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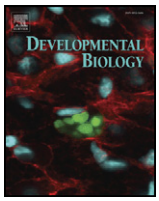

Review

Function of reactive oxygen species during animal development: Passive or active?

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ABSTRACT

Oxidative stress is considered causal of aging and pathological cell death, however, very little is known about its function in the natural processes that support the formation of an organism. It is generally thought that cells must continuously protect themselves from the possible damage caused by reactive oxygen species (ROS) (passive ROS function). However, presently, ROS are recognized as physiologically relevant molecules that mediate cell responses to a variety of stimuli, and the activities of several molecules, some developmentally relevant, are directly or indirectly regulated by oxidative stress (active ROS function). Here we review recent data that are suggestive of specific ROS functions during development of animals, particularly mammals.

Introduction

The evolution of chlorophyll-containing cyanobacteria ~2.5 billion years ago provoked radical changes in the composition of our planet's atmosphere. Carbon dioxide concentrations fell as oxygen concentrations rose, and rapidly the inhabitants of this changing environment evolved to exploit this new resource. Yet while essential for energy generation, incorporating oxygen into metabolic processes had side-effects, namely, the production of reactive by-products that damage the very molecules required for life. Under these circumstances, mechanisms to protect organisms from oxidative damage or to repair the damage caused by oxygen evolved. And remarkably, apart from simply seeking to minimize and reduce their damaging effects, organisms adapted and evolved to exploit these 'harmful' molecules in myriad biological processes. This is most evident and widely-studied in 'adult' organisms, however this review will focus on the role of such oxygen derivatives in the developmental phases leading to adulthood.

Control of ROS levels and generation of oxidative stress

Reactive oxygen species (ROS) is a generic name given to a variety of molecules and free radicals derived from molecular oxygen. The reduction of oxygen produces relatively stable intermediates. One electron-reduction produces superoxide anion, which is the precursor of most ROS. As most commonly used, ROS in this review refer to superoxide, hydrogen peroxide and their derivatives such as the hydroxyl radical. However, other special ROS with biological effects exist. Nitric oxide, for example, is a short-lived molecule with a free electron that regulates many physiological functions by itself, some of which are associated with development (Kuzin et al., 1996; Regulski et al., 2004). Hydrogen peroxide is not as reactive as the hydroxyl radical yet the latter is readily generated when the former is in the presence of Fe^{3+} (Fenton reaction). Superoxide is also not very reactive but can react with nitric oxide to produce the very potent oxidant peroxynitrite (Estevez et al., 1998). Singlet oxygen, an electronically excited form of oxygen, is very reactive and produces clear effects on cells (Klotz et al., 2003), but its biological relevance waits for more *in vivo* studies.

ROS production

ROS are formed as unavoidable by-products of aerobic respiration and various other catabolic and anabolic processes (Halliwell, 1991) (Fig. 1). For example, the respiratory chain produces essentially super-

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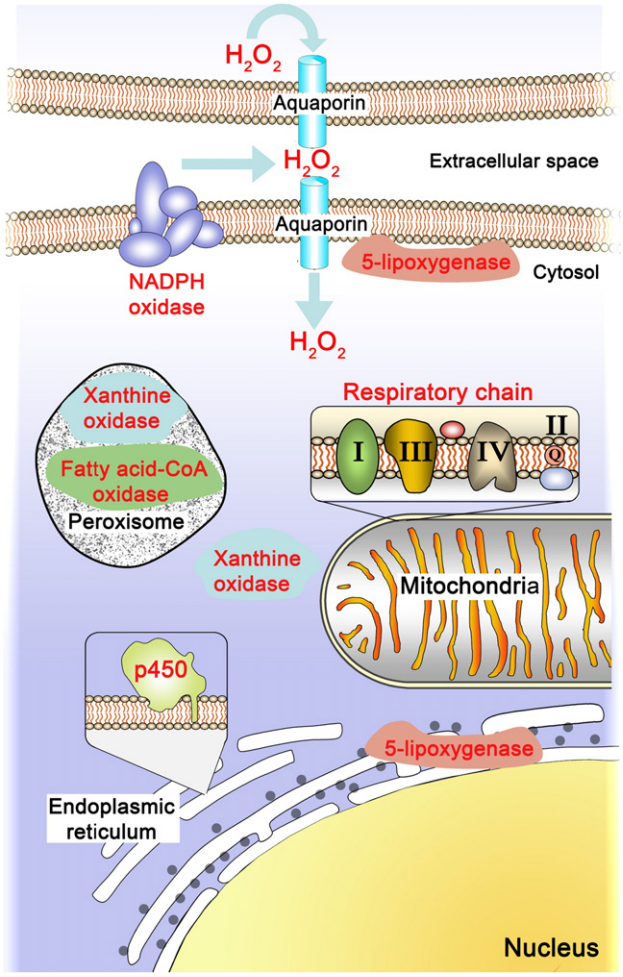

Fig. 1. Places in the cell where ROS are produced. Major organelles that are known to be sources of ROS are depicted. The activity of the respiratory chain in the mitochondria is responsible of most ROS produced in aerobiosis. On the other hand, the metabolic pathway that drives the degradation of long chain fatty acids (i.e., β-oxidation) in the peroxisome is also an important ROS source, though the amount produced depends on the activity of this metabolic pathway, a property that is cell-type specific. The function of ROS produced by cytochrome p450 or NADPH oxidases may be restricted to the area where they are located. Specific cytochrome p450 are involved in the synthesis and degradation of steroid hormones and retinoic acid, relevant molecules in development. Peroxisomal or cytosolic xanthine oxidase is an enzyme that produces ROS from molecular oxygen, whose best-characterized function is in the final catabolism of purines. 5-lipoxygenase, an enzyme involved in the synthesis of leukotrienes, can be found associated with membranes or with the nuclear envelope. ROS can also function as paracrine signals, as hydrogen peroxide can cross from one cell to another through aquaporins.

oxide that can be converted to peroxide by superoxide dismutases (Turrens, 2003). Among the enzymes that produce ROS by-products are (Fig. 1): fatty acyl-CoA oxidase, xanthine oxidase, cytochrome p450 systems, cycloxygenases and lipoxygenases (Wanders and Waterham, 2006; Soberman and Christmas, 2003). ROS are directly produced from oxygen by NADPH oxidases, a major family of enzymes whose catalytic subunits are encoded by *Nox1–5* and *Duox1–2* (Lambeth, 2004). Although this activity was initially detected during phagocytosis, it is now known that these enzymes are broadly distributed among many tissues (Lambeth, 2004). Interestingly, Nox enzymes are almost exclusive to multicellular organisms (Lalucque and Silar, 2003).

