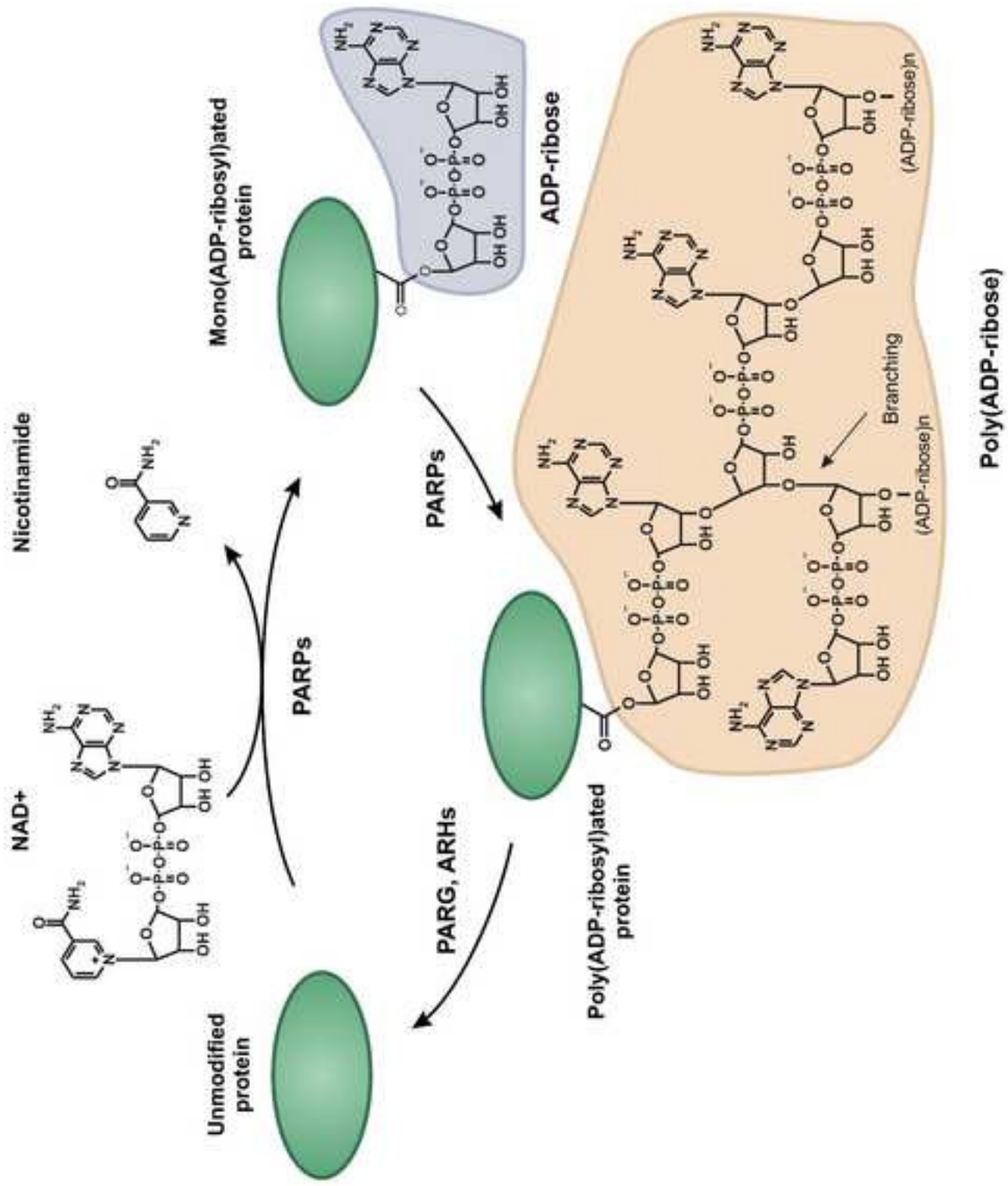
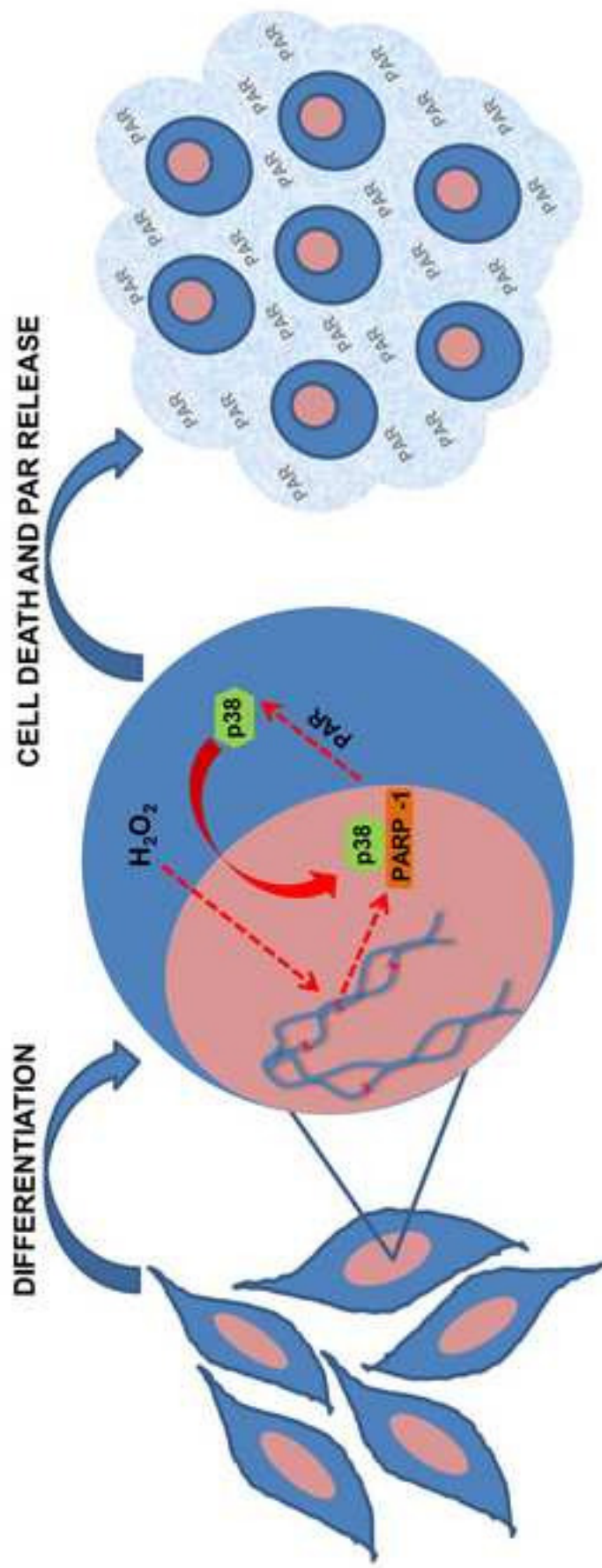