Due to the high reactivity of some ROS, the location where they are produced is critical for their biological effects (Fig. 1). Nonetheless, although it is common to think that ROS are cell autonomous signals produced within the affected cell, there are examples in which ROS holding low reactivity, such as hydrogen peroxide, appear to mediate intercellular communication. Paracrine communication could result when ROS are released from normal (i.e., myofibroblasts) or apoptotic cells and affect neighboring cells (Pletjushkina et al., 2005; Waghray et al., 2005). It is frequently considered that hydrogen peroxide diffuses freely through biological membranes; however, its water-like electrostatic characteristics suggest a limited simple passive diffusion. Recently, it was found that specific aquaporins, initially described as water channels that are present in all living cells, facilitate hydrogen peroxide diffusion across cell membranes (Bienert et al., 2007) (Fig. 1). Diffusion of hydrogen peroxide also plays a role in the autocrine signaling mediated by NADPH oxidases (Fig. 1). The diffusibility of ROS is a property that may contribute to determine the redox state of a community of cells or the propagation of ROS signals, mechanisms that could coordinate developmental events such as massive cell death.

ROS elimination

Levels of ROS are not only determined by production, but also by the rate of ROS degradation or inactivation. In general terms, the ultimate effect of antioxidants is to decrease the amount of active ROS. Cells have many ways to respond against ROS, including enzymatic and non-enzymatic antioxidants. It is the balance between the production and degradation of ROS that maintains the cellular homeostasis.

Common non-enzymatic antioxidants are glutathione and thioredoxin (Holmgren et al., 2005; Wu et al., 2004). Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) synthesis is catalyzed by the sequential action of γ-glutamylcysteine synthetase and GSH synthetase. (Lu, 1999). Once GSH is oxidized (GSSG), the reduced form can be regenerated by the GSH reductase (Gr). The balance between GSH and GSSG is a way to determine the redox state within the cell. On the other hand, thioredoxins (Trx), as well as glutaredoxins (Grx), are small proteins containing an active site with a redox-active disulfide (Holmgren et al., 2005). These proteins maintain a reduced intracellular redox state in mammalian cells by the reduction of protein thiols. Two Trx and three Trx reductases (TrxR) are present in mammals, each with a distinct intracellular location (Nakamura, 2005). Trx1 and TrxR1 are cytosolic or nuclear proteins, whereas Trx2 and TrxR2 are targeted to the mitochondria. Of the two Grx present in mammals, Grx2 appears to be in mitochondria and the nucleus (Lundberg et al., 2001).

Antioxidant enzymes act in concert to remove various ROS produced by free radical reactions. Superoxide dismutases (Cu/Zn-Sod and Mn-Sod) scavenge the superoxide radical, converting it into hydrogen peroxide and oxygen. In mammals there are three Sod: Sod1 (cytoplasmic) and Sod3 (secreted) are Cu/Zn-dependent, whereas Sod2 (mitochondrial) is Mn-dependent (Maier and Chan, 2002). Catalase and the peroxidases, on the other hand, convert hydrogen peroxide into water (Kirkman and Gaetani, 2007; O'Brien, 2000). Mammals have many peroxidases but only one catalase (Jin et al., 2003).

GSH and Trx, in addition to forming part of the antioxidant systems described above, are also specific substrates of a group of peroxidases. The glutathione peroxidases (Gpx) are a group of selenoproteins that catalyze the reduction of peroxides generated by ROS at the expense of GSH (Arthur, 2000). Four Gpx have been identified in mammalian systems: Gpx1 (the most abundant peroxidase), Gpx2 (mostly expressed in the gastrointestinal tract), Gpx3 (the plasma Gpx form), and Gpx4 (a membrane-associated protein also called phospholipid glutathione peroxidase; PHGPx). Gpx4 is unusual in that, besides being active as a monomer, it is the only enzyme capable of reducing the peroxidized lipids present in cell membranes (Imai and Nakagawa, 2003). The Trx-dependent peroxidases are known as peroxiredoxins (Prx). Six Prx have been described in mammals, each with a characteristic intracellular distribution (Wood et al., 2003). Prx1 and Prx2 are found in the cytoplasm; Prx3 is specific of mitochondria; Prx4 is found in the endoplasmic reticulum, lysosomes and in the

extracellular space; and Prx6 exists in both the cytoplasm and mitochondria. All but Prx3 can be found in the nucleus.

Generation of oxidative stress

An oxidative stress condition is generated when a cell accumulates an excessive concentration of ROS. This occurs when ROS production exceeds cellular defenses. All active cells produce a certain amount of ROS, but antioxidant systems maintain the levels low. As a passive effect, ROS can be detrimental to cells due to oxidative damage to lipids, proteins, and DNA. Among the resulting products of this oxidation are lipid hydroperoxides (Rikans and Hornbrook, 1997), carbonylated proteins (Dalle-Donne et al., 2003), and DNA with oxidized bases (e.g., 7,8-dihydro-8-oxoguanine; David et al., 2007). The following sections describe the possible active roles of ROS during development.

ROS regulation of signaling molecules

It is now well accepted that ROS are signaling molecules that, as with other second messengers, transduce messages from the extracellular milieu to generate a specific cellular response (active ROS function). ROS play defined functions through redox modifications of a great diversity of molecules participating in almost every signaling pathway described up to date. Interestingly, many proteins relevant for development are sensitive to oxidative stress conditions (Figs. 2, 3). Proteins are known to undergo redox modifications mainly at cysteine (Cys) residues, although other amino acids can be oxidized (e.g., Tyr, Trp, His) (Droge, 2002). Cys thiols undergo different degrees of oxidation by different ROS at the –SH groups resulting in generation of sulphenic acid (–SOH), sulphinic acid ($-SO_2H$) or sulphonic acid ($-SO_3H$). Alternatively, glutathionylation is the modification incorporated in the protein after oxidation. The variety of macromolecules sensitive to redox modification by ROS is particularly striking, from extracellular matrix molecules, to phosphatases, kinases and a great number of transcription factors. It is important to mention that redox regulation in some cases is indirect through, for instance, the interaction with Trx (Fig. 3).

Kinases and phosphatases

Regulation of protein phosphorylation is fundamental in many developmental processes; thus, the effect of intracellular redox states on the activity of protein kinases and protein phosphatases must be considered of particular interest. Activation of tyrosine kinases is recurrent in the control of proliferation and differentiation by several growth factors. Several protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) have been found to be redox sensitive (Fig. 3). Possible targets for oxidation are cysteines. In fact, 81 out of 82 PTK have a conserved Cys987, and 65 out of those 81 PTK have this residue within the CXXXXXXXMXXCW motif (Nakashima et al., 2005). On the other hand, PTP have the conserved motif CXXXXXR (Rhee, 2006). Serine–threonine kinases are also regulated by redox via thiol

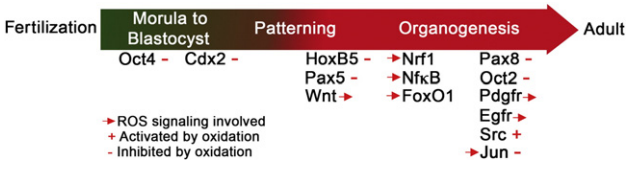

Fig. 2. Redox regulation of developmentally relevant molecules. Inhibition (−) or activation (+) can result from direct oxidation of a particular molecule. In addition, a ROS-mediated signaling pathway can act upstream (arrow at the left) or downstream (arrow at the right) of a molecule and determine its activity. The scheme shows molecules whose developmental function has been genetically demonstrated and their activity is regulated by ROS. The arrow represents embryo development transiting from anaerobic (green) to oxidative (red) metabolism; molecule position along the arrow indicates the approximate stage at which the specific molecule or members of the same family appear to be essential.

modifications that result in activation or inhibition of kinase activity (Cross and Templeton, 2004; Giannoni et al., 2005; Humphries et al., 2005; Park et al., 2000).

Regulation of kinase activity by a redox-regulated phosphatase is illustrated by the action of a MAP kinase phosphatase (Mkp) on Jun kinase (Jnk) (Fig. 3). In this case, the inactivation of Jnk by Mkp is abrogated by the oxidation of the latter (Kamata et al., 2005a). Another example is Pten, a PIP3 phosphatase that antagonizes the PI3 kinase activity and which, upon oxidation, leads to an up-regulation of this activity (Lee et al., 2002) (Fig. 3). Trx can also directly inhibit Ask1, a MAP kinase kinase kinase; ROS activate Ask1 by oxidizing and inducing the dissociation of Trx (Saitoh et al., 1998) (Fig. 3).

Transcription factors

Transcription factors have been identified acting at a decisive point in almost every developmental process. Commonly, transcription factors are visualized as the end target of all signaling pathways. Transcriptional activation of specific genes always involves a multiprotein complex in which the contribution of an individual transcription factor can result from increased stability, protein processing and posttranslational modifications. The classical posttranslational modification that regulates the activity of transcription factors is phosphorylation. However, in recent years redox regulation of transcription factor activity has been widely documented. In this part of the review, we describe the redox regulation of transcription factors with potential developmental implications (Fig. 2). For more detailed description of transcription factor redox regulation, we direct readers to some specific reviews on the topic (Allen and Tresini, 2000; Dalton et al., 1999; Sun and Oberley, 1996).

An interesting feature of various redox-regulated transcription factors is that they possess highly evolutionarily conserved redox-sensitive Cys residues. For example, paired domain-containing proteins (Pax), known to play important roles during animal development (Robson et al., 2006), bear two conserved cysteines: Cys37 (with the exception of hydra Pax-A) and Cys49. A highly basic local environment surrounds both conserved Cys making these residues ideal targets for redox regulation (Tell et al., 1998a; Tell et al., 1998b; Tell et al., 2000). Homeodomain proteins represent another family of transcriptional factors important for development that can be regulated by redox conditions (Manak and Scott, 1994). HoxB5 have been found to be redox sensitive *in vitro* and, opposite to the effect in other transcription factors, its DNA-binding ability is enhanced upon oxidation (Galang and Hauser, 1993). The redox-sensitive amino acid is also a Cys at position 232. As in the case for Pax proteins, many other homeobox proteins in several animal species described up to date (e.g., Ant, Scr, HoxA5, HoxB5, HoxC5, HoxA6, HoxB6, HoxC6, HoxA7, HoxB7) contain a highly conserved Cys residue (corresponding to Cys39 in the homeobox) (Gehring et al., 1994). These highly conserved Cys suggest that the activity of several Hox proteins is also redox regulated. Cdx2, another homeobox containing protein, binds *in vitro* as a dimer to its regulatory element in a redox-dependent manner (Suh et al., 1994).

Oct proteins, members of the POU domain-containing transcription factors, possess a highly conserved Cys at position 50 (Herr and Cleary, 1995). Experimental evidence has demonstrated that Cys50 is redox sensitive, at least in Oct2 and Oct4 proteins (Rigoni et al., 1993; Smith et al., 1998). Oct4 is of special interest because of its essential function in the maintenance of stem cell pluripotency. Oct4 binding to the DNA promoter region of *Fgf4* is redox-sensitive (Lickteig et al., 1996) and its transcriptional activity can be increased by direct interaction with Trx (Guo et al., 2004).

A common mechanism to maintain the reduced state of several transcription factors is via the combined action of Trx and Ape1/Ref1 (Fig. 3). Ref1 is a multifunctional protein with endonuclease and oxidoreductase activity (Evans et al., 2000; Xanthoudakis et al., 1992). Trx can reduce oxidized Ref1 that in turn, in the nucleus, reduces the