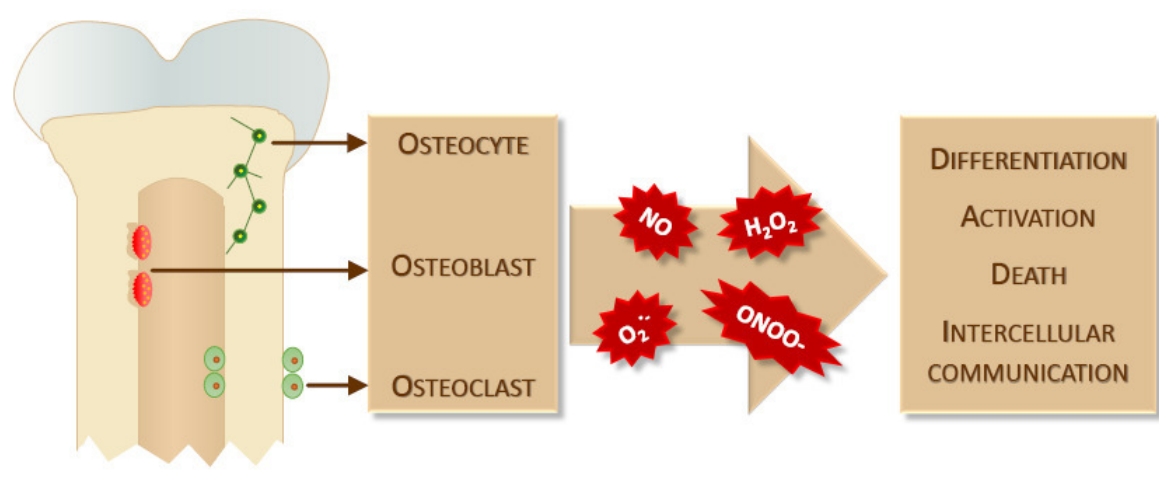
Figure 1







Graphical abstract



Accepted man