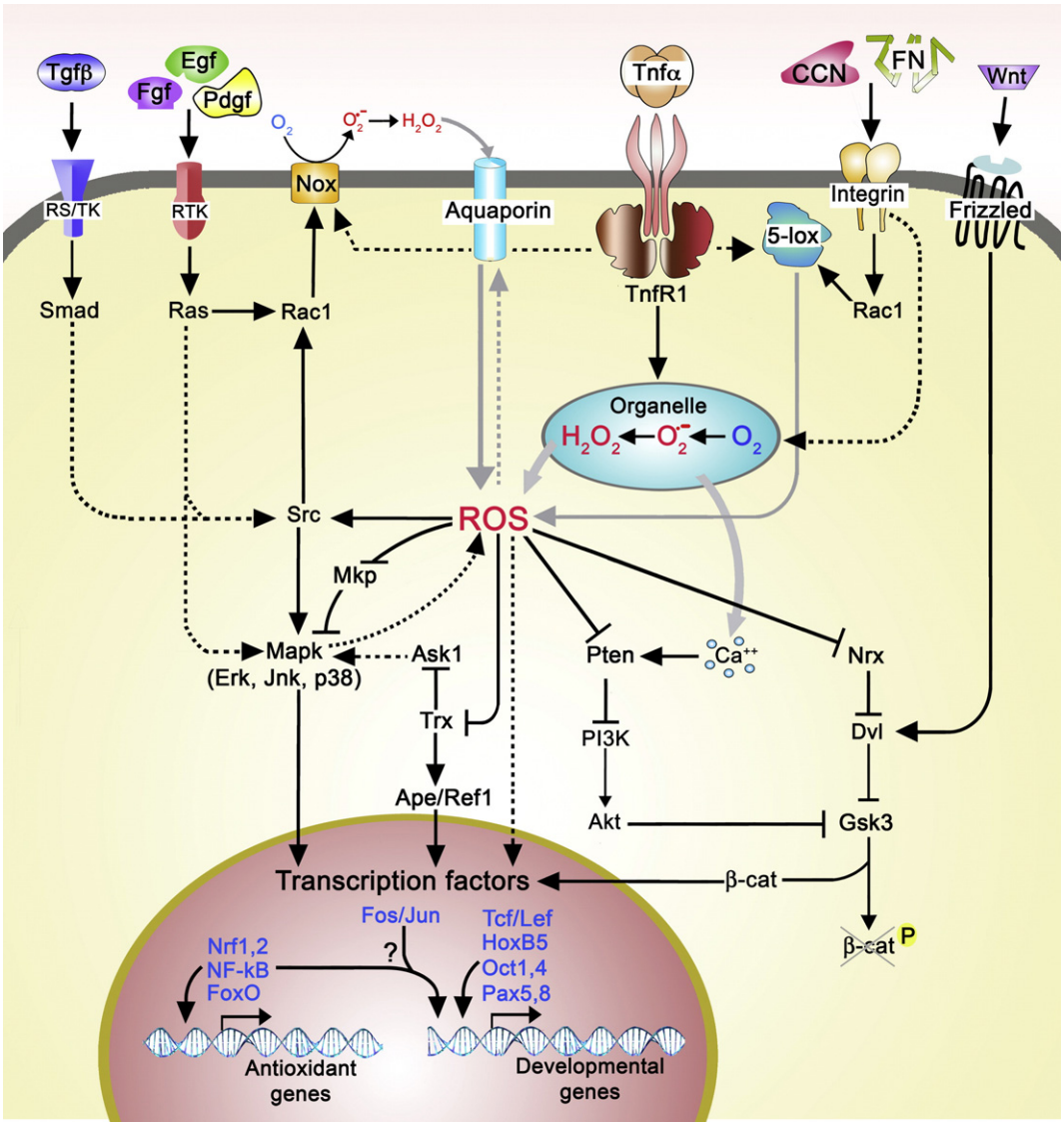

Fig. 3. An integrative overview of signalling pathways modulated by redox. Extracellular signals such as growth factors promote initial ROS production mainly through NADPH oxidases at the plasma membrane. Rac1 controls the activity of such enzymes by regulating its assembly, hence this molecule acts as modulator of signal transduction in several pathways. Tnfα induce ROS production via mitochondria as well. Other membrane proteins like integrins induce the production of ROS from mitochondria and 5-lipoxygenase (5-lox). Trx is a key protein that can act as a negative (Ask) or, in combination with Ref, as a positive regulator. Wnt signalling is negatively regulated by reduced Nrx, which binds to Dvl. Several kinases and phosphatases are redox sensitive. Expression and/or product activity of some specific transcription factor genes are regulated in response to oxidative stress, which in turn determine the transcription levels of genes (developmental genes) that control proliferation, differentiation, or cell death. Positive (e.g., Mapk-mediated) and negative (e.g., antioxidant response) regulatory circuits determine the final levels of ROS. Not all the pathways included occur at the same time in the same cell. See text for a more detailed description of the pathways. Continuous arrows indicate direct or apparent direct interactions, whereas dotted arrows indicate indirect interactions (in some cases the intermediate proteins are known but were not included for simplification). RS/TK, receptor with serine–threonine kinase activity; RTK, receptor with tyrosine kinase activity; FN, fibronectin.

Cys of the DNA-binding domain of transcription factors enhancing their activity (Hirota et al., 1997). This mechanism may be relevant for the restoration of transcriptional activity after oxidative stress. Interestingly, *Ref1*-deficient mice die shortly after implantation (Xanthoudakis et al., 1996). Besides the well-described case of AP1 transcription complex (Xanthoudakis et al., 1992), experimental evidence has shown that at least Pax-5 and Pax-8 DNA-binding activity is increased by interaction with Ape1/Ref1 (Cao et al., 2002; Tell et al., 1998a; Tell et al., 1998b; Tell et al., 2000).

ROS-induced transcriptional activation

In addition to the posttranslational modifications induced by ROS on signaling proteins, ROS can induce the transcriptional activation of specific genes (Figs. 2, 3). Interestingly, transcription factors that have been identified for their role in oxidative stress response also appear to play a fundamental role in developmental processes. For example, NFκB is induced in response to an increase in ROS concentration (Martindale and Holbrook, 2002) and its activity is regulated by the Trx/Ref1 system (Evans et al., 2000; Xanthoudakis et al., 1992). NFκB activation is in part associated with an antioxidant response, since Sod2 and Trx are among its target genes (Djavaheri-Mergny et al., 2004). Dominant-negative inactivation of NFκB in the chick limb blocks growth (Bushdid et al., 1998; Kanegae et al., 1998) and, in zebrafish, notocord development is affected (Correa et al., 2004). In mice, different defects have been observed depending on the NFκB member or members affected; defects include fetal liver degeneration, altered hematopoiesis, and abnormal formation of several epidermal appendices (Grossmann et al., 1999; Pohl et al., 2002; Schmidt-Ullrich et al., 2001). Dorsal, the homologous gene in *Drosophila*, is essential for dorso-ventral axis formation (Bergmann et al., 1996). Mice deficient in *FoxO1*, another gene activated by oxidative stress, is

essential for embryogenesis, apparently due to a vasculature defect (Hosaka et al., 2004), and a combined lack of three FoxO members (1, 3 and 4) causes hematopoietic stem cell resistance to oxidative stress (Tothova et al., 2007).

The coordinated expression of antioxidant genes upon oxidative stress is controlled, at least in part, by the antioxidant response element (ARE) (Favreau and Pickett, 1995). The transcription factors mediating this induction appear to be Nrf1 and Nrf2, members of the evolutionarily conserved subfamily of bZIP proteins CNC-bZIP (Jaiswal, 2004). *Nrf1* and *Nrf2* are widely expressed and their overexpression transactivates ARE-containing promoters. *Nrf1* mutant embryos have an abnormal liver and are anemic, which is the likely cause of lethality at midgestation (Farmer et al., 1997). *Nrf2*-deficient mice, on the other hand, are apparently normal but the expression of some antioxidant enzymes is decreased and they are sensitive to oxidative stress (Chan et al., 1996). Cells lacking Nrf1 are also sensitive to treatment with prooxidants. The analysis of double mutants suggest that *Nrf1* an *Nrf2* are partially redundant in mediating ARE function and antioxidant response during embryogenesis (Leung et al., 2003).

Signaling pathways

As briefly highlighted in the previous section abundant *in vitro* and *in vivo* evidence exists that numerous components of different signaling pathways undergo redox modifications. In addition, it is now known that stimulation of several receptors of growth factors and cytokines actively promote the production of ROS as a downstream signaling event (Fig. 3). Therefore, different receptor-mediated signaling pathways not only respond to cellular redox changes, but they also actively participate in promoting redox changes inside the cells. The most studied example is the oxidative burst that occurs in phagocytic cells. Here, we highlight evidence reported in non-phagocytic cells.

Mitogen signaling

Different growth factors with mitogenic activity such as Pdgf, fibroblast growth factor (e.g., Fgf2), and epidermal growth factor (Egf)/transforming growth factor α (Tgfα) activate signaling pathways that promote the production of intracellular superoxide and hydrogen peroxide (Fig. 3). The increased mitogenic activity of Pdgf in the absence of Prx2 reveals the relevance of ROS in the regulation of cell proliferation by this growth factor (Choi et al., 2005). NADPH oxidases have been shown to be responsible for the production of ROS in these pathways. Interestingly, overexpression of a dominant-negative form of Ras or Rac1 is capable of blocking the mitogen-induced production of intracellular superoxide (Cheng et al., 2006; Thannickal et al., 2000). This demonstrates that Ras and Rac1 are downstream of Pdgf, Fgf and Egf receptor signaling for the stimulation of ROS production. In agreement with this proposal, constitutively-active Ras or Rac1 also activate NADPH oxidase. ROS production induced by constitutively-active Ras is inhibited by a dominant-negative form of Rac1, suggesting that Rac1 is downstream of Ras (Cheng et al., 2006). Importantly, Egf-stimulated ROS production induces the phosphorylation of different cellular proteins that includes the receptor itself, indicating the presence of a regulatory feedback loop (Thannickal and Fanburg, 2000). Ngf and Tgfβ also promote the production of ROS; the former is mediated by Ras and Rac (Suzukawa et al., 2000), whereas the latter is not (Thannickal et al., 2000).

Integrin signaling

An integrin-dependent signaling mechanism involving ROS production has been recently identified (Fig. 3). Initially, after the extracellular matrix (e.g., fibronectin, CCN) contacts the cell, ROS are released from the mitochondria, and later, a second intense ROS burst is detected which is dependent on 5-lipoxygenase, an enzyme involved in the synthesis of leukotrienes from arachidonic acid (Chen et al., 2007; Taddei et al., 2007). This has two important implications. First, a single type of receptor is capable of promoting ROS formation from two different sources, and second, ROS formation can occur at different times upon the same signaling input. Src appears to be one relevant ROS target leading to its activation and subsequent focal adhesion and cytoskeleton rearrangement. Focal adhesion kinase (Fak) activation by ROS might explain the role of ROS in cell migration as well as other developmental processes requiring extracellular matrix–cell adhesion (Ben Mahdi et al., 2000).

Wnt signaling

Wnt proteins are among the most important molecules essential for animal development (Clevers, 2006). Different signaling pathways are used by Wnt to play multiple roles in embryogenesis, from pattern formation to specific cellular processes such as proliferation and differentiation (Clevers, 2006). The canonical Wnt signaling pathway results in the accumulation of β-catenin and its translocation to the nucleus, where it activates the transcription factor Tcf. Cytosolic accumulation of β-catenin occurs after Wnt binds to its receptor Frizzled causing the inhibition of the complex that continuously promotes β-catenin degradation. Dishevelled (Dvl) mediates this process. Recently, it was determined that a Trx-related protein named nucleoredoxin (Nrx) could play a regulatory role in this Wnt signaling pathway by directly controlling Dvl activity (Funato et al., 2006). Nrx binds to Dvl in its reduced form suppressing Wnt signaling (Fig. 3). In *Xenopus* embryos, increasing or reducing Nrx protein level leads to embryo abnormalities that directly relate to Wnt signaling inhibition or activation, respectively. Hydrogen peroxide can oxidize Nrx, which releases Dvl and promotes β-catenin accumulation in the absence of Wnt ligand. On the basis of these data, it is predicted that ROS levels are determinant for the activation of the Wnt signaling pathway and, consequently, should be tightly regulated for correct development.

Cellular processes regulated by ROS

As described above, many molecules relevant for development are sensitive to the action of ROS. However, is the oxidation of these particular proteins relevant in defining the fate of a developing cell? The answer to this question is not known, but many studies in cell culture have shown the ability of ROS to regulate cellular processes fundamental for development such as proliferation, differentiation, death, and migration (Fig. 4). Another question is how are the varied responses to the same signal (e.g., hydrogen peroxide) generated? ROS may function as a 'classical' second messenger; that is, the specific response depends on the cell type, the intracellular compartment where ROS are produced, and the specific ROS and dose. Indeed, for

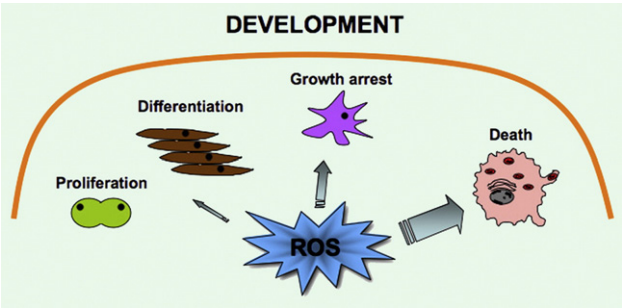

Fig. 4. Different levels of ROS induce specific cellular processes. In general, low levels of ROS are mitogenic or promote differentiation; higher amount of ROS favor growth arrest, while even higher ROS concentrations activate apoptosis. See text for specific examples.

instance, low and high hydrogen peroxide concentrations produce a contrasting p53-dependent effect on cells, in that the former causes antioxidant-mediated survival, whereas the latter promotes prooxidant-mediated apoptosis (Sablina et al., 2005).

Proliferation

Proliferating mammalian cells exhibit a broad spectrum of responses to oxidative stress (Fig. 4). Very low levels of hydrogen peroxide or superoxide stimulate proliferation of smooth muscle cells (Rao and Berk, 1992), fibroblasts (Burdon and Rice-Evans, 1989), amnion cells (Ikebuchi et al., 1991), and aortic endothelial cells (Ruiz-Gines et al., 2000), among others. Overexpression of antioxidant enzymes supports the notion of ROS as signals to maintain the growth of cells. For example, overexpression of catalase and/or Sod2 inhibits proliferation of vascular smooth muscle cell in response to Egf, which is accompanied by a reduction in Erk1/2 phosphorylation (Brown et al., 1999; Shi et al., 2004). On the other hand, higher concentrations of hydrogen peroxide can temporarily arrest growth, with housekeeping gene expression halted, and stress-related genes induced (Davies, 1999). After temporary growth arrest, many cells exhibit a transient adaptive response in which genes for oxidant protection and DNA repair are preferentially expressed. At even higher hydrogen peroxide concentrations mammalian fibroblasts are not able to adapt, but instead enter into a permanently growth-arrested state in which they appear to perform most normal cell functions but cease to divide, a state resembling cellular replicative senescence (Davies, 1999). If the oxidative stress level is further increased, cells die in an organized manner resulting in apoptosis (see below). As described above, several mitogens can mediate their effect through the production of ROS.

Differentiation

Major metabolic changes occur as cells specialize within a specific lineage. ROS production or elimination must adapt to those changes. For instance, newborn neurons but not astrocytes acquire high level of ROS upon differentiation from progenitors (Tsatmali et al., 2005; Tsatmali et al., 2006). Does the increase in ROS participate in promoting efficient differentiation during development? The answer to this question is not known but some observations suggest that it is possible. Cells with low level of ROS isolated from E15 brains differentiate into neurons that maintain higher ROS levels. Later, these immature neurons differentiate into large pyramidal-like neurons and smaller neurons that express nuclear calretinin. Antioxidant treatment does not alter neuron number but shifts differentiation towards generating a greater proportion of smaller neurons (Tsatmali et al., 2006). In addition, ROS have been shown to be essential for NGF-induced differentiation of PC12 cells (Kamata et al., 2005b), and may influence mesencephalic (Studer et al., 2000), neural crest (Morrison et al., 2000) and oligodendrocyte type-2 (Smith et al., 2000) precursor cell differentiation.

Neurogenesis is not the exception in ROS-regulated differentiation. Osteoclast differentiation is also modulated by ROS (Lee et al., 2005). Signaling through the receptor activator of NFkB (Rank) promotes differentiation of bone marrow monocyte-macrophage lineage (BMM) cells into osteoclasts. The binding of Rank ligand to its receptor leads to the recruitment of the Tnf receptor-associated factor 6 (Traf6) to the cytoplasmic domain of Rank, thereby activating a distinct Mapk signaling cascade involving Jnk, p38, and Erk. Rank ligand stimulation of BMM cells transiently increases the intracellular level of ROS by activating a NADPH oxidase. A deficiency in Traf6 blocks Rank-mediated ROS production, and antioxidants or blocking Nox1 activity inhibit the responses of BMM cells to Rank ligand, including ROS production, activation of Mapk, and osteoclast differentiation. A similar ROS-regulated mechanism has been observed during *Caenorhabditis elegans* germ line and vulval development (Shibata et al., 2003). ROS also regulate adipocyte differentiation although in a negative way. By up-regulating the expression of the gene encoding the adipogenic repressor Chop-10/Gadd153, mitochondrial ROS inhibit adipocyte differentiation (Carriere et al., 2004).

Embryonic stem (ES) cells are well suited for studying differentiation within all lineages. Using ES-derived embryoid bodies, it has been shown that cardiomyogenesis is regulated by the intracellular redox state. During differentiation, NADPH oxidase catalytic subunit Nox4 and the regulatory subunit $p67^{Phox}$ are transiently expressed and ROS are generated (Li et al., 2006; Sauer et al., 2000). PI3 kinase inhibitors or Nox4 down-regulation results in a reduction of ROS with a concomitant decrease in the number of beating foci and a reduction in the size of the area with cardiomyocytes (Sauer et al., 2000). Continuous exposure to ROS results in inhibition of cardiomyogenesis and vasculogenesis, whereas a low-level ROS pulse enhances differentiation toward the cardiomyocyte as well as vascular cell lineages (Li et al., 2006; Sauer and Wartenberg, 2005). Mechanical stress is an inducer of cardiovascular differentiation of ES cells that is also associated with NADPH oxidase activity and an increase in ROS. Treatment with free radical scavengers results in reduction in expression of genes necessary for cardiovascular differentiation such as Hif and vascular endothelial growth factor (Vegf) (Schmelter et al., 2006). It is interesting that during spontaneous differentiation of human ES cells there is also ROS generation. The expression of various antioxidant enzymes including mitochondrial and cytoplasmic superoxide dismutases, catalase, and peroxiredoxins shows a dramatic change during early differentiation (Cho et al., 2006).

Cell death

ROS, rather than being a consequence of cell death, are signaling molecules with the ability to turn on the cell death machinery (Fig. 4). As in the regulation of proliferation, different ROS levels can result in different types of cell death. In general, at the lowest death promoting ROS level, type 1 cell death (i.e., apoptosis) is observed; at intermediate levels, ROS cause type 2 cell death (i.e., autophagy) (Scherz-Shouval and Elazar, 2007); and at the highest concentrations, ROS provoke necrotic cell death (Bras et al., 2005). Two main pathways can initiate apoptotic cell death: the intrinsic, mitochondrial pathway, and the extrinsic, receptor-mediated pathway. The generation of ROS can result in mitochondrial-dependent cell death through the activation of the Mapk pathway and the proapoptotic Bcl-2 proteins Bax or Bak (Ueda et al., 2002). The source of ROS does not seem to be important in the activation of the intrinsic pathway, since mitochondrial as well as NADPH oxidase-generated ROS can induce cell death depending on the primary signal, the cell type, or the culture conditions employed (Fleury et al., 2002; Tammariello et al., 2000). Furthermore, Bax can regulate ROS production in mitochondria, suggesting a positive feedback loop between ROS production and Bax apoptotic activation (Kirkland et al., 2002).

Cell death triggered upon activation of cell surface death receptors, the extrinsic pathway, is also ROS modulated. The tumor necrosis factor alpha (Tnfα) receptor can initiate two death pathways: one directly activates caspases through adaptor proteins, and a distinct pathway involves the production of ROS (Shen and Pervaiz, 2006). Similar to the intrinsic pathway, ROS from different sources contribute to cell death (Hughes et al., 2005). Mitochondria appear to be the main ROS source, although in some cases NADPH oxidase and 5-lipoxygenase have been shown to contribute (Chen et al., 2004). Different ROS levels could elicit different effects. For instance, ROS generated from the NADPH oxidase (low level) protect, whereas mitochondria-derived ROS (high level) promote apoptosis (Deshpande et al., 2000). NFκB is the key regulator of the survival response, at least in part, by the transcriptional activation of antioxidant enzyme genes. The best-described apoptotic pathway regulated by ROS involves Jnk activation (Kamata et al., 2005a). Interestingly, there is a positive feedback loop

between Jnk activation and ROS production: Jnk contributes to Tnfα-stimulated ROS production, which in turn induces Jnk activation. Tnfα-induced elevated ROS levels are found only in wild-type mouse fibroblasts but not in *Jnk*$^{-/-}$ cells (Ventura et al., 2004). Redox regulation of several members of the MAPK pathway described above has been shown to influence the outcome in this ROS-mediated cell death mechanism (Shen and Pervaiz, 2006).

Natural motoneuron death during spinal cord development is an *in vivo* example of cell death regulated by ROS (Sanchez-Carbente et al., 2005). In this case, caspases associated to the extrinsic and intrinsic pathways appear to interact to promote motoneuron death. In addition, a caspase-independent death also contributes to this natural neurodegenerative process.

Developmental processes regulated by ROS

In mammals preimplantation and early postimplantation embryogenesis occurs under almost anaerobic conditions (i.e., a hypoxic environment; 3–5% oxygen). Thus, it is likely that during this period the embryo is very sensitive to exogenous factors that could cause oxidative stress. Accordingly, high ROS levels are detrimental for growth of embryos in culture, and administering free radical scavengers improves *in vitro* embryo development (Gardiner and Reed, 1994; Rodriguez-Gonzalez et al., 2003). It is interesting that *Oct4*, which is expressed in the embryo proper throughout the entire hypoxic period before restricting to the germ line, is positively regulated by the hypoxia-inducible factor Hif2α (Covello et al., 2006). The transition from anaerobic glycolysis to oxidative metabolism appears to occur at E9 in the mouse, coincident with the time at which chorioallantoic circulation is established and the heart starts to beat (Clough, 1985). Oxygen toxicity to the embryo is well known, but direct evidence that indicates a function for ROS in a specific developmental process is scarce.

Spermatogenesis and oogenesis

Germ cells appear to be particularly sensitive to changes in redox conditions. During spermatogenesis, spermatogenic cells and spermatozoa are protected by several antioxidant enzymes (Gu and Hecht, 1996; Nonogaki et al., 1992; Puglisi et al., 2005). Interestingly, three thioredoxins are exclusively expressed in the spermatids: *Sptrx1*, *Sptrx2*, and *Sptrx3*. Sptrx1 has a distinctive distribution in the fibrous sheath during sperm tail elongation at late spermatogenesis (Yu et al., 2002), whereas Sptrx2 is also localized in the fibrous sheath during spermatogenesis, but remains in mature epididymal spermatozoa (Miranda-Vizuete et al., 2003). Sptrx3 is localized in the Golgi apparatus during spermiogenesis (Jimenez et al., 2004). The high concentrations of polyunsaturated fatty acids in sperm cells make them highly susceptible to ROS (Alvarez and Storey, 1995). Membrane oxidation may affect motility and morphology of the sperm, finally reducing the efficiency of fusion between gametes (Imai et al., 2001).

In mammals, antioxidant enzymes such as Sod1, Sod2, and Gpx are present during different stages of oogenesis (El Mouatassim et al., 1999). All peroxiredoxins are expressed in the oocytes; particularly, Prx6 is upregulated during *in vitro* maturation (Leyens et al., 2004). Interestingly, *Sod1*-deficient female mice have drastically compromised fertility, with oogenesis halted at the middle of follicle development (Matzuk et al., 1998). The lack of Sod1 in *Drosophila* also causes reduction in fertility (Philips et al., 1989).

Fertilization and early development

GSH appears to have an important role in the preparation of the oocyte to receive the sperm and in the initiation of embryo development. An increase in GSH concentration in oocyte is associated with maturation (Luberda, 2005); this high concentration of glutathione is maintained during the first divisions followed by a continuous decrease during *in vivo* preimplantation development (Gardiner and Reed, 1994). The expression of the peroxiredoxins is sustained during the first cell division; latterly, expression declines at 16-cell embryo stage, before increasing once more at the blastocyst stage (Leyens et al., 2004). Developmental arrest of mammalian embryos cultured from just after fertilization frequently coincides with the initiation of embryonic gene expression. Such arrest at the two-cell stage in mice is released when embryos are treated with antioxidants (Natsuyama et al., 1993). All these data are consistent with normal early development occurring at very low ROS levels. This condition may apply to other organisms. In *Drosophila*, the loss of Trx homolog gene affects oogenesis and early development (Salz et al., 1994).

One interesting case where ROS apparently have a specific function is in the hatching of the mouse blastocyst from the zona pellucida (Thomas et al., 1997), at which time the embryo undergoes a superoxide burst. Different superoxide scavengers prevent the blastocyst from hatching, supporting the essential role of ROS in this process.

Morphogenesis

Among the cellular processes essential for morphogenesis is cell death. It is interesting that many of the mouse embryo regions where there is abundant cell death, also show high ROS concentrations (Salas-Vidal et al., 1998). In particular, high ROS concentration is associated to the cell death in the interdigital regions of the developing limb. ROS levels in the limb appear to be determined by *Gpx4*, whose expression is restricted to the digits (Schnabel et al., 2006). Antioxidants are also expressed in the interdigital regions, which could be part of an antioxidant response due to the high ROS concentrations in these regions (Schnabel et al., 2006; Shan et al., 2005). In agreement with a role of ROS in limb morphogenesis, interdigital cell death and interdigit regression decrease when cultured limbs are treated with antioxidants (Salas-Vidal et al., 1998; Schnabel et al., 2006). Supporting a physiological role of ROS in limb morphogenesis, retinoic acid induces cell death that is accompanied by an increase in ROS concentration and a decrease in peroxidase activity (Schnabel et al., 2006). Retinoic acid is involved in the control of cell death in other embryo regions (Cuervo et al., 2002), thus it is possible that ROS participate widely in embryo morphogenesis. In addition, the cell death that causes cavitation of ES-derived embryoid bodies also requires ROS, suggesting that ROS are essential for one of the earliest morphogenetic processes in mammalian development, the formation of the proamniotic cavity (Hernández-García et al., 2008).

Angiogenesis and cell migration

Angiogenesis is a complex process necessary for embryonic vascular development that, through controlled endothelial cell migration and proliferation, leads the growth of new capillaries from preexisting vessels. Endothelial cell-specific factors, like Vegf, Pdgf, and angiopoietin (Ang1) are critical for angiogenesis and are regulated by redox (Lassegue and Clempus, 2003). ROS contribute to Vegf- and Pdgf-induced phosphorylation of Akt, Erk1/2, and p38 Mapks in endothelial cells. The activation of Tie2 receptors by Ang-1 in human umbilical vein endothelial cells induces rapid and transient production of ROS, particularly superoxide anions. ROS production is attenuated by inhibition of the NADPH oxidase assembly and is Rac1-mediated. Interestingly, Ang1-induced cell migration was strongly inhibited by overexpression of antioxidants, by a Rac1 dominant-negative mutant, and by selective NADPH oxidase inhibitors (Harfouche et al., 2005). Rac1 also mediates Vegf-induced proliferation and migration of endothelial cells, suggesting that NADPH oxidase plays an important functional role in promoting angiogenesis (Ushio-Fukai et al., 2002). Interestingly, Nox1 is a potent trigger of angiogenesis

involving the induction of *Vegf* expression (Arbiser et al., 2002). It is worth mentioning that hypoxia is a major condition that regulates angiogenesis, and at least Hif1α is regulated by the Trx/Ref system (Harris, 2002). Thus, hypoxia and ROS regulatory networks may interact to finely control angiogenesis.

ROS-regulated migration may not be unique to endothelial cells. Recently, a genetic screen in *Drosophila* for genes that alter germ cell migration identified a thioredoxin peroxidase gene as essential in this process (DeGennaro and Lehmann, 2007). Strikingly, overexpression of this gene promotes early transepithelial migration of germ cells into the midgut primordium. These observations strongly suggest that a redox mechanism controls germ cell migration.

Redox regulators in development

The studies in culture indicate that ROS can regulate the fundamental cellular processes of development. However, as mentioned above, only few evidences support an actual developmental role of ROS. Although the activity of the respiratory chain, or of other ROS sources, and the presence or absence of antioxidants during development are not indicative of a specific ROS function, they could serve as signs of regions and/or times at which ROS potentially have a function. Unfortunately, the instability of most ROS species *in vivo* precludes a confident determination of their concentration in developing cells. High ROS concentration can be determined by staining with redox-sensitive dyes (Curtin et al., 2002). This strategy has been mainly applied to cells in culture, though it has been demonstrated to be useful for detection of high ROS levels in mouse embryos at different developmental stages (Salas-Vidal et al., 1998; Sanchez-Carbente et al., 2005; Schnabel et al., 2006). Taking advantage of the damage caused by oxidative stress, an alternative indirect way to determine ROS levels is by measuring the oxidation level of macromolecules. In this case, it is important to consider the half-life of the damaged molecule and repair mechanisms, as these will determine if the measurements indicate a short-term increase in ROS or an accumulative damage due to continuous ROS production. This strategy has been used to determine ROS damage during the adult lifespan, but its use to estimate ROS level in different embryo regions is limited by the necessity to dissect the embryo and the amount of tissue needed. In addition, the enormous dynamics of developing cells may limit the accumulation of the oxidized macromolecule to below detectable levels. Reliable determinations of ROS concentration await development of new methods that are applicable to live embryos. Recently, a protein whose fluorescence depends upon oxidation by hydrogen peroxide was developed (Belousov et al., 2006). Transgenic animals with this ROS-sensitive protein may allow determining the redox status of live cells within an organism.

The expression pattern of antioxidant genes is interesting because they could either define the areas of high and low ROS levels or reveal the areas under oxidative stress, as several of those genes respond to this condition. However, at this time, very little is known about the regulation of genes involved in the control of the intracellular redox state during normal development. This lack of information is compensated by data coming from studies where many genes regulating ROS levels have been mutated or overexpressed, especially in the mouse (Table 1). Development is affected by mutation in several genes regulating ROS levels, however, interpretation of their function needs to be taken with caution. For instance, due to the essential requirement of the respiratory chain for ATP production, null mutations in genes encoding its essential components (e.g., cytochrome *c*, apoptosis inducing factor) are lethal very early in embryogenesis (Li et al., 2000; Brown et al., 2006). Therefore, decreasing ROS production by altering the respiratory chain is not possible. On the other hand, abnormal embryo phenotypes after targeted disruption of genes

Table 1
Phenotype of mice homozygous for null alleles of genes encoding antioxidant and prooxidant proteins[a]

Phenotype	Genotype	Stage[b]	Brief description of defect	Reference
Early developmental lethality	*Aif*$^{-/-}$	E9	Small size caused by abnormal cell death	Brown et al. (2006)
	Cyt c$^{-/-}$	E8.5	Embryo developmental delay and deficient cell growth	Li et al. (2000)
	Ref1$^{-/-}$	E5.5	Abundant cell death and expanded proamniotic cavity	Xanthoudakis et al. (1996)
	Gpx4$^{-/-}$	E7.3	Failed to form well-organized embryonic structures	Yant et al., 2003; Imai et al. 2003
	γGcs$^{-/-}$	E8.5	Defects in gastrulation	Shi et al. (2000)
	Trx1$^{-/-}$	E5	Failure to hatch, proliferation affected on ICM cells	Matsui et al. (1996)
	Trx2$^{-/-}$	~E10.5	Anterior neural tube open	Nonn et al. (2003)
	TrxR1$^{-/-}$	E9.5–10.5	Development retardation and reduced proliferation	Jakupoglu et al. (2005)
	TrxR2$^{-/-}$	E13.5	Reduced size and dysplasia of cardiac tissue	Conrad et al. (2004)
Postnatal defects	*Sod1*$^{-/-}$	20 months	Shorten life span probably due to the development of hepatocarcinomas	Elchuri et al. (2005)
	Sod1$^{-/-}$; *Sod3*	20 months	Idem and increased oxidative damage	Sentman et al. (2006)
	Sod2$^{-/-}$	10 days	Neurodegeneration and enlarged heart	Lebovitz et al. (1996)
	Sod2$^{-/-}$; *TgSod1*	16 days	Idem	Copin et al. (2000)
	Gpx1$^{-/-}$; *Gpx2*$^{-/-}$	3 months	Growth retardation, intestine mucosal inflammation, and hypothermia	Esworthy et al. (2001)
	Prdx1$^{-/-}$	9 months	Short life span, anemia and cancer	Neumann et al. (2003)
	Prdx2$^{-/-}$	6 weeks	Anaemic and bigger spleen	Lee et al. (2003)
Infertility	*Sod1*$^{-/-}$	Adult female	Reproductive performance affected causing postimplantation embryo death	Matzuk et al. (1998)
Viable	*Cas*$^{-/-}$		Frequently show increased sensitivity to oxidative stress in adult animals	Ho et al. (2004)
	Sod3$^{-/-}$			Carlsson et al. (1995)
	Gpx1$^{-/-}$			Ho et al. (1997)
	Gpx2$^{-/-}$			Esworthy et al. (2001)
	Gr$^{-/-}$			Rogers et al. (2004)
	Prdx3$^{-/-}$			Li et al. (2007)
	Prdx6$^{-/-}$			Wang et al. (2003)
	Nox1$^{-/-}$			Gavazzi et al. (2006)
	Nox2$^{-/-}$			Pollock et al. (1995)
	Nox3$^{-/-}$			Paffenholz et al. (2004)

[a] The proteins considered here are those directly producing or eliminating ROS. Proteins that indirectly contribute to the control of ROS level and whose deficiency cause embryo lethality are included in Fig. 2.
[b] The earliest stage at which the defect was determined.

encoding antioxidant enzymes might indicate a relevant function during development (Table 1), but it cannot definitively establish a role for ROS. The lack of an antioxidant enzyme could increase ROS, damaging cells or turning on a ROS-mediated pathway out of context, consequently altering development. Conversely, mice deficient in several prooxidant and antioxidant enzymes are viable and mild phenotypes are only observed during adulthood (Table 1). However, since animals have evolved multiple systems to control ROS levels, redundancy among different ROS regulatory genes will not be unexpected. Redundancy may occur within the same gene family. For example, since mice null for individual *Nox* are viable, it is possible that different *Nox* are redundant for a specific developmental function.

Most gene function studies of redox regulation have not been directed to understand developmental processes. It has been observed that antioxidant gene overexpression frequently has protective effects upon oxidative stress insults; however, it is important to note that all transgenic mice produced up to date were selected from among viable offsprings (see for instance Raineri et al., 2001 and Schriner et al., 2005). Therefore, disruption of embryo development caused by a decrease in ROS, due to antioxidant enzyme overproduction, has not been evaluated. Furthermore, new genetic strategies have not been applied to study the role of ROS in development. It will be interesting, for instance, to generate conditional mutations in those genes showing early embryo lethality (Table 1) as well as controlled induction in time and space of genes encoding antioxidant enzymes.

Conclusions

Marked metabolic changes occur in cells during development. The ROS production levels in a cell are likely determined by the metabolic activity associated to a particular cellular process. On the other hand, antioxidant activity may be a consequence of the ROS production level. The complex mechanisms that cells have to determine the redox state make it difficult to assign a definitive developmental function to ROS based only upon altering ROS or antioxidant levels. Considering a passive ROS function, it is clear that cells during development are sensitive to damage by ROS. Disruption of development may result from general macromolecular damage that can cause cell death, and also from the oxidation of specific key developmental molecules.

Nearly a century ago, it was proposed that metabolic gradients guided early development (reviewed in Coffman and Denegre, 2007). In aerobiosis, metabolic activity mirrors mitochondrial activity since this organelle produces most of the energy the cell requires. Under this condition, mitochondria are the major ROS source in cells. And although counter-intuitive, hypoxia also induces the production of ROS from mitochondria (Turrens, 2003). Interestingly, mitochondria is asymmetrically distributed in early embryos of several animals, and in some instances this distribution has been associated to axis specification (Coffman and Denegre, 2007). ROS may be the mitochondrial signal that contributes to the correct development of embryos from very early stages. Although this observation supports an active role of ROS in development, a convincing determination of a developmental function depends on evidences indicating a requirement of ROS in specific processes during embryogenesis. Genetic manipulations directed to determine the active functions of ROS may bring light in this difficult task.

The potential influence of metabolic activity and the resulting ROS production on development cannot be dismissed. A function of ROS in development is likely, due to the large amount of evidence showing that ROS can regulate fundamental cellular processes. Furthermore, it is possible that oxidation of specific molecules changes their activity, and in such a way defines the fate of a developing cell. We expect that future research will reveal the reciprocal regulation of signaling cascades and metabolic pathways during animal development, in which ROS will be a key player.

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